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Research Article

RESEARCH ARTICLE

# Melatonin Attenuates Memory Impairment Induced by Klotho Gene Deficiency Via Interactive Signaling Between MT2 Receptor, ERK, and Nrf2-Related Antioxidant Potential

Eun-Joo Shin*, PhD; Yoon Hee Chung*, PhD; Hoang-Lan Thi Le, MSc; Ji Hoon Jeong, PhD; Duy-Khanh Dang, BSc; Yunsung Nam, PhD; Myung Bok Wie, DVM, PhD; Seung-Yeol Nah, DVM, PhD; Yo-Ichi Nabeshima, MD, PhD; Toshitaka Nabeshima, PhD; and Hyoung-Chun Kim, PhD

*These authors contributed equally to this work.

Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200–701, Republic of Korea (Drs Shin, Le, Dang, Nam, and Kim); Department of Anatomy, College of Medicine, Chung-Ang University, Seoul 156–756, Republic of Korea (Dr Chung); Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156–756, Republic of Korea (Dr Jeong); School of Veterinary Medicine, Kangwon National University, Chunchon 200–701, Republic of Korea (Dr Wie); Ginseng Research Laboratory, Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul 143–701, Republic of Korea (Dr Nah); Laboratory of Molecular Science, Institute of Biomedical Research and Innovation, Foundation for Biomedical Research and Innovation, Kobe 650-0047, Japan (Dr Y-I Nabeshima); Department of Regional Pharmaceutical Care and Science, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468–8503, Japan (Dr T Nabeshima)

Correspondence: Hyoung-Chun Kim, PhD, Neuropsychopharmacology and Toxicology Program, College of Pharmacy, Kangwon National University, Chunchon 200–701, Republic of Korea (kimhc@kangwon.ac.kr).



## Abstract

**Background:** We demonstrated that oxidative stress plays a crucial role in cognitive impairment in *klotho* mutant mice, a genetic model of aging. Since down-regulation of melatonin due to aging is well documented, we used this genetic model to determine whether the antioxidant property of melatonin affects memory impairment.
**Methods:** First, we examined the effects of melatonin on hippocampal oxidative parameters and the glutathione/oxidized glutathione (GSH/GSSG) ratio and memory dysfunction of *klotho* mutant mice. Second, we investigated whether a specific melatonin receptor is involved in the melatonin-mediated pharmacological response by application with melatonin receptor antagonists. Third, we examined phospho-extracellular-signal-regulated kinase (ERK) expression, nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation, Nrf2 DNA binding activity, and glutamate-cysteine ligase (GCL)

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mRNA expression. Finally, we examined effects of the ERK inhibitor SL327 in response to antioxidant efficacy and memory enhancement mediated by melatonin.
**Results:** Treatment with melatonin resulted in significant attenuations of oxidative damage, a decrease in the GSH/GSSG ratio, and a significant amelioration of memory impairment in this aging model. These effects of melatonin were significantly counteracted by the selective MT2 receptor antagonist 4-P-PDOT. Importantly, 4-P-PDOT or SL327 also counteracted melatonin-mediated attenuation in response to the decreases in phospho-ERK expression, Nrf2 nuclear translocation, Nrf2 DNA-binding activity, and GCL mRNA expression in the hippocampi of *klotho* mutant mice. SL327 also counteracted the up-regulation of the GSH/GSSG ratio and the memory enhancement mediated by melatonin in *klotho* mutant mice.
**Conclusions:** Melatonin attenuates oxidative stress and the associated memory impairment induced by *klotho* deficiency via signaling interaction between the MT2 receptor and ERK- and Nrf2-related antioxidant potential.

**Keywords:** hippocampus, *Klotho* mutant mice, memory, melatonin MT2 receptor/ERK/Nrf2, oxidative stress



## Introduction

*Klotho* mutant mice, which are defective in *klotho* expression even at 4–5 weeks of age, develop multiple age-related syndromes, including growth retardation, cognition impairment, hearing disturbances, and motor neuron degeneration, and die prematurely at ~2 months of age (Kuro-o, 2010). In contrast, 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 lifespan (Kurosu et al., 2005). Thus, *klotho* may function as an aging suppressor gene that extends the lifespan when overexpressed and accelerates aging when disrupted (Kuro-o, 2008). Although *klotho* mutant mice are considered to be a novel animal model of accelerated human aging, these mice do not exhibit certain phenotypes usually observed in older human subjects, such as brain atrophy with deposition of amyloid or senile plaques (Kuro-o et al., 1997; Nagai et al., 2003; Anamizu et al., 2005).

Our group was the first to report that oxidative stress plays a crucial role in the aging-associated cognition 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 up-regulated, in the hippocampi of *klotho* mutant mice (Nagai et al., 2003). A potent antioxidant, α-tocopherol, prevented cognitive impairment and lipid peroxide accumulation and decreased the number of apoptotic cells in *klotho* mutant mice, suggesting that the Klotho protein may be involved in the regulation of antioxidative defenses. Our recent study suggested that inactivation of the JAK2/STAT3 signaling axis and M1 muscarinic cholinergic receptor (M1 mAChR) down-regulation plays a mechanistic role in cognitive impairment in *klotho* mutant mice (Park et al., 2013). Previous studies demonstrated that Klotho-induced activation of the Forkhead box class O (FoxO) depended primarily on its ability to inhibit the insulin/IGF-1/PI3K/Akt signaling cascade (Yamamoto et al., 2005), and Klotho increased the resistance to oxidative stress by a mechanism associated with nuclear factor erythroid 2-related factor 2 (Nrf2) activation *in vivo* (Hsieh et al., 2010).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone synthesized mainly in the pineal gland and released in blood and cerebrospinal fluid, which plays regulatory roles in seasonal and circadian rhythms (Hardeland, 2009; Zawilska et al., 2009). In the central nervous system (CNS), melatonin exerts neuroprotective effects due to its direct free-radical-scavenging properties (Tan et al., 1993; Reiter et al., 2001; Baydas et al., 2003) and indirect antioxidant activities by stimulating major antioxidant enzymes (Rodriguez et al., 2004). G-protein–coupled melatonin MT1 and MT2 receptors are expressed in the CNS (Imbesi et al., 2006) and multiple signaling systems are linked to melatonin receptors, including the extracellular-signal-regulated kinase (ERK) pathway, a member of the mitogen-activated protein kinases (MAPKs; Cui et al., 2008). Recent reports have also shown that melatonin activates the Nrf2-antioxidant responsive element (Nrf2-ARE) pathway in experimental diabetic neuropathy (Negi et al., 2011), a subarachnoid hemorrhage model (Wang et al., 2012), and ischemic stroke (Parada et al., 2014).

The substantial reduction in melatonin that occurs with aging may be related to aging itself and to age-related neurodegenerative conditions (Reiter et al., 1980, 1981, 1997; Karasek and Reiter, 2002). However, the role of melatonin in the oxidative burden and memory impairment in *klotho* mutant mice, a specific aging model, is unclear. Therefore, we investigated whether a specific melatonin receptor is involved in the melatonin-mediated pharmacological response to oxidative stress and memory impairment in *klotho* mutant mice.

It is recognized that C3H/HeJ mice are regarded as a mouse model of melatonin proficiency (Torres-Frafan et al., 2006) and that *klotho* mutant mice originated from a C3H/HeJ background (Nagai et al., 2003). Thus, we examined whether the circadian cycle affects memory dysfunction mediated by genetic inhibition of *klotho*. Because we found here that the circadian cycle does not significantly affect memory function in either C3H/HeJ (wild-type) or *klotho* mutant mice (Supplementary Figure S1), we have focused on the light cycle for further experiment in the present study.

We proposed that melatonin attenuates oxidative stress and the associated memory impairment in *klotho* mutant mice via the melatonin MT2 receptor by stimulating ERK-mediated Nrf2-dependent antioxidant potentials.

## Method

### 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 guidelines for the care and use of laboratory animals. Mice were maintained under a 12h light-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 (C3H/HeJ; Kuro-o et al., 1997; Nagai et al., 2003). Prior to weaning, tail specimens were collected from each animal, and DNA was extracted to genotype wild-type and *klotho*-mutant mice. More details on the gene characterization are described in the Supplementary Materials.

## Drug Treatment

Melatonin (10 mg/mL in 5% dimethyl sulfoxide (DMSO); Sigma-Aldrich), luzindole (a non-specific MT1/MT2 receptor antagonist; 1 mg/mL in 20% DMSO; Sigma-Aldrich), 4-P-PDOT (a specific MT2 receptor antagonist; 1 mg/mL in 20% DMSO; Tocris Bioscience), and prazosin hydrochloride (an MT3 receptor antagonist; 1 mg/mL in 20% DMSO; Sigma-Aldrich) were dissolved in DMSO and then diluted in sterile saline. SL327 (an ERK inhibitor; Sigma-Aldrich) was dissolved in DMSO. All reagents were prepared immediately before use.

In our earlier study (Nagai et al., 2003), it was observed that α-tocopherol treatment (150 mg/kg, per os) significantly attenuates oxidative stress and memory impairments in *klotho* mutant mice. However, α-tocopherol treatment did not significantly alter body weight gain and life-span in *klotho* mutant mice. At that time, α-tocopherol was administrated once a day for 18 days from postnatal day (PND) 35. After that, *klotho* mutant mice begin to show growth retardation, gradually became inactive and marasmic, and died prematurely (Kuro-o et al., 1997; Nagai et al., 2003; Park et al., 2013). In order to achieve maximal efficacy of melatonin in the present study, administration of melatonin (10, 20, or 30 mg/kg, i.p.) was performed twice a day for 17 days from PND 35 to 51. The dosing regimen of melatonin was based on previous studies (Yamamoto and Mohanan, 2003; Yahyavi-Firouz-Abade et al., 2007) and our pilot study (Dang et al., 2014).

Mouse body weights and survival rates were recorded throughout the experimental period (Supplementary Figure S2). On the days of the novel object recognition test (NORT; PND 52 and 53) or passive avoidance test (PAT; PND 54 and 55), mice received melatonin 45 min prior to the behavioral test. Luzindole (0.5 or 1.0 mg/kg, i.v.; Domínguez-López et al., 2012; Fink et al., 2014; Dang et al., 2014), 4-P-PDOT (0.5 or 1.0 mg/kg, i.v.; Domínguez-López et al., 2012; Fink et al., 2014; Dang et al., 2014), or prazosin (0.5 or 1.0 mg/kg, i.v.; Yu and Koss, 2002) was injected 5 min before the memory trial. SL327 (5 or 10 mg/kg, i.p.; Selcher et al., 1999) was injected 30 min before the memory trial.

Mice were sacrificed 30 min after the PAT retention trial on PND 55 for neurochemical assays, Western blot analyses, reverse transcription-PCR (RT-PCR), and Nrf2 DNA-binding activity assays.

## Novel Object Recognition Test and Passive Avoidance Test

The novel object recognition test and passive-avoidance test were performed as described previously (Jin et al., 2009; Hwang et al., 2012). The detailed procedure is described in the Supplementary Materials.

## Determination of Malondialdehyde

The amount of lipid peroxidation in the hippocampus was determined by measuring the level of thiobarbituric acid-reactive substance in homogenates and is expressed in terms of malondialdehyde (MDA) content. The MDA level was measured using the HPLC-UV/VIS detection system (model LC-20AT and SPD-20A, Shimadzu) according to the method of Richard et al. (1992) with a slight modification (Shin et al., 2012; Tran et al., 2012). Additional details on the determination of malondialdehyde are provided in the Supplementary Materials.

## Determination of Protein Carbonyl

The extent of protein oxidation was assessed by measuring the content of protein carbonyl groups, which was determined spectrophotometrically with the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure (Shin et al., 2012; Tran et al., 2012) as described by Oliver et al. (1987). The results are expressed as nmol of DNPH incorporated/mg protein based on the extinction coefficient for aliphatic hydrazones of 21 $mM^{-1}$ $cm^{-1}$. Protein was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce).

## Synaptosomal Preparation

The synaptosomal fraction was prepared as described previously (Eyerman and Ymamoto, 2007; Shin et al., 2012). Hippocampal tissue was homogenized in 10 volumes of ice-cold 0.32 mol/L sucrose and centrifuged at 800 × *g* for 12 min at 4°C. The resulting supernatant was centrifuged at 22 000 × *g* for 20 min at 4°C to obtain pelleted synaptosomes. Hippocampal synaptosomes were resuspended in phosphate-buffered saline for measuring synaptosomal reactive oxygen species (ROS). Protein concentration of the synaptosomal fraction was determined using the BCA protein assay kit (Pierce).

## Determination of Synaptosomal ROS

Determination of the formation of ROS was performed according to the method described by Lebel and Bondy (1990). Hippocampal synaptosomes were incubated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) for 15 min at 37°C. The excess unbound probe was removed by centrifugation at 12 500 × g for 10 min. The fluorescent intensity due to the ROS was measured at an excitation wavelength of 488 nm and emission wavelength of 528 nm.

## Determination of GSH and GSSG by HPLC

Glutathione (GSH) and oxidized glutathione (GSSG) were immediately measured from dissected hippocampal tissues as described previously (Reed et al., 1980; Tran et al., 2012) using the HPLC-UV/VIS detection system (model LC-20AT and SPD-20A, Shimadzu). The detailed procedure is described in the Supplementary Materials.

## Western Blot Analysis

Hippocampi were dissected immediately after decapitation and frozen in liquid nitrogen. Hippocampal tissues were homogenized in lysis buffer, containing 200 mM Tris HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM ethylenediaminetetraacetic acid, 10% glycerol, 1X phosphatase inhibitor cocktail I (Sigma-Aldrich), and 1 × protease inhibitor cocktail (Sigma-Aldrich). Lysate was centrifuged at 12 000 x g for 30 min and supernatant fraction was used for Western blot analysis as described previously (Tran et al., 2012; Park et al., 2013). Additional details on the procedure and antibody are provided in the Supplementary Materials.

## Analysis of Nuclear Translocation of Nrf2

Nuclear and cytosolic fractions of hippocampal lysates were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, hippocampal tissues were homogenized in the provided cytoplasmic extraction reagent using a Dounce homogenizer. The homogenate was centrifuged at 16 000 × *g* for 5 min, and the supernatant (cytosolic) fraction was immediately transferred to a pre-chilled tube. The pelleted fraction was suspended in the provided nuclear extraction reagent (pre-chilled) and the resulting suspension was centrifuged at 16 000 × *g* for 10 min. The supernatant (nuclear) fraction was immediately transferred to a pre-chilled tube. The cytosolic and nuclear fractions were subjected to 8% SDS-PAGE (20–50 µg protein/lane), and the separated proteins were transferred onto a polyvinylidene difluoride membrane.

To detect Nrf2, the membrane was immunoblotted with an anti-Nrf2 antibody (1:5 000; Epitomics, Inc.). An anti-histone H4 antibody (1:1 000; Cell Signaling Technology, Inc.) was used as an internal loading control for the nuclear fraction, and an anti-β-actin antibody (1:5 000, Sigma–Aldrich) was used as an internal loading control for the cytosolic fraction (Tran et al., 2012).

### Nrf2 DNA-Binding Activity

The nuclear fraction was extracted using a nuclear extraction kit (#40410; Active Motif) according to the manufacturer's instructions. The detailed procedure of nuclear extraction is described in the Supplementary Materials.

Nrf2 DNA-binding activity was measured using the TransAM Nrf2 transcription factor ELISA kit (Active motif; Narasimhan et al., 2011) according to the manufacturer's instructions. Briefly, 10 µg of each nuclear protein extract were added to wells coated with oligonucleotides containing an ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3'). The plate was incubated for 1 h at room temperature and then washed with the 1 × wash buffer provided in the kit. After incubation with the primary antibody against Nrf2 for 1 h at room temperature, the plate was incubated with a horseradish peroxidase–conjugated secondary anti-rabbit IgG for 1 h. The colorimetric reaction was initiated using the developing solution provided in the kit. The absorbance at 450 nm was measured using a microplate reader (Spectra Max Plus 384, Molecular Devices).

### RT-PCR

Expression of the modifier and catalytic subunits of GCL (GCLm and GCLc, respectively) was assessed using semi-quantitative RT–PCR to analyze the mRNA level. Total RNA was isolated from hippocampal tissues using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were carried out using the RNA to cDNA EcoDry Premix (Clontech) with a 1 h incubation at 42°C. Additional details on the primer sequences and PCR amplification conditions are provided in the Supplementary Materials. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light. The quantitative analysis of mRNA was performed using PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat; Tran et al., 2012).

### Statistical Analyses

Data were analyzed using IBM SPSS version 21.0 (IBM). Two-way analyses of variance (ANOVAs) were performed for the effect of *klotho* mutation and melatonin. One-way ANOVAs were employed for the effect of melatonin receptor antagonists (or SL327) and post hoc Fisher's least significant difference pairwise comparisons tests were performed. A value of $p < 0.05$ was taken to indicate statistical significance.

## Results

### Effect of Melatonin Receptor Antagonist on Oxidative Stress and Imbalance

In our previous publication we suggested that oxidative stress plays a crucial role in the memory impairment of *klotho* mutant mice (Nagai et al., 2003). Thus, we examined whether melatonin attenuates oxidative stress in the hippocampi of *klotho* mutant mice and identified the melatonin receptors that are involved in the melatonin-mediated attenuation. Since the antioxidant effect produced by high doses of melatonin (30 mg/kg, i.p.) is comparable to that produced by medium doses of melatonin (20 mg/kg, i.p.) in this study, we employed mild doses of melatonin for further study (Figures 1 and 2).

Two-way ANOVAs showed significant effects of *klotho* mutation and melatonin (the level of synaptosomal ROS, malondialdehyde, and protein carbonyl) and a significant interaction between *klotho* mutation and melatonin (synaptosomal ROS formation and protein carbonyl; Supplementary Table S1). A post hoc test revealed that melatonin (20 or 30 mg/kg) significantly attenuated the increases in the oxidative stress markers (synaptosomal ROS and protein carbonyl: $p < 0.01$; malondialdehyde: $p < 0.05$; Figure 1A–C). One-way ANOVA revealed a significant effect of melatonin receptor antagonists on the level of oxidative stress markers in the hippocampi of *klotho* mutant mice treated with melatonin (20 mg/kg), and the post hoc test indicated that 4-P-PDOT (1.0 mg/kg), a selective MT2 receptor antagonist, significantly reversed ($p < 0.01$) antioxidant effects mediated by melatonin. Luzindole (1.0 mg/kg), a non-selective melatonin MT1/MT2 receptor antagonist, also appeared to counteract the antioxidant effect of melatonin in the hippocampi of *klotho* mutant mice (synaptosomal ROS: $p = 0.145$; malondialdehyde: $p = 0.146$; protein carbonyl: $p < 0.05$). However, prazosin, an MT3 receptor antagonist, did not significantly affect the levels of oxidative stress markers in the hippocampi of *klotho* mutant mice in the presence of melatonin (Figure 1D–F, Supplementary Table S1).

Melatonin consistently and significantly attenuated the homeostatic imbalance of the endogenous GSH system (i.e. decreases in the GSH level and GSH/GSSG ratio) in the hippocampi of *klotho* mutant mice. Two-way ANOVA showed significant effects of *klotho* mutation and melatonin (the level of total GSH, GSH, and GSSG and the GSH/GSSG ratio), and a significant interaction between *klotho* mutation and melatonin (GSSG level; Supplementary Table S2). The post hoc test revealed that melatonin (20 or 30 mg/kg) significantly attenuated the changes in the levels of total GSH ($p < 0.05$), GSH ($p < 0.05$), and GSSG ($p < 0.01$) and the GSH/GSSG ratio ($p < 0.01$) in the hippocampi of *klotho* mutant mice (Figure 2A–D). One-way ANOVA revealed significant effects of melatonin receptor antagonists on the total GSH and GSH levels and the GSH/GSSG ratio in the hippocampi of *klotho* mutant mice treated with melatonin (20 mg/kg). The post hoc test indicated that 4-P-PDOT (1.0 mg/kg) significantly reversed the changes in the levels of these GSH-related parameters (total GSH and GSH: $p < 0.05$; GSH/GSSG ratio: $p < 0.01$). Luzindole (1.0 mg/kg) also appeared to counteract the effect of melatonin on the level of GSH-related parameters in the hippocampi of *klotho* mutant mice (total glutathione: $p = 0.073$; GSH: $p = 0.195$; GSSG: $p = 0.066$; GSH/GSSG ratio: $p < 0.05$; Figure 2E–H, Supplementary Table S2).

In addition, the effects of melatonin antagonists on cell viability and oxidative change in the SH-SY5Y and PC12 cell lines in the presence of melatonin are shown in Supplementary Figures S3 and S4.

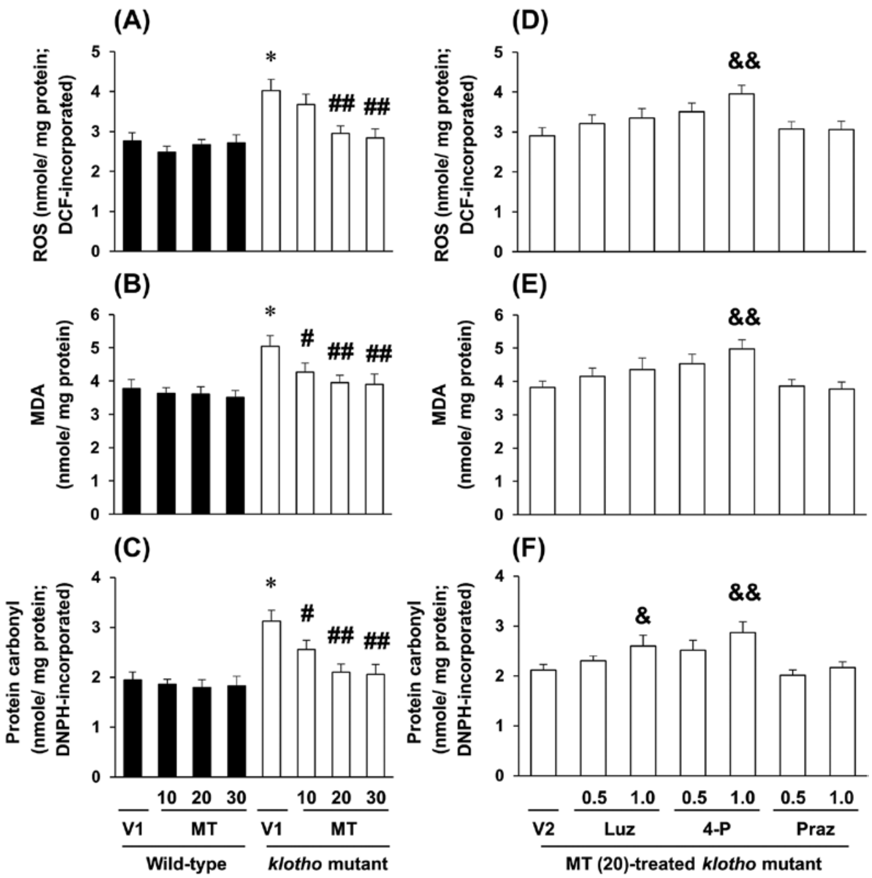


**Figure 1.** Effect of melatonin receptor antagonists on the melatonin-mediated attenuation of the formation of synaptosomal reactive oxygen species (ROS; A and D), lipid peroxidation (as determined by malondialdehyde; B and E), and protein oxidation (as determined by protein carbonyl level; C and F) in the hippocampi of *klotho* mutant mice. 4-P, 4-P-PDOT, a selective MT2 receptor antagonist (0.5 or 1.0 mg/kg, i.v.); DCF, 2',7'-dichlorofluorescein; DNPH, 2,4-dinitrophenylhydrazine; Luz, luzindole (0.5 or 1.0 mg/kg, i.v.); MDA, malondialdehyde; MT, melatonin (10, 20, or 30 mg/kg, i.p.); Praz, prazosin (0.5 or 1.0 mg/kg, i.v.); V1, Vehicle 1 (5% dimethyl sulfoxide [DMSO] in saline, the solvent for melatonin); V2, Vehicle 2 (20% DMSO in saline, the solvent for melatonin receptor antagonists). Each value is the mean ± standard error of the mean of 8 animals. $^{*}p < 0.01$ vs. wild-type mice treated with V1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. *klotho* mutant mice treated with V1; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. *klotho* mutant mice treated with V2 + MT (20; two-way analysis of variance (ANOVA; A–C) or one-way ANOVA (D–F) followed by post hoc Fisher's least significant difference pairwise comparisons test).

## Effect of Melatonin Receptor Antagonist on Learning and Memory Functions

Because oxidative stress and imbalances in the GSH system were observed in the hippocampi of *klotho* mutant mice in this study, we employed two behavioral tests, the NORT and PAT, to evaluate hippocampus-dependent memory function (Zola-Morgan et al., 1986; Impey et al., 1998; Pan et al., 2013). As the memory-enhancing effect induced by high doses of melatonin (30 mg/kg, i.p.) appeared to be comparable to that by mild doses of melatonin (20 mg/kg, i.p.), we have applied mild doses of melatonin for further study (Figure 3).

Two-way ANOVA showed significant effects of *klotho* mutation and melatonin, and a significant interaction between *klotho* mutation and melatonin, in the NORT and PAT (Supplementary Table S3). The post hoc test indicated that melatonin (20 or 30 mg/kg) significantly attenuated the memory impairments of *klotho* mutant mice in the NORT and PAT ($p < 0.01$; Figure 3A–B). One-way ANOVA revealed a significant effect of melatonin receptor antagonists on the performance of *klotho* mutant mice treated with melatonin (20 mg/kg) in the NORT and PAT (Supplementary Table S3), and the post hoc test indicated that 4-P-PDOT (1.0 mg/kg) significantly reversed the memory function of melatonin-treated *klotho* mutant mice (NORT: $p < 0.01$; PAT: $p < 0.05$). However, luzindole (1.0 mg/kg) did not significantly alter the memory-enhancing effects of melatonin in *klotho* mutant mice (NORT: $p = 0.450$; PAT: $p = 0.216$; Figure 3C–D).

## Antagonism by 4-P-PDOT or SL327 on ERK Phosphorylation in the Hippocampus

Subsequently, the effect of melatonin on the hippocampal changes in ERK phosphorylation of *klotho* mutant mice was examined because the phospho-ERK–related signaling cascades are important for the hippocampus-dependent memory formation (Adams and Sweat, 2002). Reportedly, phospho-ERK is an important signaling molecule modulating the receptor-mediated actions of melatonin in the CNS (Kilic et al., 2005; Imbesi et al., 2008). Because the attenuation in ERK phosphorylation induced by high doses of melatonin (30 mg/kg, i.p.) was comparable to that by mild doses of melatonin (20 mg/kg, i.p.), we have applied mild doses of melatonin for further study (Figure 4).

Two-way ANOVA showed significant effects of *klotho* mutation and melatonin and a significant interaction between *klotho* mutation and melatonin (Supplementary Table S3). The post hoc test revealed that melatonin (20 or 30 mg/kg) significantly attenuated ($p < 0.01$) the decrease in ERK phosphorylation in the hippocampi of *klotho* mutant mice (Figure 4A). One-way ANOVA indicated a significant effect of 4-P-PDOT or SL327, an ERK inhibitor, on ERK phosphorylation in the hippocampi of *klotho*

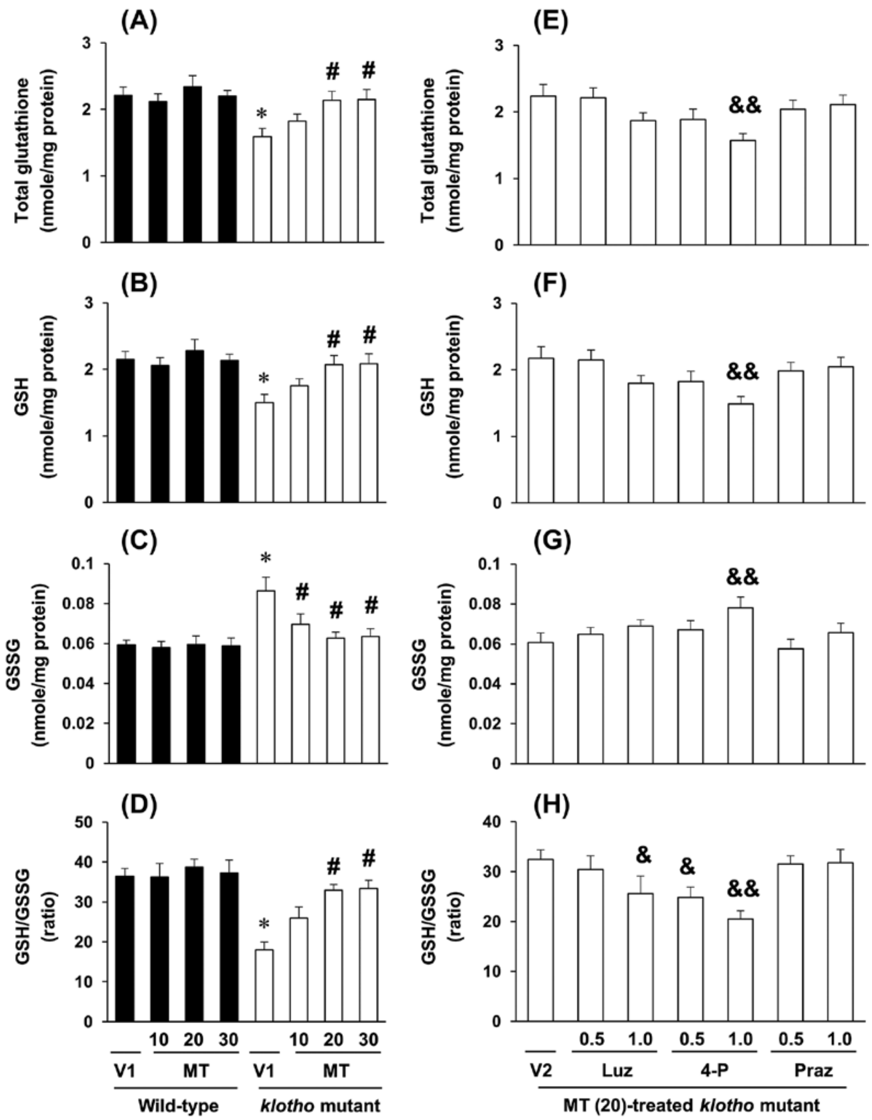


**Figure 2.** Effect of melatonin receptor antagonists on the melatonin-mediated attenuation of the changes in total glutathione (A and E), reduced glutathione (GSH; B and F), oxidized glutathione (GSSG; C and G), and GSH/GSSG ratio (D and H) in the hippocampi of *klotho* mutant mice. 4-P, 4-P-PDOT, a selective MT2 receptor antagonist (0.5 or 1.0mg/kg, i.v.); Luz, luzindole (0.5 or 1.0mg/kg, i.v.); MT, melatonin (10, 20, or 30mg/kg, i.p.); Praz, prazosin (0.5 or 1.0mg/kg, i.v.); V1, Vehicle 1 (5% dimethyl sulfoxide [DMSO] in saline, the solvent for melatonin); V2, Vehicle 2 (20% DMSO in saline, the solvent for melatonin receptor antagonists). Each value is the mean ± standard error of the mean of 6 animals. $^{*}p < 0.01$ vs. wild-type mice treated with V1; $^{\#}p < 0.01$ vs. *klotho* mutant mice treated with V1; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. *klotho* mutant mice treated with V2 + MT (20; two-way analysis of variance (ANOVA; A–D) or one-way ANOVA (E–H) followed by post hoc Fisher's least significant difference pairwise comparisons test).

mutant mice treated with melatonin (20mg/kg); the post hoc test showed that this effect on ERK phosphorylation was significantly reversed by 4-P-PDOT ($p < 0.01$ at 1.0mg/kg) or SL327 ($p < 0.01$ at 5 and 10mg/kg; Figure 4B, Supplementary Table S3).

## Antagonism by SL327 on Hippocampal Oxidative Stress

Next, we examined involvement of phospho-ERK in the MT2 receptor-mediated pharmacological effect of melatonin on the hippocampal oxidative stress of *klotho* mutant mice. One-way ANOVA showed a significant effect of SL327 on the levels of oxidative stress markers in the hippocampi of *klotho* mutant mice treated with melatonin (20mg/kg). The post hoc test revealed that SL327 (10mg/kg) significantly counteracted ($p < 0.05$) the effects on the synaptosomal ROS ($p < 0.05$), MDA ($p < 0.05$), and protein carbonyl ($p < 0.01$) levels in the hippocampi of melatonin-treated *klotho* mutant mice (Figure 5, Supplementary Table S4).

In addition, the effects of SL327 on cell viability and oxidative change in the SH-SY5Y and PC12 cell lines in the presence of melatonin are shown in Supplementary Figures S4 and S5.

## Antagonism by 4-P-PDOT or SL327 on Nuclear Translocation, DNA Binding Activity, and mRNA Expression

As the homeostatic imbalance in endogenous GSH system in the hippocampi of *klotho* mutant mice was significantly attenuated by melatonin, we examined the effect of melatonin on the nuclear translocation and DNA-binding activity of Nrf2. Nrf2 mediates the transcriptional regulation of genes

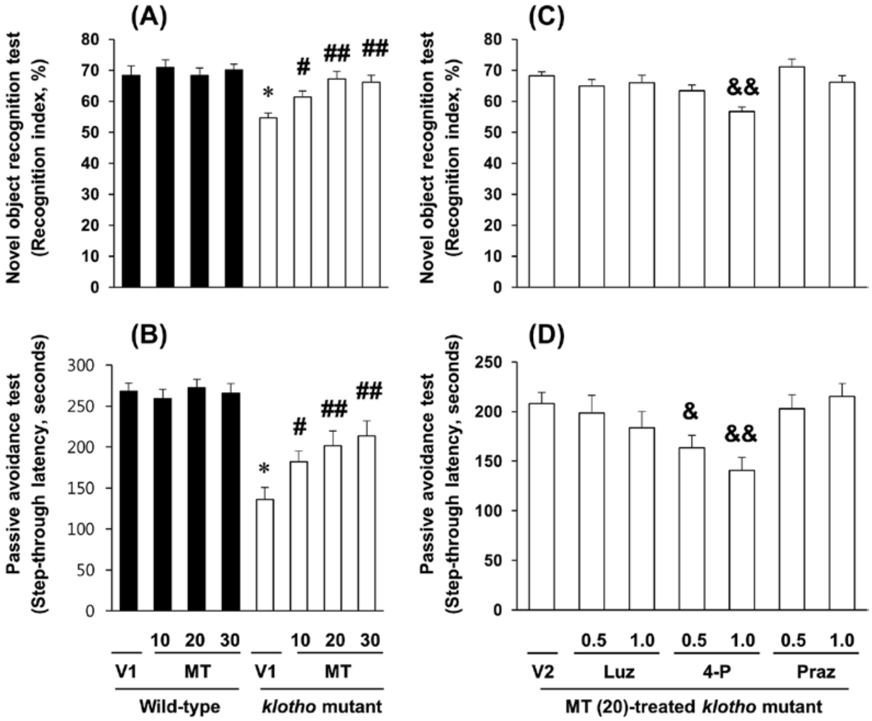


**Figure 3.** Effect of melatonin receptor antagonists on the melatonin-mediated attenuation of memory impairment as evaluated by the novel object recognition test (A and C), and passive avoidance test (B and D) in *klotho* mutant mice. 4-P, 4-P-PDOT, a selective MT2 receptor antagonist (0.5 or 1.0 mg/kg, i.v.); Luz, luzindole (0.5 or 1.0 mg/kg, i.v.); MT, melatonin (10, 20, or 30 mg/kg, i.p.); Praz, prazosin (0.5 or 1.0 mg/kg, i.v.); V1, Vehicle 1 (5% dimethyl sulfoxide [DMSO] in saline, the solvent for melatonin); V2, Vehicle 2 (20% DMSO in saline, the solvent for melatonin receptor antagonists). Each value is the mean ± standard error of the mean of 10 animals. $^{*}p < 0.01$ vs. wild-type mice treated with V1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. *klotho* mutant mice treated with V1; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. *klotho* mutant mice treated with V2 + MT (20; two-way analysis of variance (ANOVA; A and B) or one-way ANOVA (C and D) followed by post hoc Fisher's least significant difference pairwise comparisons test).

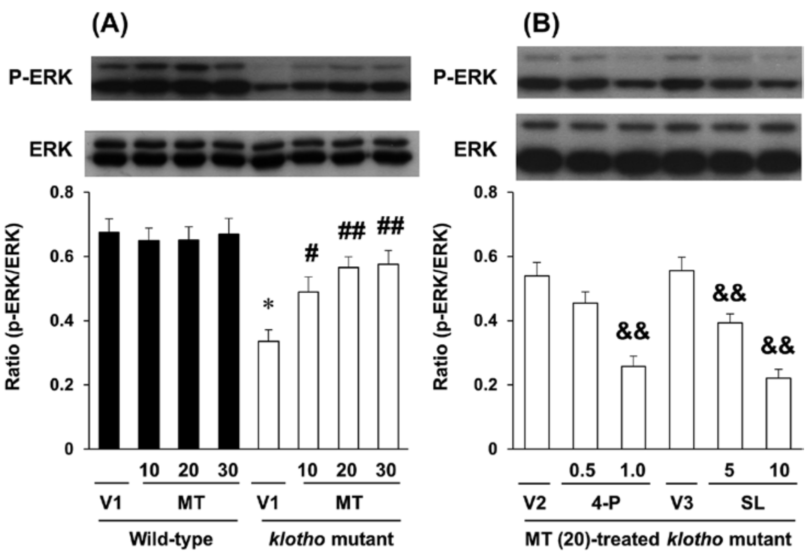


**Figure 4.** Effect of 4-P-PDOT, an MT2 receptor antagonist, or SL327, an extracellular-signal-regulated kinase (ERK) inhibitor on the melatonin-mediated attenuation of the decrease in ERK phosphorylation in the hippocampi of *klotho* mutant mice. 4-P, 4-P-PDOT (0.5 or 1.0 mg/kg, i.v.); MT, melatonin (10, 20, or 30 mg/kg, i.p.); p-ERK, phospho-ERK; SL, SL327 (5 or 10 mg/kg, i.p.); V1, Vehicle 1 (5% dimethyl sulfoxide [DMSO] in saline, the solvent for melatonin); V2, Vehicle 2 (20% DMSO in saline, the solvent for 4-P-PDOT); V3, Vehicle 3 (100% DMSO, the solvent for SL327). Each value is the mean ± standard error of the mean of 6 animals. $^{*}p < 0.01$ vs. wild-type mice treated with V1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. *klotho* mutant mice treated with V1; $^{\&}p < 0.01$ vs. *klotho* mutant mice treated with corresponding V2 or V3 + MT (20; two-way analysis of variance (ANOVA; A) or one-way ANOVA (B) followed by post hoc Fisher's least significant difference pairwise comparisons test).

encoding various antioxidant enzymes and phase 2 detoxification enzymes—including GCL, the rate-limiting enzyme in GSH biosynthesis—by binding to the *cis*-acting antioxidant response element (ARE; Wild et al., 1999). Since positive modulation in Nrf-2 and GCL levels by high doses of melatonin (30 mg/kg, i.p.) was comparable to that by mild doses of melatonin (20 mg/kg, i.p.), we have applied mild doses of melatonin for further study (Figure 6).

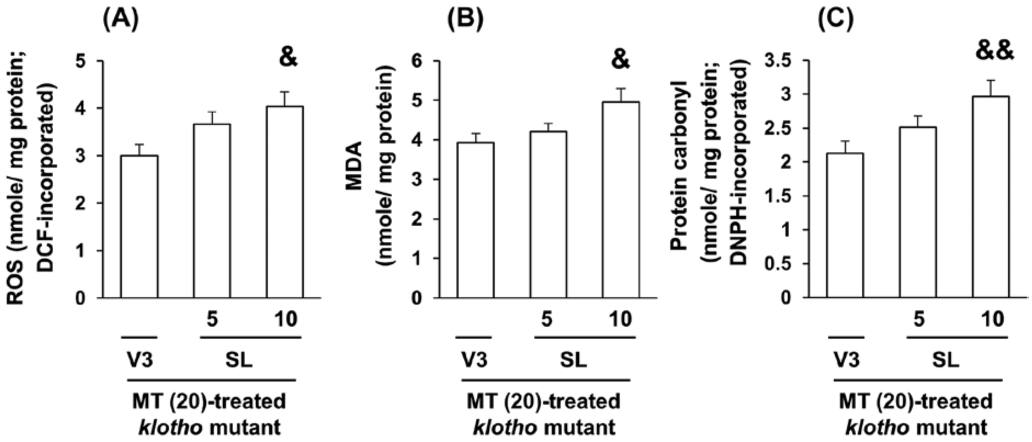


**Figure 5.** Effect of SL327, an extracellular-signal-regulated kinase inhibitor on the melatonin-mediated attenuation of the formations in the synaptosomal reactive oxygen species (ROS; A), lipid peroxidation (as determined by malondialdehyde [MDA]; B), and protein oxidation (as determined by protein carbonyl; C) in the hippocampi of *klotho* mutant mice. DNPH, 2,4-dinitrophenylhydrazine; MT, melatonin (20 mg/kg, i.p.); SL, SL327 (5 or 10 mg/kg, i.p.); V3, Vehicle 3 (100% dimethyl sulfoxide, the solvent for SL327). Each value is the mean ± standard error of the mean of 6 animals. $^{\&}p < 0.05$, $^{\&\&}p < 0.05$ vs. *klotho* mutant mice treated with V3 + MT (20; one-way analysis of variance followed by post hoc Fisher's least significant difference pairwise comparisons test).

Two-way ANOVA showed significant effects of *klotho* mutation and melatonin on the nuclear Nrf2 protein level and Nrf2 DNA-binding activity (Supplementary Table S5). The post hoc test revealed that melatonin (20 or 30 mg/kg) significantly attenuated the decrease in nuclear protein level and DNA-binding activity of Nrf2 in the hippocampi of *klotho* mutant mice ($p < 0.01$; Figure 6A–C). One-way ANOVA indicated a significant effect of 4-P-PDOT or SL327 on the nuclear translocation and DNA-binding activity of Nrf2 in the hippocampi of *klotho* mutant mice treated with melatonin (20 mg/kg); the post hoc test confirmed that these effects were significantly reversed ($p < 0.01$) by 4-P-PDOT (1.0 mg/kg) and SL327 (10 mg/kg; Figure 6, Supplementary Table S5).

Consistently, two-way ANOVA showed significant effects of *klotho* mutation and melatonin (GCLc and GCLm) and a significant interaction between *klotho* mutation and melatonin (GCLc; Supplementary Table S5). The post hoc test indicated that melatonin (20 or 30 mg/kg) significantly attenuated the decrease in hippocampal mRNA expression of GCLc ($p < 0.01$) and GCLm ($p < 0.05$) in *klotho* mutant mice (Figure 6D–E). One-way ANOVA indicated significant effects of 4-P-PDOT and SL327 on the mRNA expression of GCLc and GCLm in the hippocampi of *klotho* mutant mice treated with melatonin (20 mg/kg); the post hoc test confirmed the effect of 4-P-PDOT (GCLc: $p < 0.05$ at 0.5 mg/kg, $p < 0.01$ at 1.0 mg/kg; GCLm: $p < 0.01$ at 1.0 mg/kg) or SL327 (GCLc: $p < 0.01$ at 5 and 10 mg/kg; GCLm: $p < 0.01$ at 10 mg/kg; Figure 6, Supplementary Table S5).

### Antagonism by SL327 on Endogenous Glutathione System

Subsequently, we examined involvement of phospho-ERK in the MT2 receptor-mediated pharmacological effect of melatonin on the decreases in GSH level and GSH/GSSG ratio in the hippocampi of *klotho* mutant mice. One-way ANOVA indicated a significant effect of SL327 on the total GSH and GSH levels and the GSH/GSSG ratio in the hippocampi of *klotho* mutant mice treated with melatonin (20 mg/kg). The post hoc test confirmed that these parameters were counteracted significantly by SL327 (10 mg/kg; total GSH: $p < 0.05$; GSH: $p < 0.05$; GSH/GSSG ratio: $p < 0.05$ at 5 mg/kg, $p < 0.01$ at 10 mg/kg; Figure 7, Supplementary Table S6). SL327 also consistently counteracted the effect of melatonin on the memory impairment in *klotho* mutant mice. One-way ANOVA indicated a significant effect of SL327 on the performance of *klotho* mutant mice treated with melatonin (20 mg/kg) in the NORT and PAT; the post hoc test confirmed this effect of SL327 (NORT: $p < 0.01$ at 10 mg/kg; PAT: $p < 0.05$ at 5 mg/kg, $p < 0.01$ at 10 mg/kg; Figure 7, Supplementary Table S6).

## Discussion

*Klotho* mutant mice exhibit the majority of human age-related disorders and are an appropriate and available model of human aging, including of the brain (Kuro-o et al., 1997; Shizaki et al., 2008). Our previous studies reported that oxidative stress plays a crucial role in the aging-associated cognitive impairment in *klotho* mutant mice (Nagai et al., 2003; Park et al., 2013). As the decline in melatonin production and altered melatonin rhythms are major contributors to the increased levels of oxidative stress and the associated neurodegenerative changes observed in the elderly (Reiter et al., 1980, 1981, 1997; Karasek and Reiter, 2002), we explored the therapeutic effect of melatonin on the memory impairment induced by *klotho* deficiency. To our knowledge, we are the first to propose that melatonin rescues oxidative burdens (i.e. increases synaptosomal ROS, lipid peroxidation, and protein oxidation and decreases GSH/GSSG ratio) and memory impairment induced by *klotho* deficiency via modulating the signaling interaction between the MT2 receptor, ERK, and Nrf2-dependent antioxidant activity.

Homeostasis of the GSH system is important for maintaining cognitive function. For example, GSH depletion by diethylmaleate greatly reduced long-term potentiation and synaptic plasticity (Almaguer-Melian et al., 2000). GSH depletion by 2-cyclohexene-1-one treatment caused disruption of short-term spatial memory in the Y-maze: the GSH precursor, N-acetyl-l-cysteine, rescued this disruption in Y-maze performance (Choy et al., 2010). Importantly, melatonin contributes to the maintenance of normal GSH levels (Subramanian et al., 2007) by stimulating GSH biosynthesis via γ-glutamylcysteine synthase and glucose-6-phosphate dehydrogenase (Kilanczyk and Bryszewska, 2003; Rodriguez et al., 2004). Since in the present study we observed that melatonin attenuated impaired GSH homeostasis and cognitive dysfunction in *klotho* mutant mice, we hypothesize that melatonin might exert memory-enhancing effects via Nrf2-dependent GSH synthesis in this model of aging.

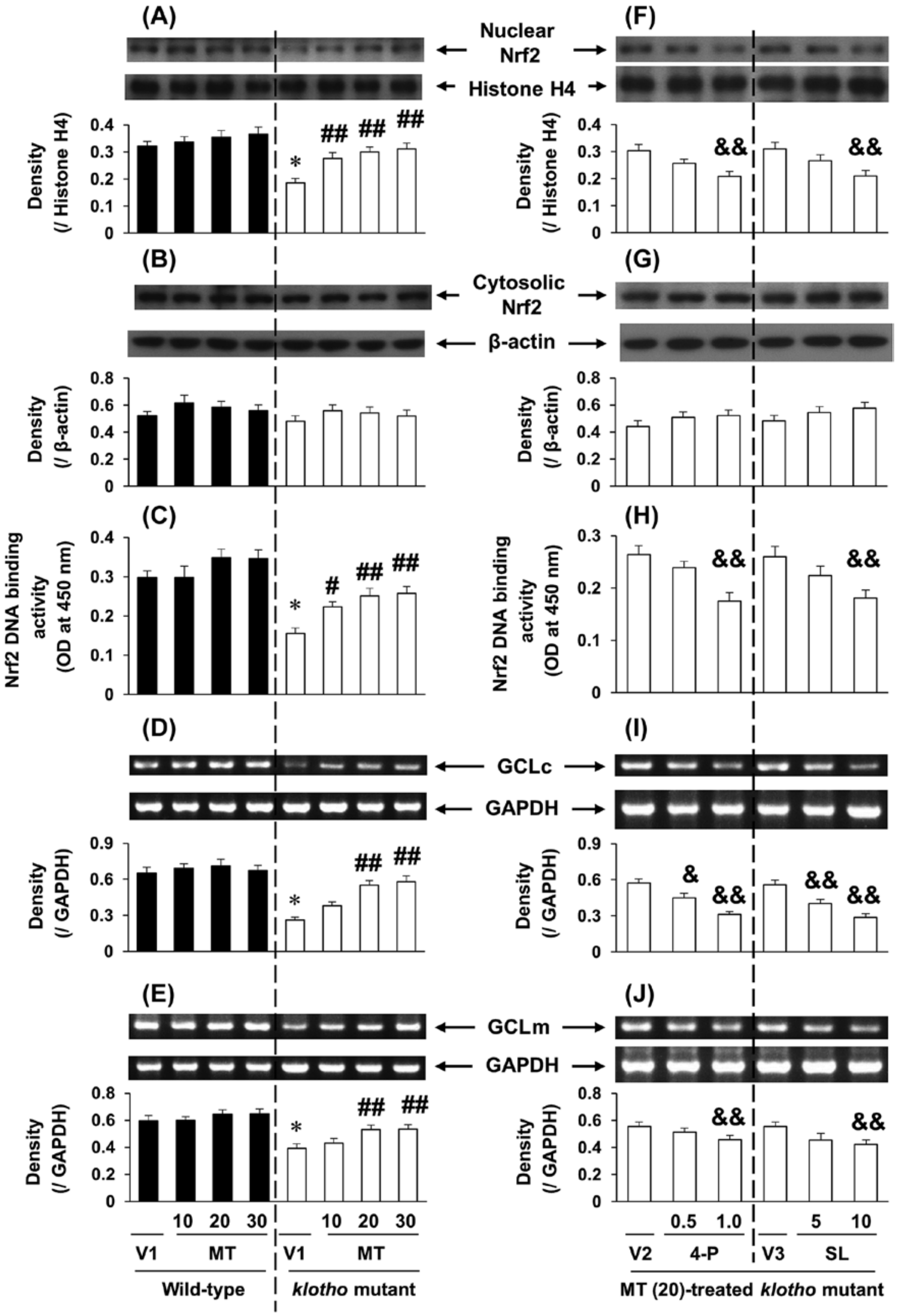


**Figure 6.** Effect of 4-P-PDOT, an MT2-receptor antagonist, or SL327, an extracellular-signal-regulated kinase inhibitor, on melatonin-mediated pharmacological activity in terms of the expression (A and F: nuclear Nrf2; B and G: cytosolic Nrf2) and DNA-binding activity (C and H) of Nrf2, and the mRNA levels of GCLc (glutamate-cysteine ligase catalytic subunit; D and I) and GCLm (glutamate-cysteine ligase modifier subunit; E and J) in the hippocampi of *klotho* mutant mice. 4-P, 4-P-PDOT (0.5 or 1.0 mg/kg, i.v.); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MT, melatonin (10, 20, or 30 mg/kg, i.p.); Nrf2, nuclear factor erythroid 2-related factor 2; OD, optical density; SL, SL327 (5 or 10 mg/kg, i.p.); V1, Vehicle 1 (5% dimethyl sulfoxide [DMSO] in saline, the solvent for melatonin); V2, Vehicle 2 (20% DMSO in saline, the solvent for 4-P-PDOT); V3, Vehicle 3 (100% DMSO, the solvent for SL327). Each value is the mean ± standard error of the mean of 6 animals. $^{*}p < 0.01$ vs. wild-type mice treated with V1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. *klotho* mutant mice treated with V1; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. *klotho* mutant mice treated with corresponding V2 or V3 + MT (20; two-way analysis of variance (ANOVA; A–E) or one-way ANOVA (F–J) followed by post hoc Fisher's least significant difference pairwise comparisons test).

Increasing evidence indicates that melatonin plays an important role in modulating learning and memory processing (Rawashdeh and Maronde, 2012). Although the mechanisms underlying its memory-facilitating effects remain unclear, melatonin exerts its action by binding to the widely-distributed MT1 and MT2 receptors in the hippocampus (Musshoff et al., 2002). Neu-P11, a novel melatonin (MT1/MT2) receptor agonist, enhanced memory performance in the NORT in rats and improved the neuronal and cognitive impairments in a rat model of Alzheimer's disease (He et al., 2013). The functional consequences of MT2

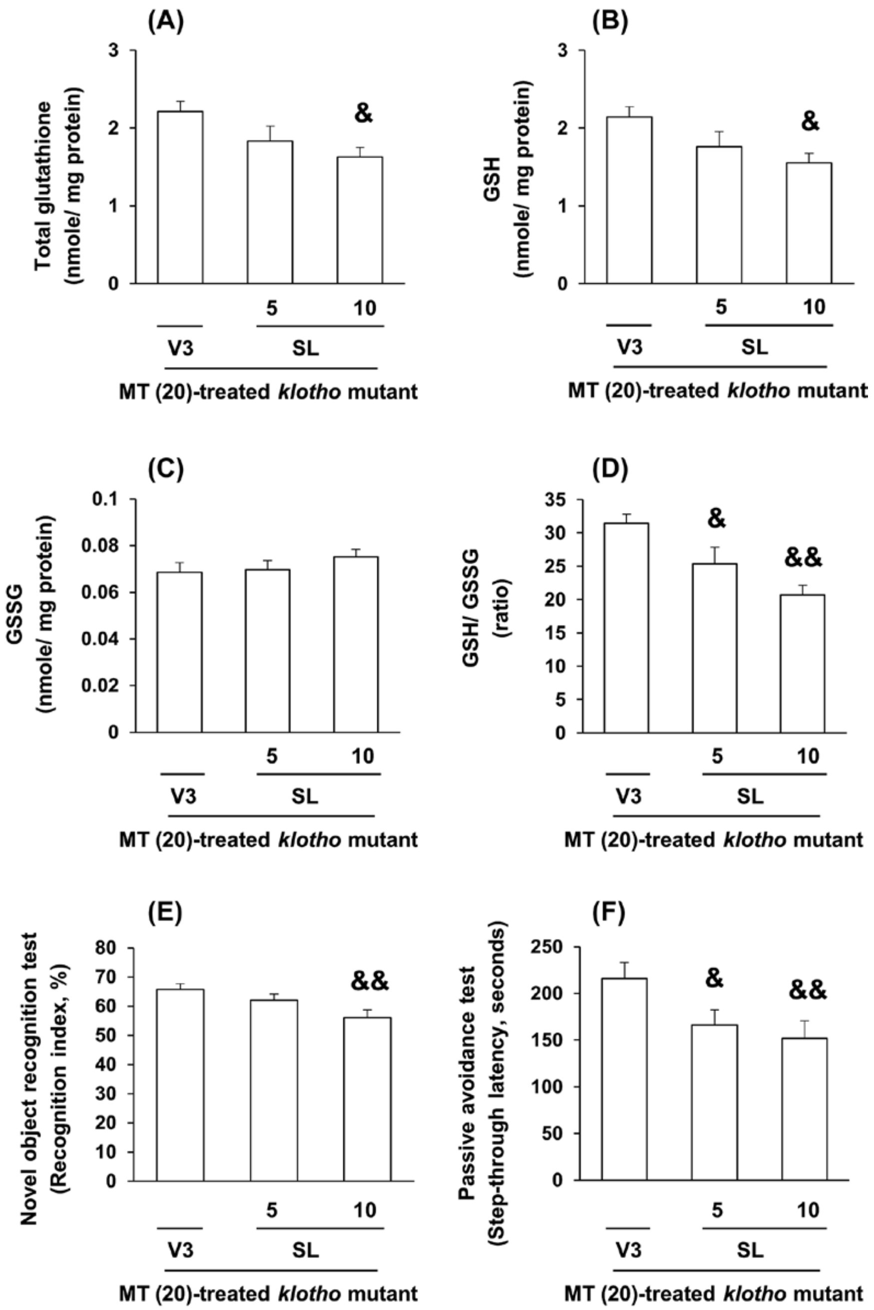


**Figure 7.** Effect of SL327, an extracellular-signal-regulated kinase inhibitor, on melatonin-mediated pharmacological activity in terms of the alteration in the hippocampal level of total glutathione (A), reduced glutathione (GSH; B), oxidized glutathione (GSSG; C) and GSH/GSSG ratio (D), and on melatonin-mediated memory function as evaluated by the novel object recognition test (E) and passive avoidance test (F) in *klotho* mutant mice. MT, melatonin (20 mg/kg, i.p.); SL, SL327 (5 or 10 mg/kg, i.p.); V3, Vehicle 3 (100% dimethyl sulfoxide, the solvent for SL327). Each value is the mean ± standard error of the mean of 6 (A–D) or 12 (E and F) animals. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. *klotho* mutant mice treated with V3 + MT (20; one-way analysis of variance followed by post hoc Fisher's least significant difference pairwise comparisons test).

receptor deficiency were observed in MT2-receptor–knockout mice, suggesting that melatonin facilitates memory through MT2-receptor–regulated hippocampal functioning (Larson et al., 2006). Although several reports indicate that luzindole, a dual MT1/MT2 receptor antagonist with higher affinity for the MT2 than the MT1 receptor (Dubocovich et al., 1998; Browning et al., 2000; Boutin et al., 2005), also facilitates memory, it has been well recognized that 4P-PDOT is a much more selective MT2 antagonist than luzindole (Dubocovich et al., 1997; Boutin et al., 2005). In this study, we observed that the counteracting effect of 4P-PDOT against protective potentials by melatonin was more pronounced than that of luzindole, suggesting that the MT2 receptor mainly mediates the effects of melatonin. Thus, our findings are considerably in agreement with those of Larson et al. (2006). In addition, we observed that the counteracting effects of luzindole were, in part, comparable to those of 4-P-PDOT against the efficacy of melatonin *in vitro* (Supplementary Figures S4 and S5).

ERK 1/2 family members were originally identified by their responsiveness to growth-factor–receptor tyrosine kinases and are activated by many G-protein-coupled receptors (GPCRs). MT receptors are one family of GPCRs that activate ERK in several systems. One important function of ERK activation is related to the localization of nuclear downstream targets, such as cAMP response element-binding protein (Sgambato et al., 1998) and Nrf2 (Shen et al., 2004). Radio et al. (2006) suggested that acute stimulation of MT2 receptors leads to ERK activation. Thus, alterations in melatonin receptor expression might influence the effects of melatonin on neuronal ERK signaling. Earlier studies demonstrated that receptor-mediated effects of melatonin on neuronal ERK pathways might be involved in the modulation of mechanisms of neuroplasticity (Bordt et al., 2001) and neuroprotection (Kilic et al., 2005). As *klotho* deficiency significantly decreased phospho-ERK and N-methyl-D-aspartate receptor-dependent long-term potentiation, and M1 mAChR stimulation by McN-A-343, an M1 mAChR agonist, activated p-ERK–dependent pathways in the hippocampi of *klotho* mutant mice (Park et al., 2013), we suggest that potentiation of ERK signaling is essential for prevention of learning and memory deficits in *klotho* mutant mice.

Nrf2-ARE is an important pathway for protection against oxidative stress (Lee and Johnson, 2004). Under oxidative stress, Nrf2 leaves Keap1, a negative regulator, and translocates to the nucleus, where it interacts with ARE, a *cis*-acting regulatory element in the promoter region of genes encoding phase II detoxification enzymes and antioxidant proteins. Subsequently, Nrf2 modulates a cytoplasmic response to oxidative stress (Ishii et al., 2000) through the transcriptional activation of genes involved in GSH synthesis, including those encoding GCLc and GCLm (Shih et al., 2003). Similar to this study, Hsieh et al. (2010) reported significantly decreased levels of both cytoplasmic and nuclear Nrf2 expression in the liver extract of *klotho* mutant mice, suggesting that this mutant down-regulates the activity of Nrf2-targeted genes, which may be related to the acceleration of aging. Therefore, our findings corroborate the hypothesis that melatonin protects against *klotho* deficiency by facilitating Nrf2-dependent signaling.

To date, limited reports on the role of Nrf2-ARE signaling in the neuroprotective mechanism mediated by melatonin are available. For example, melatonin was shown to modulate neuroinflammation by decreasing oxidative stress via increasing Nrf2 expression in an experimental diabetic neuropathy model (Negi et al., 2011). Wang et al. (2012) demonstrated that the therapeutic advantage of melatonin in response to subarachnoid hemorrhage might be due to its positive modulation of the cerebral Nrf2-ARE pathway and antioxidant signaling. Additionally, Parada et al. (2014) emphasized the potential role of the Nrf2 gene and heme oxygenase-1 overexpression in the neuroprotective effects of melatonin in the organotypic hippocampal slice culture model or photothrombotic stroke model.

Several upstream signaling cascades may activate Nrf2, either individually or in combination. These include selective effects on a number of protein kinase and lipid kinase signaling cascades, most notably the PI3K/Akt and MAP kinase pathways that regulate prosurvival transcription factors and gene expression (Shen et al., 2004). Reports from several laboratories also strongly suggest the involvement of MAPK pathways in ARE-mediated transcription through Nrf2 (Yu et al., 2000; Zipper and Mulcahy, 2000; Nguyen et al., 2003). Previous studies demonstrated that among the MAPK pathways, both the ERK and c-Jun N-terminal kinase pathways unequivocally up-regulated the activity of Nrf2 transactivation domains (Shen et al., 2004), and inhibition of the ERK pathway blocked hyperoxia-enhanced Nrf2 nuclear accumulation and ARE-driven reporter expression (Papaiahgari et al., 2004). Although numerous studies have linked melatonin to antioxidant, anti-inflammatory, and anti-apoptotic effects, as well as other neuroprotective signaling potentials (Hardeland, 2013; Pandi-Perumal et al., 2013), the current study is the first to determine the role of ERK in Nrf2 activation of melatonin via the MT2 receptor.

In conclusion, to our knowledge, this is the first study to demonstrate the protective effects of melatonin on memory impairments induced by *klotho* deficiency. We showed that melatonin attenuated oxidative stress and loss of homeostasis in the GSH system and significantly increased ERK phosphorylation, thereby enhancing the expression of antioxidant enzymes such as GCLc and GCLm in a Nrf2-related MT2- or ERK-dependent manner in the hippocampi of *klotho* mutant mice. Finally, we propose that melatonin requires interactive signaling events among the MT2 receptor, ERK, and Nrf2-related antioxidant potentials to protect against oxidative burden and memory impairment in a genetic model of aging (Figure 8).

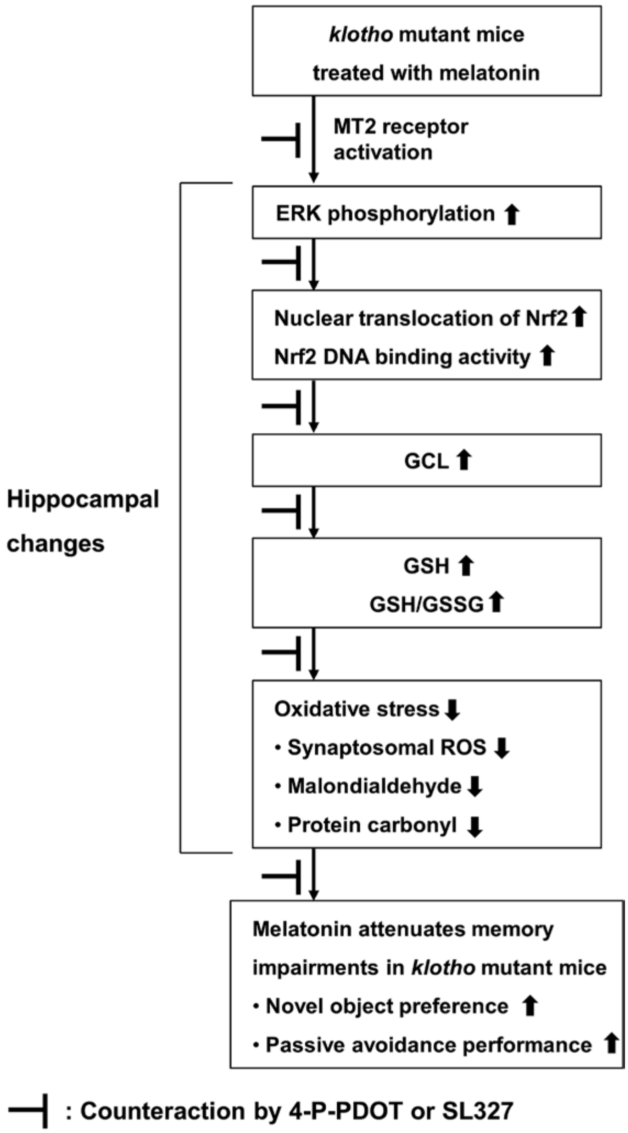


**Figure 8.** A schematic depiction of melatonin-mediated protective potential in response to memory impairment of *klotho* mutant mice. Melatonin significantly attenuated decreases in ERK phosphorylation, Nrf2 nuclear translocation, Nrf2 DNA-binding activity and GCL expression in the hippocampi of *klotho* mutant mice. Consistently, decreases in GSH/GSSG ratio and subsequent oxidative stress and memory impairment in *klotho* mutant mice were significantly attenuated by melatonin. These melatonin-mediated effects were significantly counteracted by 4-P-PDOT, an MT2 receptor antagonist, and SL327, an ERK inhibitor. Therefore, melatonin requires activation of the MT2 receptor and its associated ERK/Nrf2 signaling process to protect against the memory impairment induced by *klotho* deficiency. ERK, extracellular-signal-regulated kinase; GCL, glutamate-cysteine ligase; GSH, reduced glutathione; GSSG, oxidized glutathione; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species.

## Supplementary Material

For supplementary material accompanying this paper, visit http://www.ijnp.oxfordjournals.org/

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## Statement of Interest

None.

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