Inactivation of JAK2/STAT3 Signaling Axis and Downregulation of M1 mAChR Cause Cognitive Impairment in *klotho* Mutant Mice, a Genetic Model of Aging

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We previously reported cognitive dysfunction in *klotho* mutant mice. In the present study, we further examined novel mechanisms involved in cognitive impairment in these mice. Significantly decreased janus kinase 2 (JAK2) and signal transducer and activator of transcription3 (STAT3) phosphorylation were observed in the hippocampus of *klotho* mutant mice. A selective decrease in protein expression and binding density of the M1 muscarinic cholinergic receptor (M1 mAChR) was observed in these mice. Cholinergic parameters (ie, acetylcholine (ACh), choline acetyltransferase (ChAT), and acetylcholinesterase (AChE)) and NMDAR-dependent long-term potentiation (LTP) were significantly impaired in *klotho* mutant mice. McN-A-343 (McN), an M1 mAChR agonist, significantly attenuated these impairments. AG490 (AG), a JAK2 inhibitor, counteracted the attenuating effects of McN, although AG did not significantly alter the McN-induced effect on AChE. Furthermore, AG significantly inhibited the attenuating effects of McN on decreased NMDAR-dependent LTP, protein kinase C β II, p-ERK, p-CREB, BDNF, and p-JAK2/p-STAT3-expression in *klotho* mutant mice. In addition, k252a, a BDNF receptor tyrosine kinase B (TrkB) inhibitor, significantly counteracted McN effects on decreased ChAT, ACh, and M1 mAChR and p-JAK2/p-STAT3 expression. McN-induced effects on cognitive impairment in *klotho* mutant mice were consistently counteracted by either AG or k252a. Our results suggest that inactivation of the JAK2/STAT3 signaling axis and M1 mAChR downregulation play a critical role in cognitive impairment observed in *klotho* mutant mice.

Neuropsychopharmacology (2013) 38, 1426–1437; doi:10.1038/npp.2013.39; published online 27 February 2013

Keywords: cognitive impairment; JAK2/STAT3; *klotho* gene; long-term potentiation; M1 mAChR; PKC/ERK/CREB/BDNF

INTRODUCTION

Klotho mutant mice, which harbor a defective *klotho* gene, have an extremely short lifespan (around 8 weeks).

At 4–5 weeks of age, the mice develop multiple age-related syndromes, such as arteriosclerosis, osteoporosis, skin atrophy, infertility, thymic atrophy, and pulmonary emphysema (Kuro-o *et al*, 1997). Further evidence suggests that the *klotho* gene is responsible for aging. Introduction of a normal *klotho* gene into these mutant mice improves their phenotypes (Kuro-o *et al*, 1997), and overexpression of this gene in normal wild-type mice significantly extends their life spans (Kurosaki *et al*, 2005). Although *klotho* mutant mice are considered to be a novel animal model for accelerated human aging, these mice do not express certain phenotypes usually seen in aged humans, such as brain atrophy with amyloid or senile plaque deposition (Kuro-o *et al*, 1997; Nagai *et al*, 2003).

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Received 19 September 2012; revised 3 January 2013; accepted 28 January 2013; accepted article preview online 6 February 2013

Our group was the first to observe cognitive impairment in *klotho* mutant mice (Nagai *et al*, 2003). We showed that anti-death genes/proteins, Bcl-2 and Bcl-xL, are down-regulated while the pro-death molecule, Bax, is upregulated in the hippocampus of *klotho* mutant mice (Nagai *et al*, 2003). In addition, Li *et al* (2004) demonstrated that synaptic structures and synaptophysin are reduced in number and expression, respectively, in the CA3 region of *klotho* mutant mice. However, very limited information is available regarding underlying mechanisms involved in significant cognitive function impairment in *klotho* mutant mice, although it is recognized that the *klotho* gene may be essential for maintaining cognitive function in aging organisms.

Earlier studies suggested that soluble signaling molecules (ie, cytokines and growth factors) and their receptors are expressed in both the developing and mature mammalian CNS (Mehler and Kessler, 1997). One intracellular signaling pathway activated by soluble signaling molecules involves two families of molecules: janus kinase (JAK) proteins and the signal transducer and activator of transcription (STAT) proteins (Ihle *et al*, 1995). Importantly, p-STAT3 immunoreactivity in hippocampal neurons of young subjects is substantially higher than that of older, cognitively normal humans and rodents (Chiba *et al*, 2009).

Because McN-A-343 (McN), a M1 agonist appeared to be more effective than either AF102B (AF; Fisher *et al*, 2003) or talsaclidine (TC; Walland and Pieper, 1998) in improving cognitive impairment in *klotho* mutant mice (Supplementary Figure S3), we applied McN in the present study. JAK2 is an upstream regulator of STAT3 activation. As inhibition of JAK2 activity by AG490 (AG) results in decreased STAT3 phosphorylation, we applied AG in the present study to inhibit the JAK2/STAT3 signaling axis (Chiba *et al*, 2009).

In the present study, we investigated whether *klotho* gene deficiency affects the p-JAK2/STAT3 signaling axis, and also examined whether *klotho* gene deficiency alters the cholinergic neuronal system. To this aim, we examined the neurochemical, electrophysiological, and behavioral parameters accompanied by aging in *klotho* mutant mice. We propose here that inactivation of the JAK2/STAT3 signaling axis and M1 mAChR downregulation lead to cognitive impairments in *klotho* mutant mice.

MATERIALS AND METHODS

Animals

All animals were treated in accordance with the National Institutes of Health (NIH) Guide for the Humane Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985; www.dels.nas.edu/ila). The present study was performed in accordance with the Institute for Laboratory Research (ILAR) guidelines for the care and use of laboratory animals. Mice were maintained under a 12-h light:12-h dark cycle and fed *ad libitum*. Since *klotho* mutant mice are infertile, wild-type and *klotho* mutant mice were generated by crossing heterozygous *klotho* mutant mice (Kuro-o *et al*, 1997; Nagai *et al*, 2003). The mice were screened by polymerase chain reaction (PCR) analysis using

DNA extracted from tail specimens. More details on the gene characterization were described in the supporting information.

Guide Cannula Implantation and Intracerebroventricular Infusion

Forty-day-old mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and a stainless steel guide cannula (AG-4; Eicom, Kyoto, Japan) was implanted into the right lateral ventricle (stereotaxic coordinates: 0.5 mm posterior to bregma, 1 mm right to the midline, and 2 mm ventral to the dura, according to the atlas of Franklin and Paxinos, 2008 and Shin *et al*, 2009). Microinfusion into the lateral ventricle was performed at a rate of 1 μ l/min. More details on the guide cannula implantation and intracerebroventricular (i.c.v.) infusion were described in the supporting information.

Drug Treatment and Animal Handling

Drug treatment started after 2 days of recovery from guide cannula implantation. Because we observed, in an earlier study (Nagai *et al*, 2003), that cognitive impairment did not occur in *klotho* mutant mice until 6 weeks of age, mice received McN-A-343 (McN, M1, mAChR agonist; Tocris Bioscience, Ellisville, MO, USA; 1.0 μ g/ μ l in saline, i.c.v.) on postnatal day (PND) 42, 46, 49, and 53. AG490 (JAK2/STAT3 inhibitor; Tocris Bioscience; 15 mg/kg/2 ml, i.p.) or k252a (TrkB inhibitor; Tocris Bioscience; 0.3 mg/kg/2 ml, i.p.) was injected 1 h after each McN treatment. Novel object recognition tests or conditioned-fear learning tests were conducted on PND 54 and 55 (Supplementary Figure S1). The dose of each drug used in this study was selected based on previous studies (Chiba *et al*, 2009; Liu *et al*, 2005; Wall *et al*, 2001) and our preliminary study (Bach *et al*, 2010). Mice were killed 30 min after the test trial for western blot analyses (p-JAK2, p-STAT3, mAChRs, nAChRs, and BDNF), autoradiography, neurochemical measurements, and electrophysiological analyses. To evaluate p-ERK and p-CREB expression, mice were killed 30 min after the training trial. Mouse body weight was recorded on a daily basis from PND 35 to 55 (Supplementary Figure S5). More details on the preparation of each reagent were described in the supporting information.

Novel Object Recognition Test and Conditioned-Fear Learning Test

Novel object recognition test and conditioned-fear learning test were performed with a slight modification as described previously (Jin *et al*, 2009; Nagai *et al*, 2003). Detailed procedure is described in the supporting information.

Western Blot Analysis and Immunoprecipitation Assay

Western blot analysis and immunoprecipitation assay were done as described previously (Jin *et al*, 2009). More details on the procedure and antibody were described in the supporting information.

Autoradiography

Autoradiograms were generated and analyzed with a slight modification as described previously (Jin *et al*, 2009; Zhang and Buccafusco, 2000). More details on the autoradiography were described in the supporting information.

Level of ACh and Activities of AChE and ChAT

Hippocampi were homogenated in ice-cold 20 mM sodium phosphate buffer (pH 7.4). These were centrifuged at 12 000 g for 30 min, at 4 °C. The supernatant was assayed for ACh levels using Amplex Red Acetylcholine/Acetylcholinesterase (AChE) Assay Kit (A-12217) (Eugene, Oregon, USA), according to the manufacturer's instruction. Detailed procedure was described in the supporting information. AChE activity was measured using Amplex Red Acetylcholine/AChE Assay Kit (A-12217) (Eugene, Oregon, USA), according to the manufacturer's instruction. For choline acetyltransferase (ChAT) activity, 5% tissue homogenates were prepared in ice-cold 20 mM sodium phosphate buffer (pH 7.4), and kept frozen overnight at -20 °C. These were thawed the following day and centrifuged at 12 000 g for 1 h, at 4 °C. The supernatant was assayed for ChAT activity according to the method of Chao and Wolfram (1972). Detailed procedure was described in the supporting information.

Electrophysiological Recordings

Transverse hippocampal slices were prepared from wild-type or *klotho* mutant mice, according to the previous report with a minor modification (Min *et al*, 2009). CA1 field potentials (FPs) were evoked by stimulating Schaffer collaterals with 0.2 ms pulses delivered through concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME, USA), and recorded extracellularly in CA1 stratum radiatum. Responses were quantified as the initial FP slope in CA1. Baseline responses were recorded using half maximal stimulation intensity at 0.033 Hz. Long-term potentiation (LTP) was examined using a conventional stimulation paradigm: four episodes of theta burst stimulation (TBS) consisting of eight bursts of four 100 Hz pulses were administered every 200 ms. Because four TBS episodes induced robust LTP that lasted for >60 min, we measured the magnitude of NMDAR-dependent LTP induced by one TBS episode. More details on the hippocampal slice preparation were described in the supporting information.

Statistical Analyses

Data were analyzed using IBM SPSS version 19.0 (IBM, Chicago, IL, USA). One-way ANOVA was applied for the two data groups (Figure 1a and b). Three-way ANOVA was performed for the effect of *klotho* gene mutation, McN, and AG490 (or k252a) (Figures 1c and d, 2, 3, and 5). The effect of k252a on the McN-mediated pharmacological actions in the *klotho* mutant mice was analyzed by two-way ANOVA, with McN and k252a as between-subjects factors (Figure 4). The statistical values are presented in Supplementary Tables S1–S4. After analysis by two- or three-way ANOVA, *post-hoc* multiple pairwise comparisons with Bonferroni's

correction were then performed. *P*-values of <0.05 were considered statistically significant.

RESULTS

p-JAK2 and p-STAT3 Expression Was Significantly Decreased in the Hippocampus of *klotho* Mutant Mice

Our experimental protocol is shown in Supplementary Figure S1. First, we investigated whether *klotho* gene deficiency affected p-JAK2/p-STAT3 expression, since p-JAK2/p-STAT3 decreases in an age-dependent manner (De-Fraja *et al*, 1998). p-JAK2 and p-STAT expression were significantly decreased (p-JAK2, *P* = 0.00257; p-STAT3, *P* = 0.000779) in *klotho* mutant mice (Figure 1a; Supplementary Table S1).

M1 mAChR Expression Was Significantly Decreased in the Hippocampus of *klotho* Mutant Mice

Next, we investigated whether mAChR expression was decreased in *klotho*-deficient mice, as M1 mAChR is downstream of the p-JAK2/p-STAT3 signaling axis (Chiba *et al*, 2009).

Of the five muscarinic acetylcholine receptors (M1–M5 mAChRs) examined, only M1 mAChR expression was significantly decreased (*P* = 0.00464) in *klotho* mutant mice. Conversely, nicotinic acetylcholine receptor subtypes (α 4 nAChR, α 7 nAChR, β 2 nAChR, or α 4 β 2 nAChR) were not significantly different in the mutant mice (Figure 1b; Supplementary Table S1).

AG490 Antagonism Against McN-Induced Effects on Reduced M1 mAChR Expression and [³H]-Pirenzepine-Binding Density in the Hippocampus of *klotho* Mutant Mice

Since M1 mAChR expression and p-JAK2/p-STAT3 signaling is decreased in animal models of Alzheimer's disease (Chiba *et al*, 2009), we examined whether McN, a specific M1 mAChR agonist, affected M1 mAChR expression or [³H]-pirenzepine-binding density in the hippocampus of *klotho* mutant mice. In addition, we investigated whether AG, an inhibitor of JAK2/STAT3 signaling, affected McN-induced changes.

Three-way ANOVA indicated significant effects of *klotho* gene mutation, McN and AG490, and a significant interaction between *klotho* gene mutation and McN (Supplementary Table S1). A *post-hoc* test revealed that McN treatment significantly attenuates (*P* = 0.00308) the reduction in M1 mAChR expression in *klotho* mutant mice. This attenuation was significantly inhibited (*P* = 0.0268) by AG490. However, AG490 *per se* did not significantly alter M1 mAChR expression in either wild-type or *klotho* mutant mice treated with vehicle and saline (Figure 1c). Representative autoradiograms showing the distribution of [³H]-pirenzepine binding are presented in Figure 1d. Three-way ANOVA showed significant effects (CA1, CA3, and dentate gyrus (DG)) of *klotho* gene mutation, McN and AG490, and a significant interaction between *klotho* gene mutation and McN (DG; Supplementary Table S1). Strong binding was noted in the CA1 region and DG, whereas mild binding was observed in the CA2/3 region in the hippocampus of wild-type mice

Figure 1 Changes in protein expression of p-JAK2, p-STAT3 (a) and cholinergic receptors (b), and AG antagonism against McN-induced effects on reduced M1 mAChR expression (c) and [3 H]pirenzepine-binding density (d) in the hippocampus of *klotho* mutant mice. Veh = Vehicle (50% DMSO in saline, the solvent for AG 490); Sal = Saline; AG = AG490 (15 mg/kg/2 ml, i.p.); McN = McN-A-343 (1.0 μ g/ μ l in saline, i.c.v.). Each value is the mean $\pm$ SEM of six animals. * P < 0.01 vs corresponding wild-type mice; # P < 0.01 vs *klotho* mutant mice treated with Veh and Sal; & P < 0.05, && P < 0.01 vs *klotho* mutant mice treated with Veh and McN (one-way ANOVA (a, b) or three-way ANOVA followed by multiple pairwise comparisons with Bonferroni's correction (c, d)).

with or without McN. However, a *post-hoc* test revealed that [3 H]-pirenzepine-binding densities were significantly lower (CA1, $P = 6.82 \times 10^{-7}$; CA2/3, $P = 3.14 \times 10^{-5}$; DG, $P = 7.59 \times 10^{-8}$) in the hippocampus of *klotho* mutant

mice. AG490 did not affect these reduced binding densities *per se* in *klotho* mutant mice. However, McN significantly reversed (CA1, $P = 0.00156$; CA2/3, $P = 0.00898$; DG, $P = 7.82 \times 10^{-4}$) the decreases in [3 H]-pirenzepine binding in the

Figure 2 AG antagonism against McN-induced changes in acetylcholine level (a), and choline acetyltransferase (ChAT) protein expression (b), ChAT activity (b), AChE protein expression (c), AChE activity (c), p-JAK2 (d), p-STAT3 expression (e), and NMDAR-dependent LTP (f, g) in the hippocampus of *klotho* mutant mice. (f) Field EPSP slope (% of baseline) was plotted against time. LTP was induced by application of one TBS episode. Insets: representative traces recorded before (thin line), 60 min (thick line) after one TBS episode. A: Wild-type/Sal + Veh ($n = 11$), B: *klotho* mutant/Sal + Veh ($n = 11$), C: *klotho* mutant/McN + Veh ($n = 10$), D: *klotho* mutant/McN + AG490 ($n = 11$), E: wild-type/McN + Veh ($n = 8$). (g) Comparison of average early-LTP magnitude 58–60 min after TBS application. AG490 blocked the reversal effects of McN on NMDAR-dependent LTP. Veh = Vehicle (50% DMSO in saline, the solvent for AG490); Sal = Saline; AG = AG490 (15 mg/kg/2 ml, i.p.); McN = McN-A-343 (1.0 μ g/ μ l in saline, i.c.v.). Each value is the mean $\pm$ SEM of six animals (a–e) or 8–11 animals (f, g). * $P < 0.05$, ** $P < 0.01$ vs corresponding wild-type mice; # $P < 0.05$, ## $P < 0.01$ vs *klotho* mutant mice treated with Veh and Sal; & $P < 0.05$, && $P < 0.01$ vs *klotho* mutant mice treated with Veh and McN (three-way ANOVA followed by multiple pairwise comparisons with Bonferroni's correction).

hippocampus of *klotho* mutant mice. AG490 significantly counteracted (CA1, $P = 0.0209$; CA2/3, $P = 0.0416$; DG, $P = 0.00960$) McN effects (Figure 1d).

AG490 Antagonism Against McN-Induced Effects on Altered Cholinergic System and p-JAK2/p-STAT3 in *klotho* Mutant Mice

Since earlier reports demonstrated that M1 mAChR agonists reverse cholinergic impairment (Calabresi *et al*, 1998; Fisher

et al, 2003; Walland *et al*, 1998), we next investigated whether McN attenuated changes in *klotho* mutant mice cholinergic parameters. We subsequently examined whether AG affected McN pharmacological effects. Three-way ANOVA showed significant effects of *klotho* gene mutation (ACh, ChAT expression ChAT activity, AChE expression, and AChE activity), McN (ACh, ChAT expression ChAT activity, AChE expression, and AChE activity), and AG490 (ACh, ChAT expression, and ChAT activity), and a significant interaction between *klotho* gene mutation and

McN (ACh, AChE expression, and AChE activity; Supplementary Table S2). A *post-hoc* test indicated that acetylcholine ACh level ($P=4.24 \times 10^{-5}$), ChAT protein

expression ($P=5.15 \times 10^{-5}$), and ChAT activity ($P=8.59 \times 10^{-4}$) were significantly decreased in the hippocampus of *klotho* mutant mice (Figure 2a and b). In contrast, AChE protein expression ($P=2.15 \times 10^{-4}$) and AChE activity ($P=6.38 \times 10^{-4}$) were significantly increased in *klotho* mutant mice (Figure 2c). McN treatment significantly attenuated (ACh level, $P=7.38 \times 10^{-4}$; ChAT expression, $P=9.87 \times 10^{-4}$; ChAT activity, $P=0.00673$; AChE expression, $P=0.0160$; AChE activity, $P=0.00998$) these alterations in *klotho* mutant mice. AG treatment significantly inhibited McN-induced effects on decreased ACh level ($P=0.0182$), ChAT expression ($P=0.0187$), and its activity ($P=0.0200$). However, AG failed to significantly inhibit McN-induced effects on increased AChE expression and its activity in *klotho* mutant mice (Figure 2a–c).

Although there is no existing evidence that an M1 mAChR agonist *per se* increases p-JAK2/STAT3 signaling in an animal model of aging or neurodegeneration, IL-6, a STAT3 activator, increases carbachol-induced p-ERK (Chiba *et al*, 2009). Additionally, carbachol plays a role as a mAChR agonist via the M1 type mAChR (Anagnostaras *et al*, 2003). Therefore, we examined whether McN altered p-JAK2/STAT3 signaling in the hippocampus of *klotho* mutant mice. We then investigated whether AG affected pharmacological effects of McN. Three-way ANOVA revealed significant effects of *klotho* gene mutation, McN and AG490 on p-JAK2 and p-STAT3 expression. In addition, a significant interaction between *klotho* gene mutation and McN was shown in the p-JAK2 expression (Supplementary Table S2). A *post-hoc* test indicated that McN significantly attenuated reduced p-JAK2 ($P=0.00191$) and p-STAT3 ($P=0.00234$) expression in *klotho* mutant mice, although McN treatment did not significantly alter p-JAK2 or p-STAT3 expression in wild-type mice. Additionally, AG significantly inhibited McN-induced effects on reduced p-JAK2 and p-STAT3 in *klotho* mutant mice (p-JAK2, $P=0.00837$; p-STAT3, $P=0.0236$). However, AG490 *per se* did not significantly affect p-JAK2 and p-STAT3 expression in either wild-type or *klotho* mutant mice (Figure 2d and e).

AG490 Antagonism Against McN-Induced Effects on Impaired NMDAR-Dependent LTP in *klotho* Mutant Mice

After behavioral testing, we measured LTP magnitude in the CA1 area of hippocampal slices prepared from *klotho* mutant or wild-type mice using a maximal LTP protocol (Supplementary Figure S2; Figure 2f and g). We tested whether the reduced cholinergic activity in *klotho* mutant mice was a possible reason for impaired LTP induction with the use of McN. McN treatment significantly reversed the LTP impairment in *klotho* mutant mice (Figure 2f and g).

Figure 3 AG490 antagonism against McN-induced effects on PKC β II (a), p-ERK (b), p-CREB (c), and BDNF (d) expression in the hippocampus of *klotho* mutant mice. Veh = Vehicle (50% DMSO in saline, the solvent for AG490); Sal = Saline; AG = AG490 (15 mg/kg/2 ml, i.p.); McN = McN-A-343 (1.0 μ g/ μ l in saline, i.c.v.). Each value is the mean $\pm$ SEM of six animals. * $P<0.05$, ** $P<0.01$ vs corresponding wild-type mice; # $P<0.01$ vs *klotho* mutant mice treated with Veh and Sal; & $P<0.05$, && $P<0.01$ vs *klotho* mutant mice treated with Veh and McN (three-way ANOVA followed by multiple pairwise comparisons with Bonferroni's correction).

Figure 4 K252a antagonism against McN-induced effects on p-JAK2 (a), p-STAT3 (b), choline acetyltransferase (c), AChE (d), and M1 mAChR (f), and the level of acetylcholine (e) in the hippocampus of *klotho* mutant mice. Veh = Vehicle (5% DMSO in saline, the solvent for k252a); Sal = Saline; k252a = k252a (0.3 mg/kg/2 ml, i.p.); McN = McN-A-343 (1.0 μ g/ μ l in saline, i.c.v.). Each value is the mean $\pm$ SEM of six animals. $^{\#}P < 0.05$, $^{##}P < 0.01$ vs *klotho* mutant mice treated with Veh and Sal; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs *klotho* mutant mice treated with Veh and McN (two-way ANOVA followed by multiple pairwise comparisons with Bonferroni's correction).

Three-way ANOVA showed significant effects of *klotho* gene mutation and AG490 (Supplementary Table S2). The magnitude of NMDAR-dependent LTP in slices from *klotho* mutant mice treated with McN was $142.38 \pm 10.04\%$ of baseline ($n = 10$) 1 h after TBS application. These values were higher than those in slices from *klotho* mutant mice treated with vehicle ($116.80 \pm 6.59\%$ of baseline, 1 h

after TBS application, $P = 0.0389$; Figure 2g). Although the magnitude of NMDAR-dependent LTP in slices from wild-type mice treated with McN alone was slightly higher than in slices from control mice treated with vehicle, the two groups were not significantly different ($154.13 \pm 15.84\%$ vs $143.82 \pm 7.71\%$ of baseline, respectively, $P = 0.434$).

Figure 5 Antagonism by AG490 or k252a against McN-induced effects on novel object recognition (a) and conditioned-fear learning performance (b) of *klotho* mutant mice. Veh = Vehicle (50% DMSO in saline, the solvent for AG490); Sal = Saline; AG = AG490 (15 mg/kg/2 ml, i.p.); k252a = k252a (0.3 mg/kg/2 ml, i.p.); McN = McN-A-343 (1.0 μ g/ μ l in saline, i.c.v.). Neither 50% DMSO nor 5% DMSO solution affect significantly memory performance; thus, shown as Veh for AG490. Each value is the mean $\pm$ SEM of 15 animals. * $P < 0.01$ vs corresponding wild-type mice; # $P < 0.01$ vs *klotho* mutant mice treated with Veh and Sal; & $P < 0.05$, && $P < 0.01$ vs *klotho* mutant mice treated with Veh and McN (three-way ANOVA followed by multiple pairwise comparisons with Bonferroni's correction).

We then investigated whether the M1 agonist required JAK2/STAT3 signaling to attenuate the NMDAR-dependent LTP impairment. In the presence of AG490, the McN effect on LTP induction was not reversed; the average LTP magnitude in slices from *klotho* mutant mice treated with McN and AG490 was 117.08 ± 4.60 , significantly lower than that in slices from *klotho* mutant mice treated with McN or vehicle ($142.38 \pm 10.04\%$ of baseline; $P = 0.0406$). These results suggest that M1 mAChR and JAK2/STAT3 interaction is required to induce normal NMDAR-dependent LTP.

AG490 Antagonism Against McN-Induced Effects on PKC β II, p-ERK, p-CREB and BDNF Expression in the Hippocampus of *klotho* Mutant Mice

M1 mAChR stimulation activates a G-protein belonging to the pertussis toxin-insensitive Gq/11 family. Gq/11 subunits stimulate phospholipase C, inducing IP3-dependent calcium release and protein kinase C (PKC) activation (Fisher et al, 2003). PKC pathways are strong candidates for mediating the molecular changes that underlie spatial learning, as they play critical roles in neurotransmitter release and synaptic plasticity, and deletion of specific

PKC genes results in learning deficits. To understand the role of PKC in our experimental system, we examined PKC isozyme protein expression in the hippocampus of *klotho* mutant mice (Figure 3a; Supplementary Figure S4). Three-way ANOVA indicated significant effects of *klotho* gene mutation, McN and AG490 on PKC β II expression (Supplementary Table S3), but not on PKC α , PKC β I, PKC δ , or PKC ζ expression (Supplementary Figure S4; Supplementary Table S6). *Post-hoc* analysis showed that PKC β II expression was significantly and selectively decreased ($P = 1.45 \times 10^{-6}$) in *klotho* mutant mice (Figure 3a), whereas PKC α , PKC β I, PKC δ , and PKC ζ (Supplementary Figure S4) were not significantly different from wild-type. McN significantly attenuated ($P = 0.00215$) the decreased PKC β II expression in *klotho* mutant mice. Consistently, AG490 significantly counteracted McN-mediated attenuation ($P = 0.0122$).

ERK1/2 signal transduction pathways are downstream of the PKC pathway (Fisher et al, 2003) and are also involved in synaptic plasticity, learning, and memory. ERK is necessary for the development of several forms of memory, such as fear conditioning, conditioned taste aversion, spatial memory, step-down inhibitory avoidance, and object recognition memory (Alkam et al, 2010). ERK regulates CREB function and BDNF expression (Williams et al, 2008). CREB is a critical mediator of activity-induced transcriptional signaling of dendrite growth in response to neuronal activity (Wayman et al, 2006). Three-way ANOVA indicated significant effects of *klotho* gene mutation, McN and AG490, and a significant interaction between *klotho* gene mutation and McN on the expression of p-ERK, p-CREB, and BDNF (Supplementary Table S3). No significant change in ERK1/2 expression was observed among the groups in this study. However, a *post-hoc* test revealed that p-ERK1/2 expression was significantly decreased ($P = 5.22 \times 10^{-8}$; Figure 3b) in *klotho* mutant mice. Similar to ERK, p-CREB and BDNF expression were significantly decreased (p-CREB, $P = 6.02 \times 10^{-8}$; BDNF, $P = 4.00 \times 10^{-6}$) in the hippocampus of *klotho* mutant mice (Figure 3c and d). McN significantly attenuated these decreases in p-ERK ($P = 4.26 \times 10^{-5}$), p-CREB ($P = 9.18 \times 10^{-5}$), and BDNF ($P = 0.00269$) expression. Consistently, AG490 significantly reversed McN-mediated attenuations in *klotho* mutant mice (p-ERK, $P = 0.00163$; p-CREB, $P = 0.0192$; BDNF, $P = 0.0306$; Figure 3b–d).

k252a Antagonism Against McN-Induced Effects on p-JAK2/p-STAT3, ChAT Protein Expression, ChAT Activity, AChE Protein Expression and Activity, ACh Level, and M1 mAChR Protein Expression in *klotho* Mutant Mice

BDNF, a neurotrophin, activates a high-affinity cell surface receptor (TrkB) that is coupled to activation of phosphatidylinositol-3-kinase and protein kinase B (Akt). BDNF is increased in the hippocampus of rats during and after performance of a spatial learning task (a radial-arm maze), and both acquisition and maintenance of spatial memory are impaired when BDNF levels are decreased using antisense methods (Mizuno et al, 2003). BDNF specifically binds to TrkB receptors and induces receptor dimerization, phosphorylation, and activation of the

intracellular tyrosine kinase domain (Mizuno *et al*, 2003). Therefore, we examined whether McN-induced effects on p-JAK2 and p-STAT3 expression and cholinergic parameters (ie ChAT, AChE, ACh, and M1 mAChR) in *klotho* mutant mouse hippocampus were via BDNF-TrkB signaling. Two-way ANOVA indicated significant effects of McN (all parameters in Figure 4) and k252a (p-JAK2, ChAT expression and activity, ACh level, and M1 mAChR expression), and a significant interaction between McN and k252a (p-JAK2) (Supplementary Table S3). A *post-hoc* test revealed that McN treatment significantly increased p-JAK2 ($P = 1.18 \times 10^{-4}$), p-STAT3 ($P = 0.00222$), ChAT expression ($P = 9.71 \times 10^{-4}$), ChAT activity ($P = 1.79 \times 10^{-4}$), ACh level ($P = 0.00709$), and M1 mAChR expression ($P = 1.97 \times 10^{-4}$) in *klotho* mutant mice, whereas McN treatment significantly decreased AChE expression ($P = 0.0109$) and its enzymatic activity ($P = 0.0307$). In contrast, k252a treatment, an inhibitor of TrkB receptor tyrosine phosphorylation, resulted in significant inhibition of McN-induced p-JAK2 ($P = 0.00233$), p-STAT3 ($P = 0.0368$), ChAT expression ($P = 0.0137$), ChAT activity ($P = 0.0107$), and M1 mAChR expression ($P = 0.0195$), and ACh level ($P = 0.0342$) in *klotho* mutant mice. However, k252a *per se* did not significantly decrease AChE expression or its enzymatic activity (Figure 4).

Antagonism of AG490 or k252a Against McN-Induced Effects on Cognitive Impairment in *klotho* Mutant Mice

As the hippocampus is important in the formation of recognition memory in both humans and animals (Zola-Morgan *et al*, 1986), we evaluated visual recognition memory in a novel-object recognition memory task. The recognition test is based on the natural tendency of rodents to investigate a novel object instead of a familiar one. The choice to explore the novel object reflects the use of learning and (recognition) memory processes. Three-way ANOVA revealed significant effects of *klotho* gene mutation and AG490 or k252a (Supplementary Table S4). A *post-hoc* test indicated a significant decrease ($P = 1.92 \times 10^{-4}$) in the exploratory preference in *klotho* mutant mice, suggesting cognitive impairment. McN attenuated this decrease ($P < 0.00558$; Figure 5a).

We then examined associative fear memory in a conditioned-fear task. In contextual fear conditioning, which assesses hippocampus-dependent memory, animals learn to fear the context in which they are trained to associate conditioned and unconditioned stimuli. Three-way ANOVA indicated a significant effect of *klotho* gene mutation, McN and AG490 or k252a, and a significant interaction between *klotho* gene mutation and McN (Supplementary Table S4). A *post-hoc* test revealed that contextual freezing was significantly lower in *klotho* mutant mice ($P = 1.97 \times 10^{-8}$ vs wild-type). McN treatment significantly attenuated ($P = 9.47 \times 10^{-5}$) the lowered contextual freezing in *klotho* mutant mice (Figure 5b). Both AG490 and k252a treatment significantly counteracted McN-mediated improvements in novel objective recognition (AG490, $P = 0.0320$; k252a, $P = 6.08 \times 10^{-4}$) and conditioned-fear learning performance (AG490, $P = 0.0443$; k252a, $P = 0.00218$) (Figure 5).

DISCUSSION

This study revealed that *klotho* gene deficiency impairs hippocampal cholinergic neural systems as evidenced by significant decreases in M1 mAChR gene expression, M1 mAChR-binding density, ACh levels, ChAT activity, and ChAT gene expression, and by significant increases in AChE activity and AChE gene expression. These changes in the cholinergic neuronal system may be due to *klotho* gene deficiency in presynaptic cholinergic nerve terminals and postsynaptic neurons expressing M1 mAChR in the hippocampus. *Klotho* gene deficiency consistently decreased PKC β II, p-ERK, p-CREB and BDNF expression, and NMDAR-dependent LTP (Supplementary Figure S6). To further understand the role of M1 mAChR in our experimental system, we examined the effects of McN, an M1 agonist.

McN-induced M1 mAChR stimulation activates PKC β II-, p-ERK-, p-CREB-, and BDNF-dependent pathways in the hippocampus of *klotho* mutant mice. BDNF activation leads to JAK2/STAT3 and TrkB signaling, followed by enhanced ChAT activity and ACh level, and further stimulation of M1 mAChR. Moreover, McN enhanced NMDAR-dependent LTP via JAK2/STAT3 signaling. Interestingly, the ChAT promoter contains a STAT consensus binding site (Cattaneo *et al*, 1999). We observed here that ChAT loss was pronounced in *klotho* mutant mice, similar to that observed in the aged brain (O'Neil *et al*, 1987). Thus, it is possible that presynaptically altered levels of ChAT and JAK2/STAT3 in *klotho* mutant mice are concomitant with basal hippocampal neuronal apoptosis (Nagai *et al*, 2003), and may contribute to both cognitive and behavioral symptoms. In contrast, although McN inhibited AChE expression and its activity, neither AG nor k252a counteracted these McN effects. Therefore, this phenomenon remains to be characterized.

In addition, we showed in the present study that *klotho* gene deficiency significantly deteriorated p-JAK2/p-STAT3 expression in hippocampal neurons, suggesting a critical interaction between the JAK2/STAT3 signaling axis and the cholinergic neurotransmitter system. This is the first evidence that JAK2/STAT3 inactivation in *klotho* mutant mice contributes to the pathogenesis of cholinergic dysfunction via dual mechanisms (ie, presynaptic down-regulation of cholinergic genes such as ChAT and postsynaptic desensitization of M1 mAChR; refer to Supplementary Figure S6).

The decreased NMDAR-dependent LTP observed in *klotho* mutant mice may result from either decreased M1 mAChR expression or from downregulation of the machinery, such as PKC coupling to M1 mAChR, since LTP induction is impaired in M1 receptor knockout mice (Anagnostaras *et al*, 2003). M1 receptors are coupled to the Gq/11 family of G-proteins and activate PKC-mediated signaling cascades (Lu *et al*, 1999). In contrast, NMDA receptors are regulated by PKC (MacDonald *et al*, 2001). Interestingly, M1 receptors co-localize with NR1 on the dendrites and soma of hippocampal pyramidal neurons, and activation of M1 receptors potentiates NMDA receptor currents (Marino *et al*, 1998). Similar to previous reports (Sun and Alkon, 2005), our data might link postsynaptic cholinergic dysfunction with other substrates (PKC β II and

NMDA receptor) known to play essential roles in cognitive processes and provide further insight into the mechanisms by which impairment of M1-mediated signaling may underlie the cognitive decline of *klotho* mutant mice. Interestingly, mice lacking PKC β suffer from deficits in both cued and contextual fear conditioning (Weeber *et al*, 2000), which may be in line with our current results.

We observed here that McN facilitated LTP induction by activation of the JAK2/STAT3 signaling axis in *klotho* mutant mice. This suggests that NMDAR activation could be a mechanism for LTP facilitation through activation of the JAK2/STAT3 signaling axis by M1 mAChR. Reduced JAK2 activation in *klotho* mutant mice may result in impaired NMDAR function. As a consequence of NMDAR dysfunction, Ca²⁺ influx and extrusion across the plasma membrane, cytosolic buffering, and uptake into organelles, may be altered in neurons. Evidence showing that calcium influx is mediated by JAKs/STATs (Orellana *et al*, 2005) supports this notion. However, Nicolas *et al* (2012) showed that a JAK2 inhibitor, AG490, does not block the induction of NMDAR-dependent LTP. These results seem to contradict our observation that AG490 treatment blocked McN-induced recovery of NMDAR-dependent LTP. It suggests that JAK2/STAT3 activation differentially regulates synaptic plasticity and/or would be affected under *klotho* gene dysfunction.

In general, compensative upregulation of M1 receptors expressed on postsynaptic sites could occur if presynaptic ACh levels and ChAT levels decreased concomitantly with increased AChE expression. Unexpectedly, in our study, hippocampal M1 mAChR was downregulated in *klotho* mutant mice. Interestingly, Nomura *et al* (1997) demonstrated that SAMP8 also show decreased hippocampal M1 mAChR-binding activity. These findings raise the possibility that it may be difficult to induce compensatory adaptation in response to cholinergic neuronal dysfunction in models of aging, although this idea remains to be fully elucidated.

mAChRs undergo desensitization or downregulation in response to prolonged muscarinic agonist stimulation, resulting in a decline in mAChR density (Svedberg *et al*, 2004). However, McN treatment in the present experimental condition led to upregulated M1 mAChR in the hippocampus of *klotho* mutant mice. Functionally selective muscarinic agonists (ie, McN, oxotremorine, pilocarpine, and AF102B) antagonize the stimulatory effect of full agonist (ie, carbachol) on adenylate cyclase in cells expressing muscarinic M1 (Gurwitz *et al*, 1994). Thus, many of the functionally selective muscarinic receptor agonists seem to fall within the category of partial agonists (Hoyer and Boddeke, 1993). Bursa *et al* (1995) demonstrated that treatment of corticostriatal neurons with McN upregulates M1 mAChR mRNA in primary rat cortical neuronal cultures, suggesting that McN as a partial agonist could exert an antagonistic effect on this receptor transcript. Moreover, rapid and transient changes of striatal M1 mAChR mRNA levels are observed after the single administration of muscarinic agents. For example, the full agonist carbachol dose-dependently upregulated M1 mAChR mRNA expression, while the antagonist trihexylphenidyl dose-dependently downregulated M1 mAChR mRNA expression (Chou *et al*, 1993), suggesting that

short-term treatment with muscarinic agents positively regulates M1 mAChR mRNA expression in the early stage after treatment, contrary to negative regulation in the long-term treatment. Our results may be, at least in part, in line with earlier findings (Bursa *et al*, 1995; Chou *et al*, 1993). In addition, we cannot rule out the possibility that McN possesses other unknown pharmacological mechanisms, which are independent of M1 mAChR-like effects (Wang *et al*, 2008).

ERK1/2 family members were originally identified by their responsiveness to growth factor receptor tyrosine kinases. However, they are also activated by many G-protein-coupled receptors (GPCRs). One family of GPCRs that activates ERK in several systems is the mAChR family (M1–M5), although limited information is available regarding how mAChR activates ERK in the brain or the receptor subtype responsible. One important function of ERK activation relates to the localization of nuclear downstream targets, such as CREB (Sgambato *et al*, 1998). Although each mAChR subtype can activate ERK in cell culture, M1 significantly activates ERK in CA1 pyramidal neurons of the hippocampus (Berkeley *et al*, 2001).

It was reported (Pepitoni *et al*, 1997) that regulatory elements of the M1 mAChR gene located at the promoter region lack a CRE sequence (TGACGTCA), but contain an activator protein-1 (AP-1) binding element sequence (TGACTCA). Since CREB and AP-1 can form a heterodimer to activate the transcription of target genes (Shaulian and Karin, 2001), CREB phosphorylation may be related to M1 receptor stimulation, as shown in our McN effects.

BDNF expression may be coupled to CREB-mediated transcription. Interestingly, we observed that CREB protein levels were decreased in neurons in the cerebral cortex and hippocampus of aged rats (Chung *et al*, 2002). Asanuma *et al* (1996) indicated that CREB DNA-binding activity is decreased in several different brain regions of aged rats compared with young adult rats. We cannot rule out the possibility that reduced BDNF levels in *klotho* mutant mice are due to impaired CREB-mediated transcription of the BDNF gene.

Klotho mutant mice have impaired visual recognition memory and associative fear memory at 7 weeks of age, while the animals have normal memory function at 6 weeks of age (Nagai *et al*, 2003). Performance in the light–dark shuttle box and elevated-plus maze box tasks is normal in *klotho* mutant mice (Nagai *et al*, 2003). There is also no apparent difference in the nociceptive threshold to electric foot shock in the conditioned-fear task (Nagai *et al*, 2003), suggesting that there are no major changes in exploratory activity, emotionality, or shock sensitivity in *klotho* mutant mice. Therefore, it is possible that the impaired performance of mutant mice is mainly due to memory. In addition, we speculate that neuropathologic changes secondary by *klotho* gene deficiency can negatively affect homeostatic regulation in the M1 mAChR system and related cognitive network (see Supplementary Discussion for more details).

In conclusion, we propose that underlying mechanisms (Supplementary Figures S6 and S7) accountings for memory impairment in *klotho* mutant mice are as follows: (1) The JAK2/STAT3 axis not only upregulated ChAT expression but also sensitized M1 mAChR and increased

NMDAR-mediated LTP in the hippocampus of wild-type mice. (2) *Klotho* gene deficiency resulted in inactivation of the JAK2/STAT3 axis, dysfunction of the cholinergic neuronal system and decreased NMDAR-mediated LTP. (3) Desensitized M1 mAChR impaired PKC β II-, ERK/CREB-, and BDNF-dependent signaling. (4) McN, an M1 agonist, attenuated these impairments induced by *klotho* gene deficiency. Thus, it is possible that the *klotho* gene may be essential for maintaining cholinergic neuronal function in aging organisms, and that an M1 agonist may be useful in treating cognitive impairment associated with aging-related disorders.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Brain Research Center from 21st Century Frontier Research Program (2012K001115) funded by the Ministry of Science and Technology, Republic of Korea. This work was, in part, supported by a Grant (#20120006020) from the Bio & Medical Technology Development Program through the National Research Foundation funded by the Ministry of Education, Science and Technology, Republic of Korea; by a Grant (#E00025) of the Korea-Japan Joint Research Program, National Research Foundation of Korea, Republic of Korea; and by grants from Ministry of Health Labour and Welfare (MHLW): Research on Risk of Chemical Substances, and Ministry of Education, Culture, Sports, Science and Technology (MEST): Academic Frontier Project. Zhengyi Li and Jae-Hyung Bach were supported by BK 21 program. The English in this manuscript has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: <http://www.textcheck.com/certificate/x6dfje>

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)