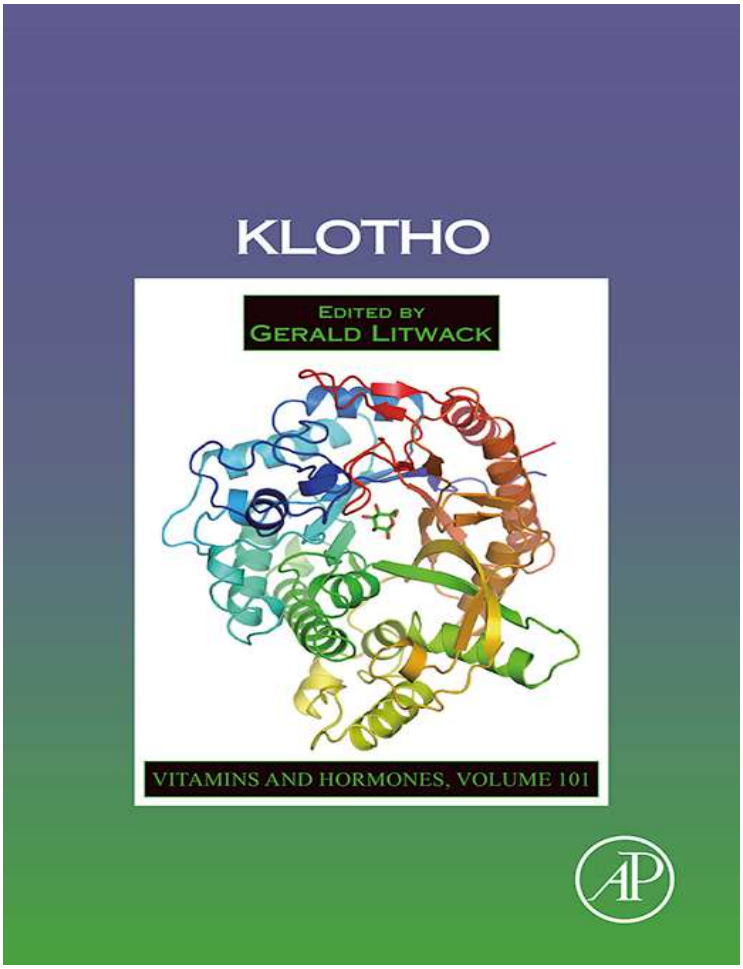

From C.R. Abraham, P.C. Mullen, T. Tucker-Zhou, C.D. Chen and E. Zeldich, Klotho Is a Neuroprotective and Cognition-Enhancing Protein. In: Gerald Litwack, editor, *Vitamins and Hormones, Vol. 101*, Burlington: Academic Press, 2016, pp. 215-238.
ISBN: 978-0-12-804819-1

Academic Press

CHAPTER NINE

Klotho Is a Neuroprotective and Cognition-Enhancing Protein

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Contents

Abstract

In this chapter, we will describe what has been learned about Klotho and its potential functions in the brain. Klotho is localized in the choroid plexus and, to a lesser extent, in hippocampal neurons. Cognitive decline is a common issue in human aging affecting over 50% of the population. This cognitive decline can also be seen in animal models such as the Rhesus monkey. A long-term study undertaken by our lab demonstrated that normal brain aging in rhesus monkeys and other animal models is associated with a significant downregulation of Klotho expression. This observation substantiates data from other laboratories that have reported that loss of Klotho accelerates the development of aging-like phenotypes, including cognitive deficits, whereas Klotho overexpression extends life span and enhances cognition in mice and humans. Klotho is a type 1 transmembrane pleiotropic protein predominantly expressed in kidney and brain and shed by ADAM 10 and 17 into the blood and cerebral spinal fluid, respectively. While the renal functions of Klotho are well known, its roles in the brain remain to be fully elucidated. We recently demonstrated that Klotho protects hippocampal neurons from amyloid and glutamate toxicity via the activation of an antioxidant enzymatic system suggesting Klotho is a neuroprotective protein. Furthermore, Klotho is necessary

Vitamins and Hormones, Volume 101
ISSN 0083-6729
http://dx.doi.org/10.1016/bs.vh.2016.02.004

for oligodendrocyte maturation and myelin integrity. Through its diverse roles in the brain, Klotho has become a new therapeutic target for neurodegenerative diseases such as Alzheimer's disease and demyelinating diseases like multiple sclerosis. Discovery of small molecule Klotho enhancers may lead to novel treatments for these incurable disorders.

1. INTRODUCTION

α-Klotho (Klotho) is an antiaging protein named after the Greek goddess Clotho, who spins the thread of life (Kuro-o et al., 1997). The Klotho gene was discovered by Kuro-o and colleagues while attempting to develop mice overexpressing the rabbit type I sodium-proton exchanger (NHE-1). Insertion of the NHE-1 transgene into the Klotho promoter resulted in a striking phenotype reminiscent of premature aging in humans. Subsequent analysis of Klotho-deficient (Klotho −/−) mice revealed systemic age-related abnormalities including gait disturbance, emphysema, osteoporosis, arteriosclerosis, hypomyelination, hippocampal neurodegeneration, and cognitive deficits (Chen et al., 2013; Kuro-o et al., 1997; Li et al., 2004; Nagai et al., 2003; Shiozaki et al., 2008). Klotho −/− mice begin to develop symptoms at 3–4 weeks and die prematurely at 2–3 months. Subsequent experiments demonstrated that Klotho overexpression confers resistance to oxidative stress, enhances cognitive performance, and extends lifespan by 20% and 30% in female and male mice, respectively (Brobey et al., 2015; Dubal et al., 2014; Kurosu et al., 2005; Zeldich et al., 2014).

A number of observations from the first report on Klotho −/− mice suggest that Klotho acts as a circulating hormone. For example, although Klotho −/− mice develop normally, several organs that do not express Klotho, including the thymus, skin, and lungs, are severely affected by Klotho depletion. Furthermore, exogenous expression of Klotho cDNA in the brain and testes of knockout mice improves aging phenotypes across all organ systems, indicating that Klotho acts in a noncell-autonomous manner. Findings from the initial study on Klotho were groundbreaking not only due to the discovery of the Klotho gene but also because the authors identified the possibility that Klotho acts as a hormone with pleiotropic and beneficial functions throughout the body (Kuro-o et al., 1997).

In humans, the Klotho gene is located on chromosome 13 and encodes a single-pass type I transmembrane protein that shares sequence homology with β-glycosidases (Tohyama et al., 2004). Klotho contains a 130-kDa

extracellular domain, a transmembrane domain, and a very short 8 or 9 amino acid intracellular domain. The large extracellular domain is shed from the cell surface by ADAM10, ADAM17, and BACE1 (Bloch et al., 2009; Chen, Podvin, Gillespie, Leeman, & Abraham, 2007). The shed ectodomain of Klotho (sKlotho) is detectable in the blood and cerebrospinal fluid (CSF), where it likely functions as a humoral factor (Imura et al., 2004; Kurosu et al., 2005). While the membrane bound form of Klotho is a coreceptor for FGF23 (Urakawa et al., 2006), sKlotho modulates ion channels (Cha et al., 2009, 2008; Chang et al., 2005), acts as an antioxidant, inhibits insulin/IGF1 signaling, and enhances oligodendrocyte maturation (Chen et al., 2013; Kurosu et al., 2005; Zeldich et al., 2014).

Klotho is predominantly found in the kidney, but is also expressed in the brain, lung, skeletal muscle, urinary bladder, testes, and ovaries (Kuro-o et al., 1997). Several groups have identified a relationship between Klotho and cognitive performance (Degaspari et al., 2015; Dubal et al., 2014; Nagai et al., 2003; Park et al., 2013), but very little is known regarding the molecular mechanisms of Klotho in the brain and a putative receptor has not been identified. Much of our mechanistic understanding comes from its function in the kidney, where Klotho regulates vitamin D, calcium, and phosphate levels by acting as a coreceptor for FGF23 (Kuro-o, 2012; Kurosu et al., 2006; Urakawa et al., 2006). Although Klotho undoubtedly plays a vital role in the kidney, investigating its neuronal function is especially important given that Klotho expression decreases with age in white matter, and sKlotho is reduced in the CSF of patients with multiple sclerosis (MS) and Alzheimer's disease (Duce et al., 2008; Emami Aleagha et al., 2015; Semba et al., 2014).

Recently, a shorter, 70 kDa secreted Klotho protein resulting from differential splicing has been identified as the major isoform in the brain with the use of specific antibodies. As we have shown previously with the long Klotho isoform (Duce et al., 2008), the shorter form also diminishes with aging and in an AD mouse model (Massó et al., 2015). Another interesting recent finding is that application of secreted recombinant Klotho together with FGF23 to hippocampal cells impacts neuronal morphology and synaptic density via the activation of the Akt pathway (Hensel et al., 2016).

2. THE EXPRESSION AND LOCALIZATION OF KLOTHO IN THE BRAIN

Klotho expression varies with age, species, and cell type. Expression in the brain is widespread throughout postnatal development with Klotho

mRNA detectable as early as postnatal day 1 (P1). The most thorough analysis of changes in Klotho expression during development was undertaken using the rat brain as a model. In most areas of the developing rat brain, Klotho mRNA levels are high early in development, decline sharply in week 2 of postnatal development then slowly climb back to adult levels (P75) that are similar to levels seen in early development. These changes were more pronounced in areas of the amygdala, while mRNA levels in the hippocampus remained relatively constant (Clinton et al., 2013). Klotho levels also fluctuate with advancing age. In aged rhesus monkeys, Klotho is reduced at both the mRNA and protein level in white matter from the corpus callosum. Analysis of white matter from mice and rats confirmed these findings indicating that reduced Klotho in the brain is common among aged mammals (Duce et al., 2008). This decline with age is thought to be due to hypermethylation of the promoter region (Duce et al., 2008; King, Rosene, & Abraham, 2012). Hypermethylation of the Klotho promoter not only increases with age, but in vitro methyl modification of the identified methylation sites was capable of reducing gene transcription. These findings are particularly significant given the recent evidence showing that age-related decreases in Klotho expression may be linked to neurodegeneration (Semba et al., 2014).

Measurements of Klotho mRNA expression by RT-PCR in mice show that Klotho is expressed in the kidney, choroid plexus, pituitary gland, skeletal muscles, urinary bladder, and reproductive organs (Kuro-o et al., 1997). Although Klotho clearly has an important function in the brain, its exact distribution in the central nervous system (CNS) remains unclear. Studies using in situ hybridization and immunohistochemistry (IHC) in mice and rats demonstrate that Klotho expression in the CNS is highest in the choroid plexus, where it is localized to the apical membrane of ependymal cells (Li et al., 2004). Soluble Klotho is found in the CSF of humans and mice, indicating that the ectodomain is released into the CSF following proteolytic cleavage from ependymal cells of the choroid plexus (Imura et al., 2004). It is thought that cleavage into the CSF and blood by ADAM 10 and 17 mediates the hormonal activity of sKlotho throughout the brain and body (Bloch et al., 2009; Chen et al., 2007).

Although expression in the CNS is highest in the choroid plexus, Klotho is also found at lower levels in other brain regions. Klotho has been identified in the cerebellar Purkinje cells of mice, where it is localized to the plasma membrane and nuclear envelope (German, Khobahy, Pastor, Kuro, & Liu, 2012). IHC in rats found that Klotho is present at the membrane of

cortical and hippocampal neurons, which may explain Klotho's influence on cognition. In this study, researchers confirmed IHC results and extended findings using in situ hybridization. Klotho mRNA was detected throughout the cortex and hippocampus, as well as various other limbic areas including amygdalar, thalamic, and hypothalamic nuclei. Outside of the choroid plexus, Klotho mRNA levels were highest in the medial preoptic area, median preoptic nucleus, ventromedial hypothalamus, and basolateral amygdala (Clinton et al., 2013). Compared to these regions, expression in cortical and hippocampal areas was slightly lower. Characterization of Klotho expression in vivo in glial cells showed that Klotho is not present in microglia or astrocytes, but is expressed in oligodendrocytes, which may be important for Klotho's influence on myelination (Clinton et al., 2013). However, primary cultured astrocytes do express Klotho (Abraham, Chen, Cuny, Glicksman, & Zeldich, 2012). In sum, results from localization studies suggest that a key function of Klotho in the brain is to act as a secreted humoral factor in the CSF, but more detailed functional analysis indicates that Klotho has varied region-specific effects throughout the CNS. For example, expression in hippocampal neurons likely influences spatial memory, while amygdalar expression may relate to fear learning. At this time a potential role of transmembrane Klotho in neurons in cell-to-cell communication with oligodendrocytes or other neurons via FGF signaling cannot be excluded and requires further investigation. With the identification of the exact cleavage site that releases sKlotho, researchers can now produce knockin mice that express only the transmembrane or the shed forms of Klotho in selected brain cells to determine the functions of each isoform (Chen et al., 2014).

3. KLOTHO AND COGNITION

A growing body of evidence points to a connection between Klotho and cognition. Mouse models demonstrate that Klotho deficiency impairs performance on tests of learning and memory, while overexpression improves cognitive function (Dubal et al., 2014; Nagai et al., 2003). Interestingly, cognition is enhanced in humans carrying a single copy of the Klotho-VS polymorphism, while homozygous Klotho-VS individuals exhibit a lower IQ from a young age (Deary et al., 2005). Biochemical analysis of Klotho mutant mice and characterization of the Klotho-VS polymorphism have led to the identification of candidate cellular and molecular mechanisms underlying Klotho's influence on cognition.

3.1 Antioxidant and Neuroprotective Role of Klotho in the Hippocampus

Klotho's impact on cognition likely involves its activity in the hippocampus and/or cerebral cortex, as these regions are crucial for the consolidation and storage of long-term memories (Huang, Nguyen, Abel, & Kandel, 1996; Scoville & Milner, 2000). Klotho −/− mice exhibit decreased synaptophysin immunoreactivity in the hippocampus, indicating a reduction in synaptic vesicles. This effect is most profound at the stratum lucidum, the tract of mossy fiber projections connecting granule cells of the dentate gyrus with pyramidal cells in the CA3 field. This pathway facilitates normal hippocampal function, which is vital for acquiring and consolidating new memories (Scoville & Milner, 2000). When researchers assessed the morphology of single neurons from the stratum lucidum of Klotho −/− mice, they found that individual axons were normal in size and number of vesicles, but the total number of synapses at the stratum lucidum was markedly decreased (Li et al., 2004). Subsequent reports have confirmed the presence of structural abnormalities in the hippocampus of Klotho −/− mice (Shiozaki et al., 2008).

Although the exact mechanism is unknown, it appears that Klotho facilitates normal hippocampal structure and function. One possible mechanism is that Klotho acts as part of the endogenous antioxidant defense system, which has an important neuroprotective role in the hippocampus. In support of this hypothesis, markers of DNA and lipid oxidation are elevated in the hippocampus of Klotho-deficient mice, and expression of the proapoptotic protein bax is increased, while expression of antiapoptotic blc-2 and bcl-Xl is decreased (Nagai et al., 2003; Shiozaki et al., 2008). Consistent with findings in mice lacking Klotho, mice that overexpress Klotho show a 50% reduction in DNA oxidation, as measured by urinary 8-OHdG levels. In CHO and HeLa cells, treatment with Klotho suppresses paraquat-induced lipid oxidation and apoptosis, via inhibition of insulin/IGF-1 signaling, activation of FOXO1, and subsequent transcription of SOD2. Treatment of mice overexpressing Klotho with paraquat revealed that Klotho is also protective against oxidative stress in vivo (Yamamoto et al., 2005). Recently, the importance of Klotho's antioxidant function for its neuroprotective role was confirmed using a hippocampus-derived cell line (HT22) and primary hippocampal neurons (Zeldich et al., 2014). The addition of sKlotho to these cells significantly enhanced the expression of the thioredoxin/peroxiredoxin (Trx/Prx) redox system. The greatest effect was seen on peroxiredoxin-2 (Prx-2), an antioxidant enzyme, that showed an increase in expression at both mRNA and protein levels.

Because the hippocampus is integrally involved in the acquisition and consolidation of episodic memories (Scoville & Milner, 2000), and Klotho −/− mice have altered hippocampal morphology and impaired hippocampal long-term potentiation (LTP) (Park et al., 2013), it is unsurprising that Klotho −/− mice score lower than wild-type (WT) littermates on tests of long-term memory (LTM). Klotho −/− mice perform more poorly on novel object recognition, as well as context-cued and tone-cued fear conditioning tasks—implicating both hippocampal and amygdalar dysfunction. Notably, researchers found that memory impairments observed in Klotho −/− mice could be ameliorated by treatment with the potent antioxidant α-tocopherol, which further implicates the importance of Klotho's antioxidant function in its neuroprotective role (Nagai et al., 2003).

It is important to point out that memory impairments observed in Klotho −/− mice are age dependent. WT mice perform better than Klotho −/− mice when assessed at 7 weeks of age, but not at 6 weeks. This effect may relate to the accumulation of oxidative cellular damage over time in the Klotho-deficient mice (Nagai et al., 2003). In addition, deficits were seen in LTM but not short-term memory (STM); mice lacking Klotho performed worse when tested 24 hours, but not 1-h posttraining, implying that Klotho depletion causes dysfunction of the hippocampal circuitry involved in LTM consolidation, but not prefrontal circuitry responsible for STM. Conversely, experiments with Klotho-overexpressing mice (Klotho-OE) show that Klotho overexpression enhances both STM and LTM in an age-independent manner. Young- and middle-aged Klotho-OE mice perform better on the Morris water maze task, a hippocampal dependent test of spatial memory, as well as in context-cued fear conditioning. Klotho-OE mice also score higher on the Y maze task, a paradigm that tests working memory, which is a type of STM (Dubal et al., 2014).

3.2 Klotho and LTP

To better understand the effect of Klotho on cognition at the molecular level, researchers examined the relative amounts of GluN2A, GluN2B, and GluN2C, *N*-methyl-D-aspartate receptor (NMDAR) subunits in the hippocampus and prefrontal cortex (PFC) of Klotho-OE mice. Hippocampal NMDARs are involved in the induction of LTP, which is thought to be the cellular correlate of LTM (Bliss & Collingridge, 1993; Malenka & Nicoll, 1993). LTP is triggered when glutamate binds NMDARs and calcium enters the cell—initiating a complex cascade of intracellular signaling pathways. Ca^{2+} activates calcium-sensitive proteins such as protein kinase C and

calcium/calmodulin-dependent protein kinases (CaMKII and CaMKIV). These kinases and many other molecules stimulate diverse biochemical pathways that ultimately alter the strength of synapses. Modulation of glutamate receptors, de novo protein synthesis, and changes in neuronal morphology are three well-characterized mechanisms that result in enhanced synaptic connectivity. For example, calcium influx stimulates CaMKII, which then modulates NMDARs by binding to GluN2B subunits (Barria & Malinow, 2005).

The NMDAR is required for many forms of LTP, and NMDAR-dependent LTP in the hippocampus is necessary for spatial memory, temporal memory, and multimodal associative learning, among other forms of memory (Huerta, Sun, Wilson, & Tonegawa, 2000; Morris, Anderson, Lynch, & Baudry, 1986; Rondi-Reig, Libbey, Eichenbaum, & Tonegawa, 2001). In Klotho-OE mice, the GluN2B subunit is enriched at the postsynaptic density in the hippocampus and PFC at the protein level, but not at the mRNA level, implicating the involvement of posttranslational modifications (Dubal et al., 2014). GluN2B currents are involved in LTP (Smith, Smith, Bredemann, & McMahon, 2015) and this subunit has an important impact on learning and memory. Depletion with age is linked to cognitive decline, and overexpression rescues these deficits (Brim et al., 2013). Because Klotho overexpression upregulates the GluN2B subunit in the hippocampus and PFC, researchers believe modulation of this subunit is involved in spatial and working memory improvements observed in Klotho-OE mice. Consistent with this hypothesis, Klotho overexpression results in enhanced hippocampal LTP, which likely mediates Klotho's impact on cognition. To highlight the involvement of the GluN2B subunit, Klotho-OE and WT mice were treated with a low dose of ifenprodil, a selective inhibitor of NMDARs containing the N2B subunit. Treatment with ifenprodil did not affect WT mice, but abolished memory improvements seen in Klotho-OE mice, revealing a vital role for the GluN2B subunit in Klotho induced cognitive enhancement (Dubal et al., 2014).

Although glutamate is the primary excitatory neurotransmitter involved in hippocampal LTP, this process is also subject to modulation by a number of other neurotransmitters and hormones including serotonin, acetylcholine (ACh), dopamine, norepinephrine, cortisol, and estrogens (Rodrigues, LeDoux, & Sapolsky, 2009; Sweatt, 2004). Modulatory inputs at the hippocampus influence learning and memory by impacting LTP, and it is possible that Klotho impacts cognition by interacting with these modulators. For example, impaired cognition and LTP in Klotho −/− mice may be related to decreased ACh-mediated JAK/STAT signaling (Park et al., 2013).

3.3 Klotho and the Neuroendocrine System

Recent evidence has raised the possibility that Klotho's impact on cognition is modulated by estradiol (E2). Like Klotho, E2 influences cognitive function, and postmenopausal decreases in E2 levels are associated with memory decrements in some postmenopausal women. E2 enhances hippocampal LTP (Foy et al., 1999; Sweatt, 2004; Woolley, Weiland, McEwen, & Schwartzkroin, 1997; Yun et al., 2007) and rescues memory deficits in ovariectomized (OVX) animals (Daniel, Hulst, & Berbling, 2006; Talboom, Williams, Baxley, West, & Bimonte-Nelson, 2008). Intriguingly, a recent study showed that E2-treated OVX rats exhibited ten times higher hippocampal Klotho expression, compared to vehicle-treated animals (Sarvari et al., 2015). This report is especially exciting because Klotho and E2 seem to act in similar ways by improving cognition and enhancing LTP by acting through GluN2B subunits (Smith & McMahon, 2006). These findings raise the possibility that Klotho and E2 may interact to enhance cognition—however, a definitive model requires additional analysis.

Since Klotho is a potent inhibitor of the IGF-I pathway, which negatively regulates the secretion of growth hormone (GH) from the pituitary gland, it was hypothesized that Klotho may enhance GH secretion. Recent studies indeed implicated Klotho, as a positive regulator of the secretion of the GH from normal somatotropes and pituitary adenomas through the possible involvement of IGF-I and bFGF pathways (Shahmoon et al., 2014). Measuring levels of soluble Klotho levels was also proposed as a marker in the follow-up of patients with acromegaly (Kohler et al., 2013).

3.4 The Klotho-VS Polymorphism and Human Cognition

In line with experiments linking Klotho and cognition in rodents, studies of human populations indicate that Klotho enhances learning and memory. Analysis of three independent cohorts—the Hillblom Aging Study, the Memory and Aging Project, and the Normal Aging Cohort—revealed that heterozygous carriers of the Klotho-VS polymorphism score significantly higher on neuropsychological tests of cognitive function (Dubal et al., 2014). These results were confirmed by meta-analysis of the three populations. The Klotho-VS polymorphism is a variant composed of six single-nucleotide polymorphisms (SNPs), with three located in exons and three within introns. Of the SNPs occurring in exons, two out of three lead to amino acid substitutions—F352V and C370S. Expression of this

polymorphism in human embryonic kidney cells leads to increased formation of Klotho–FGF receptor complexes and higher levels of FGF23 signaling (Tucker Zhou, King, Chen, & Abraham, 2013). Heterozygous carriers of this variant also have higher sKlotho levels in the serum than noncarriers, which is thought to be a result of enhanced shedding that has been reported in HeLa cells transiently transfected with a truncated Klotho-VS construct (Arking et al., 2002). Increased Klotho levels in the serum of carriers and noncarriers tend to correlate with higher scores on tests of semantic fluency, category fluency, and modified trials (Dubal et al., 2014). On the other hand, women homozygous for the V/V genotype had lower nonverbal reasoning scores at age 79, after adjustment for cognitive ability at age 11 (Deary et al., 2005). Taken together, these results strongly suggest that increasing Klotho levels enhances cognition in both humans and rodents. Although the precise mechanism is not fully understood, two leading theories posit:

1. Klotho promotes memory formation and hippocampal function by acting as an antioxidant and neuroprotective agent.
2. Klotho influences memory formation by modulating LTP via upregulation of GluN2B NMDAR subunits, resulting in improved hippocampal and prefrontal dependent memory.

Given Klotho's pleiotropic function, it is likely that both of these processes contribute to Klotho's effect on learning and memory. In addition, modulators such as ACh and E2 may be related to Klotho's cognition-enhancing properties. Although significant progress has been made regarding our understanding of Klotho's impact on cognition, further work is required for a more precise and detailed characterization of the mechanisms and interactions involved.

3.5 The White Matter, Aging, and Cognition

In addition to modulating cognition through its antioxidant and LTP enhancing activity, it is possible that Klotho influences learning and memory by counteracting cognitive impairments associated with "normal" aging. Although no studies to date have specifically addressed Klotho's impact on normal cognitive aging, Klotho expression and levels decrease with age (Duce et al., 2008; Semba et al., 2014), as does cognitive function. These correlations may represent a relationship between aging, decreased Klotho, and cognitive decline, but causal evidence is needed to confirm and better understand this relationship.

Age-related cognitive decline (ARCD) is a well-documented phenomenon characterized by impairments in neural processing, executive function, and memory acquisition (Makris et al., 2007). Initially, neuronal death was thought to underlie ARCD, but as imaging methodologies improved, a number of reports were published describing no significant loss of neurons with age (Giannaris & Rosene, 2012). Studies have demonstrated that aged monkeys do not display a neurodegenerative phenotype in cognitively relevant areas such as the PFC or the hippocampal CA1 field (Peters, Leahu, Moss, & McNally, 1994; Peters, Nigro, & McNally, 1997; West, 1993). While significant loss of gray matter is not apparent in the aged brain of healthy animals, age-related white matter loss and myelin abnormalities have been observed in disease-free monkeys and humans. Ultrastructural analysis of white matter in the aged brain shows abnormal thickness, splitting, and redundancy of myelin sheaths, as well as reduced white matter volume and thinning in layer I of the PFC (Hinman & Abraham, 2007). These age-related white mater changes may yield severe functional consequences, as myelination facilitates normal neuronal communication, and permits high speed information processing required for normal brain function. Although age-related white matter changes are heterogeneous and complex, myelin breakdown and reduced white matter volume correlate with both age and cognitive decline (Peters, 1996).

White matter refers to the bundles of myelinated projections that connect various brain regions and was named because of its characteristic white appearance—the result of an abundance of myelinated axons. Myelin is a lipid- and protein-containing membrane that spirals around axons to form the myelin sheath, which provides electrical insulation for axons. In the CNS, oligodendrocytes are responsible for myelination, and this process results in markedly faster speeds of action potential (AP) propagation. By increasing the velocity of AP propagation, myelination facilitates the remarkable information processing capacity of the brain. When myelin is disrupted, normal AP conduction cannot occur, and neuronal communication is impaired. As a result, specific brain functions are impaired, depending on the regions and pathways affected. This process can be seen on a pathological level in MS, as demyelination and white matter lesions lead to neurological symptoms that depend on lesion location. For example, demyelination and cell death in pathways important for learning and memory results in symptoms related to cognition, while lesions in other brain areas may cause sensory, motor, and/or sexual dysfunction. Because

demyelination occurs in both MS and normal brain aging, albeit to vastly different extents, it is possible that cognitive dysfunction in MS and ARCD both involve white matter changes and circuit level brain dysfunction despite differing underlying molecular mechanisms. White matter loss is common in neurodegenerative disorders such as MS, AD, and Parkinson's disease (PD), and may relate to cognitive dysfunction in these conditions. Although ubiquitous gray matter loss makes it difficult to study the cognitive consequence of white matter loss in AD, a recent study in humans diagnosed with PD shows that white matter loss correlates with cognitive deficits (Galantucci et al., 2015).

3.6 Klotho, Aging, and Neuroinflammation

It appears that during normal brain aging, the number of neurons does not decrease, but cellular structure and function are altered by aging processes. For example, increases in neuroinflammation with age are associated with the activation of microglia and astrocytes in white matter (Sloane, Hollander, Moss, Rosene, & Abraham, 1999; Sloane et al., 2000). When activated, these cells phagocytose myelin (Duce, Hollander, Jaffe, & Abraham, 2006) and release inflammatory and oxidant molecules, both of which are processes previously associated with aging. Microglial activation in aged monkeys correlates with cognitive deficits, and inflammation is associated with impaired cognition in chronic kidney disease (Madero, Gul, & Sarnak, 2008), suggesting a key role for inflammation in ARCD. Notably, Klotho inhibits the proinflammatory cytokine TNFα, and cognitively impaired nephrectomized rats show decreased levels of Klotho and increased levels of TNFα in the PFC (Degaspari et al., 2015). In parallel, Klotho was shown to be downregulated by TNFα via the transcription of nuclear factor kappa B (NFkB) in animal models of chronic kidney disease and colitis (Moreno et al., 2011; Thurston et al., 2010). These findings were confirmed in a study that demonstrated that Klotho was downregulated in the temporal lobe of epilepsy patients, TNFα upregulation in epilepsy patients is associated with chronic hippocampal inflammation and the expression of Klotho in the area was inversely correlated with the expression of NFkB and TNFα (Teocchi, Ferreira, da Luz de Oliveira, Tedeschi, & D'Souza-Li, 2013). We have demonstrated in vitro that endogenously secreted Klotho rescues cocultures of oligodendrocyte precursor cells and astrocytes from TNFα-induced cytotoxicity (Abraham et al., 2012).

4. KLOTHO, OLIGODENDROCYTE BIOLOGY, AND MYELINATION

A number of processes other than inflammation, such as oxidative stress and altered gene expression, also influence white matter degeneration and ARCD. Findings of decreased Klotho expression, white matter abnormalities, and cognitive deficits in both aged monkeys and Klotho-deficient mice support the hypothesis that Klotho is involved in ARCD and white matter degeneration in humans. Following the discovery of reduced Klotho expression in the white matter of aged mammals (Duce et al., 2008), recent efforts in our lab have been aimed at elucidating the role of Klotho in myelination, and the effect of Klotho on oligodendrocyte biology. We have found that Klotho impacts oligodendrocytes in vitro and myelination in vivo. Klotho activates Akt and Erk1/2 signaling, and enhances maturation of primary oligodendrocyte progenitor cells (OPCs) and the oligodendrocytic cell line, MO3.13 (Chen et al., 2015, 2013). Analysis of protein and mRNA levels shows that Klotho also enhances expression of myelin-associated proteins in OPCs and MO3.13 cells. In OPCs, treatment with Klotho upregulated the two major protein constituents of myelin, proteolipid protein (PLP), and myelin basic protein, as well as myelin-associated glycoprotein (MAG) and oligodendrocyte-specific protein. Analysis of Klotho-induced OPC maturation following treatment with the Akt inhibitor, LY294002, and the Erk-1 inhibitor, UO126, revealed a vital role for Akt signaling and a less important role for Erk-1 (Chen et al., 2013). Consistent with the involvement of Akt signaling, Klotho induced Akt phosphorylation at S473 in OPCs and MO3.13 cells (Chen et al., 2015, 2013).

Recent evidence from mouse models has provided further insights into the relationship between Klotho, oligodendrocytes, and myelination in vivo. Klotho-deficient mice have a markedly lower percentage of myelinated fibers in the corpus callosum and optic nerve, as well as fewer total and mature oligodendrocytes in the fimbria (Chen et al., 2013). As expected, analysis of Klotho −/− mice revealed a significant reduction in both protein and mRNA levels of myelin-related proteins MAG, PLP, and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase. Klotho −/− mice also show impaired myelin at the node of Ranvier, as the paranodal region is diminished and the nodal region is enlarged. In sum, these findings strongly suggest that Klotho enhances myelination both in vitro and in vivo (Chen et al., 2013). In line with this hypothesis, a recent study using the cuprizone model

of MS demonstrates that spontaneous remyelination is significantly enhanced in Klotho-OE mice (Zeldich, Chen, Avila, Medicetty, & Abraham, 2015). Further evidence for Klotho's therapeutic potential in MS comes from the finding that CSF levels are significantly lower in MS patients compared to healthy age-matched controls (Emami Aleagha et al., 2015), which may relate to the failure of remyelination mechanisms seen in MS patients. Given that Klotho enhances oligodendrocyte maturation and myelination and has antioxidant and neuroprotective properties, Klotho modulators represent powerful and promising therapeutics for demyelinating disorders such as MS. The ability of Klotho to stimulate remyelination may be especially important, because normal repair mechanisms are disrupted in MS patients (Franklin, 2002). Due to Klotho's therapeutic potential, development and optimization of small molecule Klotho modulators is a current focus in our laboratory (Abraham et al., 2012; King, Chen, et al., 2012).

5. KLOTHO, OXIDATIVE STRESS, AND NEURODEGENERATION

While upregulating Klotho to induce remyelination represents a promising treatment for MS (Zeldich et al., 2015), Klotho modulators may also be useful for treating other neurodegenerative diseases given Klotho's antioxidant and neuroprotective properties (Yamamoto et al., 2005; Zeldich et al., 2014). Cellular oxidative damage is thought to play a role in aging and has been implicated in numerous age-related brain diseases (Popa-Wagner, Mitran, Sivanesan, Chang, & Buga, 2013). Oxidative stress is mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated as a result of normal cellular metabolism. These species include molecules such as superoxide, hydroxy radicals, hydrogen peroxide, nitric oxide, and peroxynitrite. ROS and RNS are not harmful at basal levels and some have functional roles as signaling molecules (Arancio et al., 1996). A suite of endogenous antioxidant enzymes including superoxide dismutase, glutathione, and catalase are responsible for neutralizing ROS and RNS as they are produced in order to prevent toxic accumulation of these species. In pathological conditions, ROS and RNS oxidize nucleic acids, proteins, and lipids causing cellular damage and eventually death. Because of the high-energy demands of neurons, the brain may be particularly susceptible to oxidative damage, and reactive oxidants are elevated in PD, amyotrophic lateral sclerosis (ALS), MS, and AD.

ROS levels are elevated during glutamate excitotoxicity, and these processes are thought to mediate neurodegeneration in disorders such as ALS, MS, and PD (Lau & Tymianski, 2010). In Klotho-treated HT22 cells and primary hippocampal neurons obtained from Klotho-OE mice, Klotho confers resistance to glutamate excitotoxicity and oxidative stress (Zeldich et al., 2014). Downregulation of the antioxidant enzyme Prx-2 via shRNA abolished the ability of Klotho to protect against glutamate toxicity, indicating a key role for Prx-2 in Klotho-mediated neuroprotection (Zeldich et al., 2014). Experiments in Klotho −/− mice have shown that lack of Klotho leads to decreased levels of Trx, which is correlated with higher p38 activation. In contrast, challenging the brains of Klotho transgenic mice with a neurotoxin, MPTP and analysis for residual neuron numbers and integrity in the substantia nigra pars compacta showed that Klotho overexpression significantly protects dopaminergic neurons against oxidative damage, partly by modulating p38 MAPK activation level. The data highlight the importance of ASK1/p38 MAPK pathway in the brain and identify Klotho as a stabilizer of the interaction of Trx with ASK1, leading to increased antioxidative resistance (Brobey et al., 2015). This recent study further confirms that Klotho regulates the Trx–Prx system to control oxidative stress. Remarkably, in cultures of human aortic vascular smooth muscle cells, Klotho-induced antioxidant defense is also associated with the increased expression of Prx (Prx-1), suggesting that the antioxidative mode of action of Klotho universally involves the regulation of this redox system (Rizzo et al., 2014).

If the brain is particularly vulnerable to oxidative stress, developing blood–brain barrier penetrant antioxidants may be especially valuable. A recent study in mice demonstrates the therapeutic benefit of reducing cellular oxidative damage by showing that antioxidant cerium oxide nanoparticles decrease ROS in the cerebellum and hippocampus and alleviate disease severity in the EAE model of MS (Heckman et al., 2013). Klotho may have a similar therapeutic mechanism, as it protects neurons from oxidative stress both in vitro and in vivo (Yamamoto et al., 2005; Zeldich et al., 2014), although there is some discrepancy with respect to the mechanism involved. In nonneuronal cell lines including COS, HeLa, and CHO cells, Klotho acts as an antioxidant by inhibiting Akt and FOXO1 resulting in increased SOD2 expression (Yamamoto et al., 2005). Conversely, in brain-derived cells such as the HT22 cell line, primary hippocampal neurons, and oligodendrocytes, Klotho induces phosphorylation of Akt (Chen et al., 2013; Zeldich et al., 2014), while in primary

hippocampal neurons the Akt activation resulted in inhibitory phosphorylation of FOXO3a (Zeldich et al., 2014).

Because Klotho can reduce oxidative stress and protect against glutamate toxicity, Klotho boosters may be effective treatments for a number of neurodegenerative diseases. For example, substantial evidence suggests that excitotoxicity and oxidative damage play a role in ALS pathogenesis and motor neurons affected in ALS are highly susceptible to excitotoxicity (Barber, Mead, & Shaw, 2006; Foran & Trotti, 2009). Klotho −/− mice have fewer large anterior horn cells (AHCs) in the spinal cord, with concomitant increases in GFAP and neurofilament phosphorylation (Anamizu et al., 2005). Changes in AHCs of Klotho −/− mice resemble the loss of motor neurons seen in ALS, and degeneration in this region may account for altered gait in mice-lacking Klotho. However, motor abnormalities seen in Klotho −/− have been described as Parkinsonian, and may be the result of Purkinje cell degeneration in the cerebellum.

Purkinje cells are also sensitive to excitotoxic insult (Slemmer, De Zeeuw, & Weber, 2005), and neurodegeneration caused by the accumulation of neurofilaments (Tu et al., 1997). Klotho-deficient mice show increased phosphorylation and expression of neurofilament subunits in the cerebellum, and denser dendritic arbors in Purkinje cells, which may be due to decreased expression of MAP2, a microtubule-associated protein necessary for proper dendritic spacing. Purkinje cell bodies were reported to have abnormal inclusions similar to lipofuscin type granules and axons also showed signs of degeneration (Shiozaki et al., 2008). These findings suggest that Klotho protects Purkinje cells from excitotoxicity in wild-type mice, while Klotho-deficient mice lack this protective mechanism leading to Purkinje cell damage and death.

Klotho also appears to play a protective role in the retina by acting as an antioxidant. Cultured retinal pigment epithelial cells treated with Klotho show decreased markers of oxidative stress, and these cells play a critical role in retinal function (Kokkinaki et al., 2013). Although the retinas of Klotho −/− mice are normal throughout development, functional deficits arise around 7 weeks of age (Reish et al., 2013). In line with these findings, Klotho levels are elevated in a model of retinal degeneration, suggesting that Klotho is involved in retinal disease states, although it is unknown whether a causal relationship exists (Farinelli et al., 2013; Kokkinaki et al., 2013).

Klotho levels in the CSF decline with age and are further decreased in age-related neurodegenerative disorders such as MS and AD (Emami Aleagha et al., 2015; Semba et al., 2014). It can be difficult to interpret this

type of correlative data because it is impossible to know if reduced Klotho is a cause or consequence of pathology. Regardless, a number of studies support the hypothesis that upregulating Klotho is useful for treating AD and MS. In line with this idea, Klotho overexpression provides therapeutic benefit in a transgenic mouse model of AD. Mice expressing human APP (hAPP) develop behavioral and neuropathological phenotypes that recapitulate features of AD such as neuritic plaques, impaired LTP, altered hippocampal spine morphology, and cognitive deficits. These mice also die prematurely, which may be a result of increased epileptiform activity in the cortex (Dubal et al., 2015). In transgenic mice overexpressing both hAPP and Klotho (hAPP × Klotho), the deleterious effects of hAPP expression on cognition and lifespan were ameliorated. Furthermore, hAPP mice had reduced levels of the GluN2A and GluN2B subunits in the hippocampus, while Klotho-OE and hAPP × Klotho mice had significantly higher levels of GluN2B when compared with either WT or hAPP mice. Consistent with results from Klotho-OE mice, LTP was enhanced in hAPP × Klotho mice. Although Klotho overexpression in hAPP mice does not alter the number or neuritic plaques, or the levels of phosphorylated tau, it does prevent loss of spinophillin staining in the hippocampus, demonstrating Klotho's neuroprotective role. These results indicate that although Klotho does not directly alter AD pathogenesis, enhancing Klotho levels in the brain may effectively counteract cognitive decline associated with dementia, either by enhancing LTP via upregulation of GluN2B or by providing neuroprotection through an antioxidant mechanism.

6. KLOTHO ENHANCERS AS A POTENTIAL TREATMENT FOR NEURODEGENERATION

Increasing Klotho appears to be a promising strategy for treating AD and MS, but due to Klotho's relatively large size, it cannot be directly used as a therapeutic. Instead, small molecule Klotho enhancers that can cross the BBB are being screened in vitro to identify lead compounds for testing in animal models of neurodegeneration (Abraham et al., 2012; King, Chen, et al., 2012).

Klotho is a large protein with antiaging and neuroprotective properties, which is downregulated in aging and Alzheimer's diseased brains. Klotho has been implicated in myelination processes, and a decline of Klotho is associated with cognitive and neuronal decline. Since Klotho is a large molecule and unlikely to cross the blood–brain barrier, it will be difficult to clinically

administer Klotho to restore Klotho levels in the brain. Instead, a primary goal is to develop novel treatments based on Klotho-enhancing small molecule compounds, focusing on neurological improvement or protection. This goal aligns well in potential treatments for AD and MS, neither of which has a cure or long-term effective treatments.

Using a high throughput screen of 150,000 compounds, a number of Klotho-enhancing small molecule hits were identified (Abraham et al., 2012; King, Chen, et al., 2012). Medicinal chemistry optimization of the drug-like properties of these compounds has led to the recent identification of a lead compound that, when administered via intraperitoneal injection into mice was well tolerated and gave excellent brain exposure. Such compounds will be tested for efficacy in animal models of neurodegeneration.

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