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Research Report

Expression of klotho mRNA and protein in rat brain parenchyma from early postnatal development into adulthood

Sarah M. Clinton[a], Matthew E. Glover[a], Astha Maltare[b], Ann M. Laszczyk[b], Stephen J. Mehi[b], Rebecca K. Simmons[a], Gwendalyn D. King[b,*]

[a]Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, AL, USA
[b]Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL, USA

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ABSTRACT

Without the age-regulating protein klotho, mouse lifespan is shortened and the rapid onset of age-related disorders occurs. Conversely, overexpression of klotho extends mouse lifespan. Klotho is most abundant in kidney and expressed in a limited number of other organs, including the brain, where klotho levels are highest in choroid plexus. Reports vary on where klotho is expressed within the brain parenchyma, and no data is available as to whether klotho levels change across postnatal development. We used *in situ* hybridization to map klotho mRNA expression in the developing and adult rat brain and report moderate, widespread expression across grey matter regions. mRNA expression levels in cortex, hippocampus, caudate putamen, and amygdala decreased during the second week of life and then gradually rose to adult levels by postnatal day 21. Immunohistochemistry revealed a protein expression pattern similar to the mRNA results, with klotho protein expressed widely throughout the brain. Klotho protein co-localized with both the neuronal marker NeuN, as well as, oligodendrocyte marker olig2. These results provide the first anatomical localization of klotho mRNA and protein in rat brain parenchyma and demonstrate that klotho levels vary during early postnatal development.

1. Introduction

Klotho (kl) is a type I transmembrane protein (Kuro-o et al., 1997; Shiraki-Iida et al., 1998) that is shed from the cell surface by ADAM 10/17 cleavage (Chen et al., 2007). Shed kl protein is detectable in serum and CSF (Imura et al., 2004), allowing humoral functions body-wide. Kl protein is expressed in a limited number of organs, with the highest

Abbreviations: CSF, cerebrospinal fluid; DAB, diamino-benzidine; FGFR, fibroblast growth factor receptor; FGF23, fibroblast growth factor 23; GFAP, glial fibrillary acidic protein; IGF-1, insulin-like growth factor 1; IHC, immunohistochemistry; ICC, immunocytochemistry; Kl, klotho; mRNA, messenger ribonucleic acid; MBP, myelin basic protein; P, postnatal; qPCR, quantitative polymerase chain reaction; TGFβ, transforming growth factor receptor beta

*Correspondence to: University of Alabama at Birmingham, Department of Neurobiology, 1825 University Boulevard, Shelby 913, Birmingham, AL 35294-2182, USA. Fax: +1 205 934 6571.
E-mail address: gdking@uab.edu (G.D. King).

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levels in kidney (Kuro-o et al., 1997) where it maintains phosphate/calcium/vitamin D homeostasis via FGF23-mediated signaling (Kurosu et al., 2006). Kl also inhibits insulin/IGF-1 signaling, wnt signaling, TGF-β signaling, and modifies ion channels through its sialidase activity (Cha et al., 2008, 2009; Doi et al., 2011; Kurosu et al., 2005, 2006; Liu et al., 2007; Utsugi et al., 2000). Through its varied functions as a humoral factor and transmembrane protein, kl impacts the aging process. In addition to a dramatically shortened lifespan, kl knockout mice develop numerous age-related pathologies (Kuro-o et al., 1997; Nagai et al., 2003). Conversely, overexpression of kl in mice extends lifespan and increases resistance to oxidative stress (Kurosu et al., 2005). Human case reports demonstrate that major disruptions in kl expression or function produce extreme illness (Brownstein et al., 2008; Ichikawa et al., 2007), while minor polymorphic changes increase risk for multiple age-related disorders (Deary et al., 2005; Kawano et al., 2002; Kim et al., 2006, 2008). Kl protein levels decrease with age in the brain of rat, mouse, and non-human primate (Duce et al., 2008), and also decrease with age in human serum (Xiao et al., 2004), rodent liver and heart (Nabeshima, 2002; Shih and Yen, 2007).

Considering kl's known roles as an age-regulating protein that affects oxidative stress, mineral homeostasis, and insulin signaling, it is critical to elucidate kl's basic function(s) in the brain, the mechanisms by which its expression and/or function vary with age, and its possible role in the pathophysiology of central nervous system degenerative disorders. While much is known about kl function in kidney, considerably less is known about kl in brain. Kl knockout mice exhibit decreased memory retention compared to wild-type controls (Nagai et al., 2003) and have altered cholinergic function (Park et al., 2013). Kl knockout mice have fewer dopaminergic neurons (Kosakai et al., 2011), hippocampal neurons, and Purkinje cells, and show evidence of neuronal degeneration in cortex and hippocampus compared to wild-type mice (Kuro-o et al., 1997; Shiozaki et al., 2008). Kl knockout mouse brains are deficient in axonal transport (Uchida et al., 2001), have decreased synaptic protein expression (Li et al., 2004; Shiozaki et al., 2008), and increased markers of apoptosis (Nagai et al., 2003; Shiozaki et al., 2008) and oxidative stress (Nagai et al., 2003). To date, several reports characterized abnormalities in the kl knockout mouse brain, yet there is no anatomical map of kl expression in the brain of any species, or an understanding of how the protein functions in brain to cause the observed alterations.

Rat kl protein is 94% homologous to mouse kl, with an identical domain structure (Ohyama et al., 1998). Expression is likewise restricted to a limited number of organs with highest expression in kidney (Ohyama et al., 1998). Kl expression patterns in rat do not completely match patterns in mouse as kl is expressed in rat but not mouse lung (Kuro-o et al., 1997; Ohyama et al., 1998). Controversy exists over whether and where kl is expressed in the brain parenchyma (German et al., 2012). Outside of the choroid plexus, while kl mRNA is detected, evidence of protein expression by immunohistochemistry is limited (German et al., 2012). This may be the result of deficient detection tools, lower overall kl expression, a result of kl's currently unknown function in brain, evidence that kl in brain is predominantly shed, and/or an indicator that although mRNA is transcribed it is not translated in brain.

Beyond the molecular cloning of rat kl, nothing is known about its mRNA or protein expression pattern in rat brain beyond confirmation that kl is age-downregulated in the rat brain as it is in rhesus monkey and mouse (Duce et al., 2008). Since rats have been used as models of brain aging for many decades (Gallagher et al., 2011), understanding and manipulating kl in rat is important and would open an array of widely used behavioral tests to more deeply understand its role in cognition. While available antibody tools clearly detect kl in kidney where its transmembrane form is most concentrated, these tools are less effective for immunohistochemistry in other organs where kl expression is anticipated to be much lower. In order to comprehensively assess kl expression in the rodent brain, we undertook a two-pronged approach of (1) detecting kl mRNA by radioactive *in situ* hybridization, and (2) confirming protein expression by immunohistochemistry with an antibody we verified would specifically detect kl protein. In addition, we report the mapping of kl mRNA expression during early postnatal rat neurodevelopment.

2. Results

2.1. *Relative quantification of kl mRNA in the adult rat brain*

Although reverse-transcriptase PCR has detected kl mRNA in rat brain (Ohyama et al., 1998), no information is available as to whether it is expressed in brain parenchyma or restricted to choroid plexus outside of the brain itself. In mouse brain, kl protein is detected from total brain homogenates (Chen et al., 2013; King et al., 2011) but localization by immunohistochemistry (IHC) remains controversial, with the exception of the choroid plexus. We used quantitative PCR (qPCR) to examine regional expression of kl and compare its expression levels across organ systems. Since kl expression is consistently highest in kidney across multiple species, detection of kl mRNA in rat kidney samples served as our positive control (Fig. 1) (Kuro-o et al., 1997; Ohyama et al., 1998). As anticipated (Ohyama et al., 1998), kl mRNA was not detectible in liver, thus providing a negative control (Fig. 1). Comparing kl mRNA expression from various brain areas, the highest kl level in brain tissue was detected in choroid plexus with lesser but clearly detectible kl mRNA in both grey matter (cortex)- and white matter (fimbria)-enriched brain samples (Fig. 1).

2.2. *Kl protein expression in adult rat brain*

While qPCR allows quantitative assessment of mRNA levels, it provides no information regarding anatomical or cell-type specific localization. In our hands, anti-kl antibody AF1819 was the only commercially available antibody that functioned in IHC and showed specific labeling in the convoluted tubules of kidney and choroid plexus of brain (Fig. 2A–D). To verify antibody specificity, 8 week old wild-type and kl knockout mouse kidney and brain were likewise assessed (Fig. 2E–G). As anticipated, specific staining consistent with