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The relevance of α-KLOTHO to the Central Nervous System: some key questions

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Highlights

- α-Klotho is essential for maintaining cognitive function and brain health.
- "Brain α-Klotho" is proposed as a distinct and vital pool of α-Klotho.
- Brain α-Klotho has probable roles in neuroprotection and in promoting myelination.
- The biggest gaps in our knowledge of α-Klotho function in the CNS are identified.
- Experimental approaches to advance understanding of brain α-Klotho are suggested.

ABSTRACT

α-Klotho is well described as an anti-aging protein, with critical roles in kidney function as a transmembrane co-receptor for FGF23, and as a soluble factor in serum. α-Klotho is also expressed in the choroid plexus, where it is released into the cerebrospinal fluid. Nonetheless, α-Klotho is also expressed in the brain parenchyma. Accumulating evidence indicates that this pool of α-Klotho, which we define as brain α-Klotho, may play important roles as a neuroprotective factor and in promoting myelination, thereby supporting healthy brain aging. Here we summarize what is known about brain α-Klotho before focusing on the outstanding scientific questions related to its function. We believe there is a need for *in vitro* studies designed to distinguish between brain α-Klotho and other pools of α-Klotho, and for a greater understanding of the basic function of soluble α-Klotho. The mechanism by which the human KL-VS variant affects cognition also requires further elucidation. To help address these questions we suggest some experimental approaches that other laboratories might consider. In short, we hope to stimulate fresh ideas and encourage new research approaches that will allow the importance of α-Klotho for the aging brain to become clear.

Abbreviated Title: Klotho signaling in the CNS

Keywords: KLOTHO; CNS; cognition; signaling; aging.

1. INTRODUCTION

α-Klotho is an antiaging protein named after Klotho, the Greek goddess who "spins the thread of life" (Kuro-o et al., 1997). Since its discovery interest in α-Klotho has grown steadily, with the idea that α-Klotho mutant animals can be instructive models for understanding aging in humans. This interest has intensified, with mutations in the human *KL* gene themselves linked to longevity (Arking et al., 2002). α-Klotho loss-of-function mice display many features consistent with accelerated aging and have already produced useful mechanistic data explaining the adverse effect of kidney damage on life expectancy, and how the kidney maintains calcium and phosphate homeostasis. Most evidence so far suggests that these results are transferrable to humans. However, some key questions remain, and although α-Klotho is expressed in the brain and is vital for normal cognitive function, the role of this protein in neural tissues remains poorly understood.

We begin this article by summarizing some of the central discoveries in the α-Klotho field. Where possible we have specified the type of α-Klotho protein that was assayed or used, although this important information is often unavailable. We then focus on the function of α-Klotho in the central nervous system (CNS). It is our contention that CNS α-Klotho plays a central role in maintaining brain health throughout life, and its loss with aging contributes to neurodegeneration and impaired cognition. As the major theme for this article, we suggest some key experimental questions that need to be addressed. Some are long unanswered, such as the reason for elevated α-Klotho expression in the choroid plexus, whereas others are inspired by more recent observations. We do not expect all suggested lines of research to be fruitful; our intention is simply to stimulate debate and help foster ideas. Nonetheless, we are convinced that α-Klotho plays a critical anti-aging role in the human CNS and as such, its study may be vital for understanding the effect of aging on the brain.

2. DISCUSSION

2.1 BACKGROUND

2.1.1 Discovery of α-Klotho as an Anti-Aging Protein

The initial discovery of the murine *kl* gene, which encodes α-Klotho, was somewhat serendipitous. Heterozygous mice carrying a non-expressing copy of a randomly inserted *slc9a1* transgene were crossed and 1-in-4 of the resultant offspring displayed many features consistent with accelerated aging, such as genital atrophy, arteriosclerosis, ectopic calcification, osteoporosis, skin atrophy, infertility, and emphysema. Most strikingly, the animals have an average life span of just 61 days (Kuro-o et al., 1997). Subsequent investigations revealed that the inserted transgene had caused an 8kb deletion 6kb upstream of the first exon of *kl*, resulting in an almost complete loss of α-Klotho mRNA. Since these animals retain some α-Klotho expression, *kl*/*kl* homozygotes are considered hypomorphs rather than knockouts.

The multi-organ *kl*/*kl* hypomorph phenotype was surprising, since the authors also reported a very restricted pattern of gene expression, with transcripts encoding α-Klotho most abundant in the kidney, brain, pituitary gland and reproductive organs of wild-type animals, but undetectable in most other tissues (Kuro-o et al., 1997). Nonetheless there can be no doubt about the profound effects of losing α-Klotho expression, as the cardinal features of the *kl*/*kl* hypomorph have been replicated independently in of other loss-of-function animal models. These include two global knockouts (Olauson et al., 2012; Tsujikawa et al., 2003) and two strains of mice with *kl* mutations identified as part of an ENU screen for genes involved in ectopic calcification (Esapa et al., 2015). The restricted tissue expression pattern has also been confirmed and appears to be conserved amongst mammals (Lim et al., 2015). Strengthening the link between α-Klotho and aging further, mice overexpressing α-Klotho have been reported to live 30% longer than their wild-type littermates (Kurosu et al., 2005).

Importantly, α-Klotho is also linked to aging in humans. The so-called KL-VS variant, which contains two co-inherited amino acid substitutions, F352V and C370S, has been reported to decrease life expectancy in homozygotes. Curiously, individuals heterozygous for this allele (i.e., KL-VS carriers) have an increased life span (Arking et al., 2002) and decreased cardiovascular disease risk (Arking et al., 2005). The reason for this contradiction is unknown (see Section 3.8). Beyond KL-VS status, total levels of α-Klotho in sera and cerebrospinal fluid (CSF) from humans and mice are reported to decrease with age (Semba et al., 2014; Yamazaki et al., 2010), whilst the expression of this protein in brain white matter is reduced in mice and monkeys (Duce et al., 2008). Taken together, these observations indicate that loss of α-Klotho is central to aging in mammals.

2.1.2 Membrane-bound, shed and secreted α-Klotho

α-Klotho protein exists in five different forms – a transmembrane protein, three shed forms, and a secreted protein (Figure 1). The transmembrane and shed forms are the product of the same mRNA transcript, which initially produces a ~130kDa single-pass transmembrane protein, referred to herein as m-KL. m-KL consists of short transmembrane and intracellular sequences at the c-terminus, as well as a large extracellular section containing two KL domains, KL1 and KL2. Each of these KL domains possesses glucosidase activity and is able to cleave sialic acid from the carbohydrates of glycoproteins (i.e. desialylation). To date, only a handful of proteins have been reported as targets of α-Klotho glucosidase activity, with the best described being the calcium channels TRPV5 and TRPV6 (Chang et al., 2005; Lu et al., 2008). Desialylation of these calcium channels allows their binding to the extracellular glycan-binding protein galectin-1, leading to their clustering and retention on the plasma membrane, with a resultant increase calcium channel activity.

The shed forms of α-Klotho are released from the cell surface by proteolytic cleavage of m-KL by the metalloproteinases ADAM10 and ADAM17 (Chen et al., 2007). Two cleavage sites have been identified: the first immediately adjacent to the plasma membrane; the second

between the KL1 and KL2 domains. Cleavage at the juxtamembrane site releases a protein containing both KL1 and KL2 domains (p-KL), whereas cleavage at the second releases a protein containing just the KL1 domain (p-KL1). Cleavage at both sites leads to the shedding of p-KL1 and a second protein containing KL2 (p-KL2) (Chen et al., 2014a). By contrast, secreted α-Klotho (s-KL) is produced by an alternative splicing event that introduces a short and unique sequence immediately after KL1, producing a protein that is almost identical to p-KL1, but with a distinct C-terminus (Matsumura et al., 1998). It is not known if this small C-terminal sequence confers any functional differences between s-KL and p-KL1. As the name would suggest, it is assumed that s-KL is secreted, and likely resides only within extracellular fluids and the lumen of intracellular vesicles.

A detailed review of our current understanding of the basic biological function of α-Klotho is beyond the scope of this article. Nonetheless, it is reasonable to summarize m-KL as a component of cell surface receptor complexes, while secreted and shed forms act as humoral or endocrine factors. However, it is worth observing that very few publications make a distinction between shed and soluble forms, instead referring to "soluble α-Klotho". It is of course highly unlikely that the different types of soluble α-Klotho will have exactly the same function. Much of this vagueness has been due to the absence of selective antibodies, but we expect technical advances will allow these key details to be filled in in future.

2.1.3 α-Klotho and the CNS

Expression in the choroid plexus. The epithelial cells of the four choroid plexuses of the brain (collectively, "the choroid plexus") were identified as sites of abundant α-Klotho expression in the original description of this protein (Kuro-o et al., 1997), an observation that has since been replicated by others. At present we do not know why α-Klotho expression is high in the choroid plexus, but based on what is known about these structures, some reasonable assumptions can be made.

The choroid plexus has three roles: i) a barrier between blood and cerebrospinal fluid (CSF); ii) the major source of CSF production; and iii) a source of secreted soluble factors for the CSF (Damkier et al., 2013). Since soluble α-Klotho protein is detectable in CSF, it is reasonable to assume this protein has been produced by the choroid plexus. As such, α-Klotho expression in the choroid plexus is likely to be analogous to α-Klotho expression in the kidney (see section 2.4); in the same way the kidney supplies soluble α-Klotho to serum, the choroid produces this protein for the CSF. This comparison is perhaps unsurprising, as the kidney and choroid plexus are so similar at the gene expression level that the choroid plexus has actually been referred to as "the kidney of the brain" (Sathyanesan et al., 2012).

Expression in the rest of the brain. α-Klotho mRNA is also detectible, albeit at lower levels, in several other brain regions, including cortex, hippocampus, cerebellum, striatum, substantia nigra, olfactory bulb and medulla (Brobey et al., 2015; Clinton et al., 2013; German et al.,

2012; Li et al., 2004). Lack of suitable antibodies have hampered studies of α-Klotho expression at the protein level, but are nonetheless consistent with expression in multiple brain regions, and in both neurons and oligodendrocytes (Brobey et al., 2015; Clinton et al., 2013; Degaspari et al., 2015; German et al., 2012; Li et al., 2004). Since there is no evidence α-Klotho can cross the blood-brain barrier and there is very little fluid exchange between the CSF and the interstitial fluid of the brain parenchyma, we can be reasonably confident that α-Klotho protein detectable in the brain ('brain α-Klotho') has been expressed locally. As such we consider brain α-Klotho a distinct pool of α-Klotho, separate from serum α-Klotho and α-Klotho produced by the choroid plexus ('CSF α-Klotho'). The role of brain α-Klotho is the major focus this review.

Fascinatingly, the relative expression of s-KL mRNA compared to m-KL mRNA is higher in the brain than other organs, and far more so in humans than mice (Massó et al., 2015; Matsumura et al., 1998), suggesting that soluble forms of α-Klotho may be most important in this organ. A role for m-KL cannot be excluded entirely though. As we describe in the next section, m-KL functions as a co-receptor for FGF23 in the kidney, and it is interesting to note that a related protein, β-Klotho, acts as a transmembrane co-receptor for FGF21 in the suprachiasmatic nucleus (Bookout et al., 2013). However, m-KL is highly selective for FGF23, and there is little evidence that FGF23 is active in the brain, beyond the intriguing study of Hensel and colleagues (Hensel et al., 2016). In this paper, mouse hippocampal neurons were reported to display altered neuronal morphology and changes in cell signaling in response to recombinant FGF23. Curiously, the observed effects appear to be modulated by soluble α-Klotho, indicating that the lack of m-KL in the brain does not necessarily preclude a role for brain α-Klotho in FGF23 signaling (Hensel et al., 2016). But in any case, the combined data point towards a primary requirement for brain α-Klotho as s-KL, and suggest that this role may be especially important more developed brains of humans.

Requirements of α-Klotho for brain function. The majority of *in vivo* studies have not distinguished between pools of α-Klotho, but the evidence suggests that this protein is required at many levels for normal brain health. The initial report of *kl*/*kl* hypomorphs described hypokinesis at 5-weeks of age caused by degeneration of Purkinje cells in the cerebellum (Kuro-o et al., 1997). Subsequent investigations in the hippocampi of these animals have reported loss of synapses, perturbations in axonal transport, alterations in neurofilaments and accumulation of lysosomes (Li et al., 2004; Shiozaki et al., 2008; Uchida et al., 2001). Suggesting a relevance of α-Klotho to Parkinson's disease, midbrain dopaminergic neurons also show evidence of neurodegeneration (Kosakai et al., 2011), while anterior horn cells present degenerative features similar to those found in humans with amyotrophic lateral sclerosis (Anamizu et al., 2005). Unsurprisingly, by seven-weeks of age the mice display severe cognitive impairments, associated with cholinergic dysfunction (Park et al., 2013), oxidative damage and apoptotic stress. The CNS phenotype of *kl*/*kl* mice, as well as the action of certain treatments reported to ameliorate the degeneration, such as

α-tocopherol (Nagai et al., 2003) and melatonin (Shin et al., 2015), are shown in Figure 2. Supporting these data, mice over-expressing α-Klotho show improved cognition and appear resistant to neurodegeneration caused by overexpression of the Alzheimer's disease protein hAPP (Dubal et al., 2014; Dubal et al., 2015), and also by treatment with MPTP, a well-established Parkinson's disease model (Brobey et al, 2015).

As we explain in Section 2.4, much of the brain phenotype of *kl*/*kl* mice appears to be secondary to kidney damage. Nonetheless there is good evidence of a specific role for brain α-Klotho in maintaining normal brain function. In particular, lower α-Klotho mRNA levels have been reported in hippocampi taken from post-mortem human epilepsy patients (Teocchi et al., 2013), and in the white matter of aged rhesus monkeys (Duce et al., 2008). Interestingly, CSF α-Klotho is also reported to decrease with age and in patients with Alzheimer's disease and relapsing-remitting multiple sclerosis (Aleagha et al., 2015; Semba et al., 2014)

A requirement for α-Klotho in human cognition has largely been corroborated through studies of the KL-VS variant. These studies suggest a discordance between the phenotypes of KL-VS hetero- and homozygotes that mirror observations for longevity. In the first such study, Deary and co-workers reported decreased IQ in individuals homozygous for KL-VS, with heterozygotes unchanged compared to controls (Deary et al., 2005). By contrast, two subsequent publications reported improved cognition in heterozygotes (Dubal et al., 2014; Yokoyama et al., 2015). Consistent with a link between α-Klotho and neurodegeneration, KL-VS carriers were also found to display increased brain volumes (Yokoyama et al., 2015). Lack of numbers prevented KL-VS homozygotes from being studied as thoroughly, but it is fascinating to note that a strong trend towards decreased brain volume was observed in the few individuals examined (Yokoyama et al., 2015). Thus, mirroring the effect of KL-VS on longevity, higher brain function appears enhanced in KL-VS carriers, but weakened in homozygous individuals.

Effects of α-Klotho on neural cells in vitro. Supporting brain α-Klotho acting as a soluble factor are the growing number of papers reporting biological effects of recombinant p-KL or p-KL1 protein on neural cell lines. Indeed, the evidence that soluble α-Klotho is neuroprotective appears overwhelming. These include resistance to oxidative and endoplasmic reticulum stresses, and protection from cytotoxicity elicited by the Alzheimer's disease protein Aβ and glutamate excitotoxicity (Cheng et al., 2015; Zeldich et al., 2014). A number of cell signaling pathways have been reported as modulated by α-Klotho and suggested to underlie these observations, including the PI3K/Akt, ASK1/p38 MAPK and PERK pathways (Banerjee et al., 2013; Brobey et al., 2015; Zeldich et al., 2014). Experiments by Xin and colleagues, showed α-Klotho participation in oxidative stress resistance in cortical neurons and cerebellar granule cells. Stimulation of α-Klotho expression by DNA demethylation, decreased apoptotic cell death under H_2O_2 treatment (Xin et al., 2015).

Conversely, the protective effect was lost with α-Klotho inhibition with shRNA. The effects α-Klotho on cell signaling pathways in neural cells are depicted in Figure 3.

Recombinant p-KL also affects the behavior of other neural cell types. In particular, work from Carmela Abraham's laboratory has identified oligodendrocytes as a likely target of soluble α-Klotho. These studies have reported that recombinant p-KL promotes the differentiation and myelination of a human oligodendrocytic hybrid cell line (Chen et al., 2015a), and the maturation of rat oligodendrocytes *in vitro* (Chen et al., 2013a), via the co-ordinate regulation of several key signaling pathways including Wnt, NFκB, p53, Akt and ERK. Since *kl*/*kl* hypomorphic mice display decreased myelination (Chen et al., 2013b) and mice over-expressing α-Klotho display enhanced remyelination after cuprizone-induced demyelination (Zeldich et al., 2015), the control of myelination seems a likely physiological role for brain α-Klotho.

2.1.4 α-Klotho and the Kidney

Although this article focuses on the CNS, α-Klotho expressed in the kidney needs some mention, since, as alluded to above, this pool of α-Klotho has a clear influence on the CNS. In the kidney the primary function of α-Klotho is in its m-KL form, acting as a co-receptor for FGF23. This role is highly conserved throughout evolution, certainly as far as zebrafish (Mangos et al., 2012) and perhaps as far as nematodes (Château et al., 2010). Through mediation of FGF23 signaling, m-KL forms part of a multi-organ regulatory mechanism that controls the levels of circulating calcium, phosphate and vitamin D (Erben and Andrukhova, 2016). Importantly, much of the *kl*/*kl* phenotype, including neuropathological and cognitive defects, can be attributed to loss of renal FGF23 signaling. Most strikingly, an animal with kidney-specific deletion α-Klotho ("*Six2-kl*") has been reported to phenocopy *kl*/*kl* mice (Lindberg et al., 2014). Although the cognitive function and neuropathology of *Six2-kl* mice is unreported, it is not unreasonable to assume an impairment since like *kl*/*kl* mice these animals have elevated vitamin D levels (Lindberg et al., 2014). Restoring vitamin D levels can rescues much of the *kl*/*kl* phenotype, including loss of dopaminergic neurons (Carpinelli et al., 2011; Kosakai et al., 2011; Leibrock et al., 2016).

Taken together, these observations indicate that by regulating vitamin D homeostasis (and perhaps also calcium and phosphate) renal m-KL is essential for health throughout the body, including the brain. However, the state of the kidney may influence brain health in multiple ways. Cognitive impairment is common in patients with chronic kidney disease (CKD), and within the general population decreased kidney function is a risk factor for future cognitive decline (Hermann et al., 2014). The mechanism by which CKD causes cognitive decline appears distinct to the neurological defects seen in *kl*/*kl* mice since CKD is an established cause of *decreased* vitamin D levels. However, the kidneys are also the source of the majority (perhaps all) of the soluble α-Klotho in serum (Lindberg et al., 2014), and increased levels of serum α-Klotho are correlated with reduced risk of cognitive decline (Shardell et al.,

2016). Intriguingly, a number of publications report decreased serum α-Klotho in CKD, while serum α-Klotho levels are also reduced in rodent kidney damage models. Thus, brain health may be subject to control via the function of renal m-KL, and also through soluble forms of α-Klotho produced in the same organ.

Data from our own study indicate another layer of complexity in the linkage between the kidney, the CNS and α-Klotho. Using 5/6-nephrectomized rats to study cognitive performance following kidney damage, we found that approximately half the nephrectomized animals developed clear memory impairment in the inhibitory avoidance test. Intriguingly, in comparison to both sham surgery controls and nephrectomized animals that did not develop memory impairment, these animals displayed a marked decrease in levels of α-Klotho in the pre-frontal cortex. Thus, in addition to causing cognitive impairment and decreasing serum α-Klotho, kidney damage also reduces α-Klotho in the brains of animals, a decrease that is associated with cognitive impairment (Degaspari et al., 2015).

2.2 OUTSTANDING QUESTIONS

2.2.1 What does brain α-Klotho do?

Current data point towards brain α-Klotho functioning as a soluble factor that alters neural cell behavior by modulating intracellular signal transduction cascades. However, this highlights one of the more conspicuous gaps in our knowledge: the missing identity of the membrane receptor(s) through which soluble α-Klotho signals. Both m-KL and soluble forms have been reported to inhibit canonical Wnt signaling by interfering with the interactions between Wnt ligands and their receptors (Liu et al., 2007; Sun et al., 2015). Suggesting that this is relevant to the brain, p-KL represses Wnt signaling in oligodendrocyte cell models (Chen et al., 2013a; Chen et al., 2013b), and the suppression of this pathway has been shown by others to promote oligodendrocyte differentiation (Guo et al., 2015). However, Wnt inhibition is highly unlikely to be the only mechanism by which brain α-Klotho signals. Both in oligodendrocyte and neurons, recombinant α-Klotho stimulates numerous other signaling pathways. Moreover, the rapid activation of these cascades is largely inconsistent with them being downstream of canonical Wnt inhibition. As such, one must assume that alternative protein targets for brain α-Klotho are present in the brain.

One intriguing possibility given the established nature of the brain as an electrically active organ, is that via its glucosidase activity α-Klotho may regulate the function of glycosylated ion channels and transporters. In principle, modulation of electrochemical gradients on neural cell membranes could affect the activity of many cell signaling cascades. At present we know very little about which glycoproteins are targeted by α-Klotho (in the brain or elsewhere), although we note that the renal α-Klotho targets TRPV5 and TRPV6 are expressed in brain (Kunert-Keil et al., 2006). Furthermore, galectin-1, which binds desialated

TRPV5 and TRPV6 and mediates their clustering, is reported to be neuroprotective (Wang et al., 2015). With this in mind it is also worth highlighting the growing links between α-Klotho and Na,K-ATPases, a family of sodium/potassium pump present on plasma membranes. This connection was first described in a report that m-KL directly interacts with the Na, K-ATPase α1 subunit in kidney, parathyroid and choroid plexus, and facilitates the recruitment of the Na, K-ATPase α1 subunit to the plasma membrane in response to Ca^{+2} oscillations (Imura et al., 2007). As extracellular Ca^{2+} levels decrease, α-Klotho expression is upregulated, increasing α1 subunit transport to the membrane and Na pump activity. This response is lost in *kl*/*kl* mice (Imura et al., 2007). Intriguingly, subsequent data suggest that p-KL also enhances Na, K-ATPase activity, and that this effect is entirely dependent on glucosidase activity (Sopjani et al., 2011). As such, if we assume brain α-Klotho acts primarily as a secreted factor, Na,K-ATPases could well be key targets of this protein. However, this story does not end here. In addition to maintaining plasma membrane electrochemical gradients, Na,K-ATPases are now established as an unusual class of transmembrane receptors that mediate signaling elicited by ouabain and related compounds (Kawamoto et al., 2012; Lima et al., 2013). Through interactions with Src tyrosine kinases and IP3-receptors, Na,K-ATPases mediate the activation of Src tyrosine kinase- and intracellular Ca^{+2}-dependent signaling cascades, respectively (Aperia et al., 2016; Madan et al., 2016). Although classically described as a compound that affects the heart, ouabain is emerging as a potent neuroprotective agent (Kinoshita et al., 2016), and the Na^+/K^+-ATPase α_3 isoform plays an important role in the control of spatial learning and memory (Holm et al., 2016). Taken together, these observations make a role for brain α-Klotho as a regulator of Na,K-ATPase-dependent cell signaling an exciting suggestion.

In any case, the functions of brain α-Klotho are a long way from being fully resolved. At present, we would argue that the lack of knowledge regarding the physiological targets of soluble forms of α-Klotho is a major limitation. Thus, we would argue that there is a need for unbiased glycomics approaches to identify substrates of α-Klotho glucosidase activity in neural tissues. One might imagine a study where isolated membrane fractions from brain are treated with and without recombinant α-Klotho, and glycoproteins affinity purified with carbohydrate-binding proteins, prior to identification by mass spectrometry. In principle, the desialylation of α-Klotho substrates will alter their affinity for the relevant carbohydrate-binding protein; for example, if galectin-1 is used, the amount of the α-Klotho substrate TRPV5 purified would increase significantly after treatment with recombinant α-Klotho. In this way, we would expect new and perhaps unexpected targets of brain α-Klotho to announce themselves, hopefully leading to more solid answers for the function of this protein in future.

2.2.2 Is brain α-Klotho involved in cognition?

The evidence from mice and from humans is clear; α-Klotho is required for normal higher brain function, with cognitive performance correlated with levels of soluble α-Klotho in serum. But can we say the same specifically for brain α-Klotho? At present, no. All studies performed to date have been unable to distinguish between the requirement for brain and other pools of α-Klotho. Indeed, brain α-Klotho may affect the behavior of neurons and oligodendrocytes *in vitro*, but when it comes to tests of learning and memory performed *in vivo*, there is perversely more evidence of a requirement for α-Klotho produced in the kidney.

However, we are now in a position where direct testing of the requirement for brain α-Klotho in higher brain function is possible, using Cre-mediated brain-specific knockout of α-Klotho (or alternatively, brain region-specific knockout). In particular, the klotho$^{flox/flox}$ mice described by Olauson and colleagues that contain Flox sites either side of *kl* exon 2 (Lindberg et al., 2014; Olauson et al., 2012), could be crossed with a suitable Cre strain. For example, crossing with Thy1-cre mice would ablate α-Klotho expression in the hippocampus and forebrain. Such an animal would be expected to express α-Klotho normally outside the brain and thus avoid the debilitating consequences of decreased kidney α-Klotho. Examining this animal in conventional rodent behavioral tests (e.g. inhibitory avoidance, Morris water maze, elevated plus maze etc.) in comparison to appropriate littermate controls, should demonstrate convincingly whether α-Klotho expressed in brain is necessary or dispensable for normal cognition.

2.2.3 Is brain α-Klotho neuroprotective?

α-Klotho is required for maintaining the health of a variety of tissues, and has been shown in *in vivo* studies of organs other than the brain to regulate processes that are known to affect neuroprotection, such as inflammation (Liu et al., 2011; Maekawa et al., 2009; Zeng et al., 2016) and metabolic sensing pathways (Château et al., 2010; Kurosu et al., 2005). Nonetheless, the most significant data suggesting a direct neuroprotective action of α-Klotho come from studies of neuronal cell death *in vitro*: we are unaware of any direct studies *in vivo*. To address this question we would suggest a brain-specific knockout strategy akin to that described in the previous section. However, rodents are relatively resistant to neurodegeneration – for example, several mutations that cause Parkinson's disease in man fail to elicit any overt phenotype in mice – and thus any loss of neurons may be below detectable thresholds. To overcome this potential problem, we would recommend also investigating the effects of brain-specific α-Klotho knockout in an established and reliable neurodegenerative disease background, such as the 3xTg Alzheimer's disease mouse model (Oddo et al., 2003). Here, with robust levels of neurodegeneration guaranteed, the consequence of losing a potential neuroprotective factor might be more easily observed.

This loss-of-function approach would, of course be strengthened if the effect of increasing brain α-Klotho were studied in parallel. At present, we do not have means to do this

genetically, but levels of soluble α-Klotho in the brain could be increased through stereotactic injection of recombinant protein or viral transduction of DNA encoding α-Klotho. In principle, combined loss- and gain-of-function data would make a powerful case for the status of brain α-Klotho as neuroprotective.

2.2.4 Does loss of serum α-Klotho cause cognitive decline?

Interestingly, impaired cognition is a consequence of both kidney damage or CKD, and decreased α-Klotho production in the kidney. However, the mechanisms appear distinct, since loss of renal α-Klotho exerts its effects, at least in part, through hypervitaminosis D, whereas CKD causes decreased vitamin D levels. Nonetheless, both situations lead to decreased levels of soluble α-Klotho in serum. Since serum α-Klotho is itself linked to cognitive decline, these observations lead to a fairly natural question: do serum α-Klotho levels affect higher brain function?

The obvious approach to answering this question would be to generate mice that specifically lack serum α-Klotho. However, this will not be easy. In the mouse, the vast majority of serum α-Klotho (perhaps all) is produced by proteolysis of renal m-KL. Any loss-of-function strategy will therefore have to reduce shedding without compromising m-KL expression and function. This is not impossible though. Chen and colleagues have described a metalloproteinase resistance m-KL mutant with severely reduced shedding (~94%), but only a minor weakening in FGF23 signaling (Chen et al., 2014a). In principle therefore, a knock-in mouse containing a metalloproteinase resistant m-KL could be generated. However, to address the importance of serum α-Klotho we would suggest the first step would be to determine whether loss of serum α-Klotho caused by kidney damage is necessary for the effects of kidney damage on cognition. Specifically, whether the administration of recombinant α-Klotho protein will rescue learning impairments in the nephrectomy model. Importantly, this strategy appears feasible, since intraperitoneal injections of α-Klotho have been used to ameliorate symptoms and extend life expectancy by 17.4% in *kl*/*kl* mice (Chen et al., 2013a).

2.2.5 How does kidney damage cause loss of α-Klotho in the brain? Is it via decreased serum α-Klotho?

Our observation of decreased α-Klotho protein in the pre-frontal cortex of nephrectomized mice (Degaspari et al., 2015) indicates that kidney damage can affect α-Klotho expression in other organs, and adds to the mounting evidence that renal impairment impacts on cognition. However, our work also raises the questions of how kidney damage is able to elicit decreased brain α-Klotho, and whether this is dependent upon loss of serum α-Klotho.

The involvement of serum α-Klotho can be answered using the same approaches described for investigating the role of this pool of α-Klotho in cognition; determining whether decreased brain α-Klotho following nephrectomy can be rescued with intraperitoneal

injections of recombinant α-Klotho. Nonetheless, when attempting to uncover the mechanism linking two biological events it is always sensible to look from both ends. Thus, an additional consideration is how brain α-Klotho expression levels are regulated. Since brain α-Klotho was measured by ELISA in our study, we are unable to say if decreased levels are due to lowered expression or increased turnover. We are unaware of any studies investigating the control of α-Klotho at the level of its translation or protein stability, although α-Klotho has been reported as being ubiquitinated (Wagner et al., 2012), suggesting protein stability could perhaps be under dynamic control. Some studies of α-Klotho gene expression have been reported, indicating that the *kl* promoter can be activated by testosterone and the androgen receptor (Hsu et al., 2014), and by the transcription factor Egr-1 (Choi et al., 2010). Perhaps most intriguingly however, α-Klotho transcription is strongly repressed by promoter methylation (Azuma et al., 2012; King et al., 2012; Sun et al., 2012). This phenomenon has been suggested to underlie the tightly controlled tissue-specific pattern of α-Klotho expression (Azuma et al., 2012) and is reported to increase with age (King et al., 2012). Interestingly, increased *KL* promoter methylation has also been described in a number of cancers, including cervical carcinoma, colorectal, breast and gastric cancers (Lee et al., 2010; Pan et al., 2011; Rubinek et al., 2012; Wang et al., 2011). Fascinatingly, promoter methylation may be important to our observations. In studies combining *in vivo* experiments in the murine kidney and *in vitro* work in human tubular cells, Sun and co-workers found that elevated levels of uremic toxins – a side-effect of kidney impairment – lead to increased expression of DNA methyltransferase (Sun et al., 2012). This, in turn, was found to cause *kl* promoter hypermethylation and down-regulation of α-Klotho transcription. Since uremic toxins are believed to act via inflammatory pathways and we see evidence of neuroinflammation in our system, it is quite possible that elevated promoter methylation underlies the decreased brain α-Klotho seen in nephrectomized rats. Supporting this idea further, *kl* promoter methylation can be modulated in the brain, since an age-dependent increase that correlates with decreased protein expression has been reported in the white matter of rhesus monkeys (King et al., 2012). Altered promoter methylation in the brains of our animals is clearly a hypothesis that can be tested relatively easily.

Finally, a very simple alternative mechanism for the control of brain α-Klotho by kidney damage should be acknowledged. As we have mentioned, there is no evidence that serum α-Klotho is able to cross the blood-brain barrier, but we are unaware of any studies testing this directly. If serum α-Klotho can cross the blood-brain barrier, it is not inconceivable that much or all of the pre-frontal cortex α-Klotho protein measured by ELISAs has in fact come from the kidney. Although this mechanism is perhaps unlikely, it is undeniable that this would be sufficient to explain the observed decrease in α-Klotho following nephrectomy.

2.2.6 Could increasing α-Klotho treat neurodegenerative disease?

As we have outlined, α-Klotho expression is linked by numerous lines of evidence to brain health, including protection from neurodegeneration in a mouse Alzheimer's disease model (Dubal et al., 2015). The relative contributions of the different pools of α-Klotho remain to be determined, but the data suggest that therapeutic strategies to increase global α-Klotho levels may have potential to halt cognitive decline and increase neuronal survival (Abraham et al., 2012).

As illustrated in Figure 4, a growing list of factors have been reported to influence α-Klotho expression, both positively and negatively. Interestingly, these include soluble Amyloid precursor protein, which upregulates α-Klotho expression (Li et al., 2010), indicating that in pathological amyloidogenic conditions such as Alzheimer's Disease, proper α-Klotho expression might be impaired. Consistently, α-Klotho is decreased in the serum and brain of AD animal models (Kuang et al., 2014; Massó et al., 2015) and also in the CSF of humans (Semba et al., 2014).

More relevant for potential treatments to elevate α-Klotho expression is the work of Kuang and colleagues, who studied the effect of the neuroprotective compound ligustilide in SAMP8 mice, an accelerated aging model that develop a number of Alzheimer's disease features, such as Aβ accumulation and tau phosphorylation (Kuang et al., 2014). Strikingly, alongside improvements in numerous measures of brain health, this report found ligustilide to elevate levels of α-Klotho in serum and in the choroid plexus (Kuang et al., 2014).

There are two limitations to the Kuang study. Firstly, the authors do not show whether α-Klotho actually contributes to the neuroprotective mechanism of ligustilide. Presumably the expression levels of other genes may be changed, some of which may also be involved in the neuroprotection. Secondly, SAMP8 mice have an aging, rather than a pure neurodegenerative phenotype – the extent to which these animals represent human Alzheimer's disease is open to debate. Thus, to test the effect of ligustilide further, we would suggest studying the effects of compounds reported to elevate α-Klotho expression in a more recognized Alzheimer's disease model. In addition, to provide some evidence that α-Klotho is involved in neuroprotection, we would suggest the need for parallel studies examining the effect of unrelated α-Klotho elevating compounds. Although such experiments do not prove a requirement for α-Klotho, they would certainly strengthen the case.

2.2.7 What is the target of Choroid Plexus / CSF α-Klotho?

As already observed, little is known about the role of choroid plexus α-Klotho, although a role as a transmembrane receptor can probably be discounted, since *in vitro* studies have shown that the only FGF receptor transmembrane α-Klotho cannot form a co-receptor complex with is FGFR2, and FGFR2 is the only FGF receptor isoform expressed in murine choroid plexus (Reid and Ferretti, 2003). Thus, it is very unlikely that α-Klotho is able to

mediate FGF23 signaling in choroid plexus epithelial cells. As such, the assumed reason for high α-Klotho expression in the choroid plexus is for it to be released into the CSF as a neuroendocrine factor.

This, though, leads to another key question: what is the target of CSF α-Klotho? Since there is little exchange between the interstitial fluid of the brain parenchyma and the CSF, the most likely targets are within the brain stem and spinal cord. In agreement with this, certain data point towards the medulla oblongata, a neuronal mass involved in regulating blood pressure. It is well established that altering the chemical composition of the CSF can affect the medulla oblongata, and a small number of publications suggest that CSF α-Klotho may be one such factor. Specifically, intracerebroventricular injection of α-Klotho siRNA has been reported to increase blood pressure in both Sprague Dawley (Wang and Sun, 2010) and WKY rats (Chen et al., 2015b). This same treatment appears to impair the baroreflex – the rapid negative feedback mechanism by which the body reacts to sudden changes in blood pressure by altering the heart rate (Chen et al., 2015b). Conversely, in animals with impaired baroreflexes, increasing CSF α-Klotho by intracerebroventricular injection of recombinant protein elicits an improved response (Chen et al., 2015b; Chen et al., 2014b). Interestingly, siRNA-mediated knockdown of α-Klotho was confirmed in choroid plexus samples by PCR and Western blotting (Wang and Sun, 2010), but only by Western blotting in the medulla oblongata (Chen et al., 2015b) . This last observation is fascinating since it is consistent with α-Klotho being able to modulate the medulla oblongata, but does not demonstrate where this protein came from. In principle, the protein is likely to be soluble α-Klotho produced in the choroid plexus, but the possibility that it was made in the medulla oblongata itself cannot be excluded. There is therefore a clear need to demonstrate α-Klotho expression in the medulla oblongata at the mRNA level. But in any case, taking these papers together, it is clear that α-Klotho is central to the maintenance of normal blood pressure. Furthermore, it is likely that this involves endocrine modulation of the medulla oblongata by α-Klotho from the choroid plexus.

Intriguingly, this hypothesis can perhaps be extended further, since rats subjected to chronic unpredictable stress show decreased α-Klotho expression in the choroid plexus (Sathyanesan et al., 2012). The levels of CSF α-Klotho were not measured in this study, but if we assume CSF α-Klotho is produced by the choroid plexus, it is likely that CSF α-Klotho is also reduced. As such, it is not unreasonable to speculate that levels of α-Klotho in CSF may be inversely correlated with physiological stress, and that decreased CSF α-Klotho may be involved in raising the blood pressure of stressed individuals. This connection between stress and reduced α-Klotho is strengthened by the observation that women with chronic psychological stress caused by raising a child on the autistic spectrum have lower levels of α-Klotho in serum than women raising a non-autistic child (Prather et al., 2015). Thus, it is quite plausible that stress decreases α-Klotho expression at multiple locations, and is therefore central to the effects of elevated stress on aging.

We would suggest that the above reports make the medulla oblongata a sensible starting point for studies aimed at identifying the targets of CSF α-Klotho. Initial experiments testing this idea could take the form of *ex vivo* electrophysiological studies on the medulla oblongata, with recordings taken in the presence or absence of recombinant α-Klotho. Parallel investigations could in principle be performed comparing explants from α-Klotho deficient and wild-type animals, although these would require prior establishment of whether α-Klotho is expressed in the medulla oblongata or not.

To confirm these data at the whole animal level, CSF α-Klotho can be knocked down by intracerebroventricular injection of siRNA, while recombinant α-Klotho can be efficiently administered via the same route (Chen et al., 2015b; Wang and Sun, 2010) . However, loss-of-function studies can perhaps be performed more cleanly with the use of choroid plexus specific knockout. In particular, Crouthamel and co-workers have reported the generation of transgenic mice with choroid plexus-specific expression of cre (Crouthamel et al., 2012). This animal can in principle be crossed with a floxed α-Klotho mouse. Thus, the tools exist to uncover further the function of CSF α-Klotho.

2.2.8 What explains the opposing phenotypes of *KL-VS* carriers and homozygotes on cognition?

The association between the KL-VS allele and altered higher brain function clearly demonstrate that α-Klotho function is conserved in humans. Nonetheless the apparent contradiction between data for heterozygous and homozygous individuals makes this evidence hard to interpret and leads to a mechanistic conundrum: how can one copy of KL-VS be good, but two copies bad?

We would suggest that the answer is in fact quite straightforward. KL-VS might enhance one function of α-Klotho while simultaneously compromising another such that when one KL-VS allele is present the benefits of the gained function outweigh the deficits of the partially lost function. However, when two copies are present the loss of function becomes too great to be overcome.

Intriguingly, certain data support this idea. KL-VS is reported to enhance secretion of s-KL (Arking et al., 2002), and in agreement, KL-VS heterozygotes display a small increase in serum α-Klotho (Dubal et al., 2014). KL-VS carries also appears to enhance m-KL function, by increasing the interaction between this protein and FGF receptors, thereby strengthening FGF23 signaling (Tucker Zhou et al., 2013). At the same time, KL-VS is reported to reduce glucosidase activity (Arking et al., 2002). Taking these observations together, a prediction can be made. In heterozygotes, KL-VS causes a decrease in glucosidase activity that can be tolerated; any negative effects are outweighed by improved FGF23 signaling and/or higher levels of s-KL. In KL-VS homozygotes however, FGF23 signaling and s-KL secretion are

increased further, but the loss of glucosidase activity now becomes a limitation, presumably to such an extent that the individual's health is jeopardized.

Testing these ideas in animal models will be difficult, as it will require separating the functions of membrane-bound from shed and soluble forms of α-Klotho, as well as distinguishing the effects of altered glucosidase activity from gains/losses of all function. However, key to this is the requirement for α-Klotho glucosidase activity. At present we have no evidence that this enzymatic activity is required for cognition.

Thus as a first step, we would contend that the need for α-Klotho glucosidase activity *in vivo* should be determined. Traditionally, the requirement for an enzymatic activity – as opposed to the requirement for the expression of an enzyme – can be demonstrated in one of two ways: by pharmacological inhibition, or with the use of a catalytically inactive mutant. To the best of our knowledge there are no specific α-Klotho inhibitors available, although the modified sugar D-Saccharic acid 1,4-lactone monohydrate has been used *in vitro* (e.g.(Sopjani et al., 2011)). Thus direct inhibition *in vivo* is currently not possible. By contrast, a knock-in mouse lacking glucosidase may be possible, as the key glutamic acid residues within the two catalytic sites have been identified (Tohyama et al., 2004). Mutating these residues for unrelated amino acids can be expected to ablate the glucosidase activity of α-Klotho. Before these mutations can be introduced into a transgenic animal one would first need to confirm *in vitro* that catalytic activity is lost but that the other functions of α-Klotho are retained. However, in principle at least, a genetic approach like this can verify the requirement for glucosidase activity *in vivo*, which will take us closer to resolving the mystery behind cognition and longevity in the KL-VS variant.

4. CONCLUSIONs

We believe the above data make a clear case for a central role for α-Klotho in controlling cognition in many mammal species, including man. In healthy animals α-Klotho appears to fulfil a homeostatic purpose, necessary for the normal survival and function of neurons and other brain cells, although the precise nature of that role remains to be elucidated. With age, and in response to various insults to the health of the organism, α-Klotho expression falls, leading to reductions in higher brain function that typically occur with aging. It is likely that these events parallel age-related changes to the functions of other bodily organs following decreases in α-Klotho.

However, a number of key experimental questions need addressing before the role of α-Klotho in cognition can be fully understood. We hope to have contributed towards defining these challenges. Nonetheless, beyond the role of brain α-Klotho, fundamental gaps in our knowledge about the basic biology of this protein remain. As mentioned, the roles of the different types of α-Klotho need to be further elucidated. Moreover, there is some evidence to suggest α-Klotho may behave differently in males and females, since levels are higher in

the CSF of men than women (Semba et al., 2014), and α-Klotho gene expression can be induced by testosterone (Hsu et al., 2014). The potential implications of these observations are intriguing and further work is clearly needed. But in any case, in light of the connections between decreased α-Klotho expression and a growing list of age related conditions, it is clear that the study of this protein will be hugely beneficial for understanding human aging, in the brain and beyond.

AUTHOR CONTRIBUTIONS

Conceived and designed the manuscript: CS, EMK and DCB. Wrote the manuscript: MML-C, CHYM and DCB. Final revision: MML-C, CHYM, CS, EMK and DCB

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LEGENDS

Figure 1. Different forms of α- Klotho protein. Two different transcripts can be generated from *klotho* gene. mRNA 1 is translated to the membrane form of α- Klotho (m-KL), which can be shed by ADAM 10 or ADAM 17 and produce a number of soluble forms. Another soluble form is obtained from the mRNA 2 (an alternative splicing variant), this protein has an additional C-terminal sequence, and is secreted to the extracellular medium.

Figure 2. α-Klotho and CNS function: Lessons from *klotho*$^{-/-}$ mice. Here we summarize the main findings from studies of *kl*/*kl* mice which are directly related to impaired synaptic function, cholinergic signaling, oxidative and apoptotic stress. All of these features are well known to be related to impaired cognitive function. Depicted in purple are therapeutic interventions that have been reported to be capable of reverting these alterations and improving cognition in *kl/kl* mice.

Figure 3. Effects of increased α-Klotho in neural cell signaling pathways. A number of cell signaling pathways have been reported to be modulated by α-Klotho. The data point to important roles in neuroprotection and myelination.

Figure 4. The modulation of different α-Klotho pools following potentially harmful or protective stimuli. A number of studies have reported factors that can up or downregulate α-Klotho levels, reinforcing its relevance as a neuroprotectant and as a therapeutic target for many diseases. But an important question remaining is if and how the different α-Klotho pools interact.

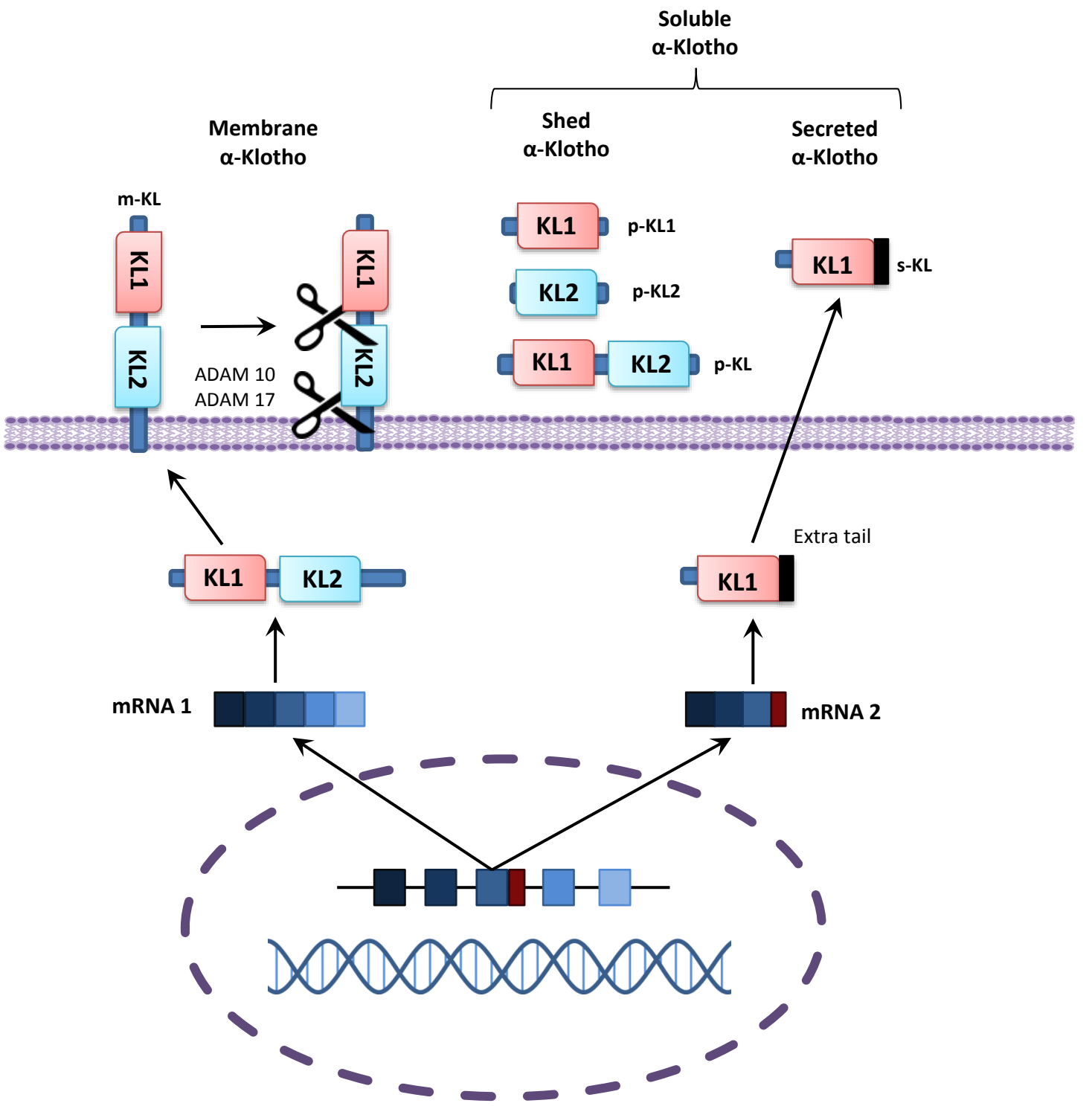


Figure 1. Different forms of α- Klotho protein. Two different transcripts can be generated from klotho gene. mRNA 1 is translated to the membrane form of α- Klotho (m-KL), which can be shed by ADAM 10 or ADAM 17 and produce a number of soluble forms. Another soluble form is obtained from the mRNA 2 (an alternative splicing variant), this protein has an additional C-terminal sequence, and is secreted to the extracellular medium.

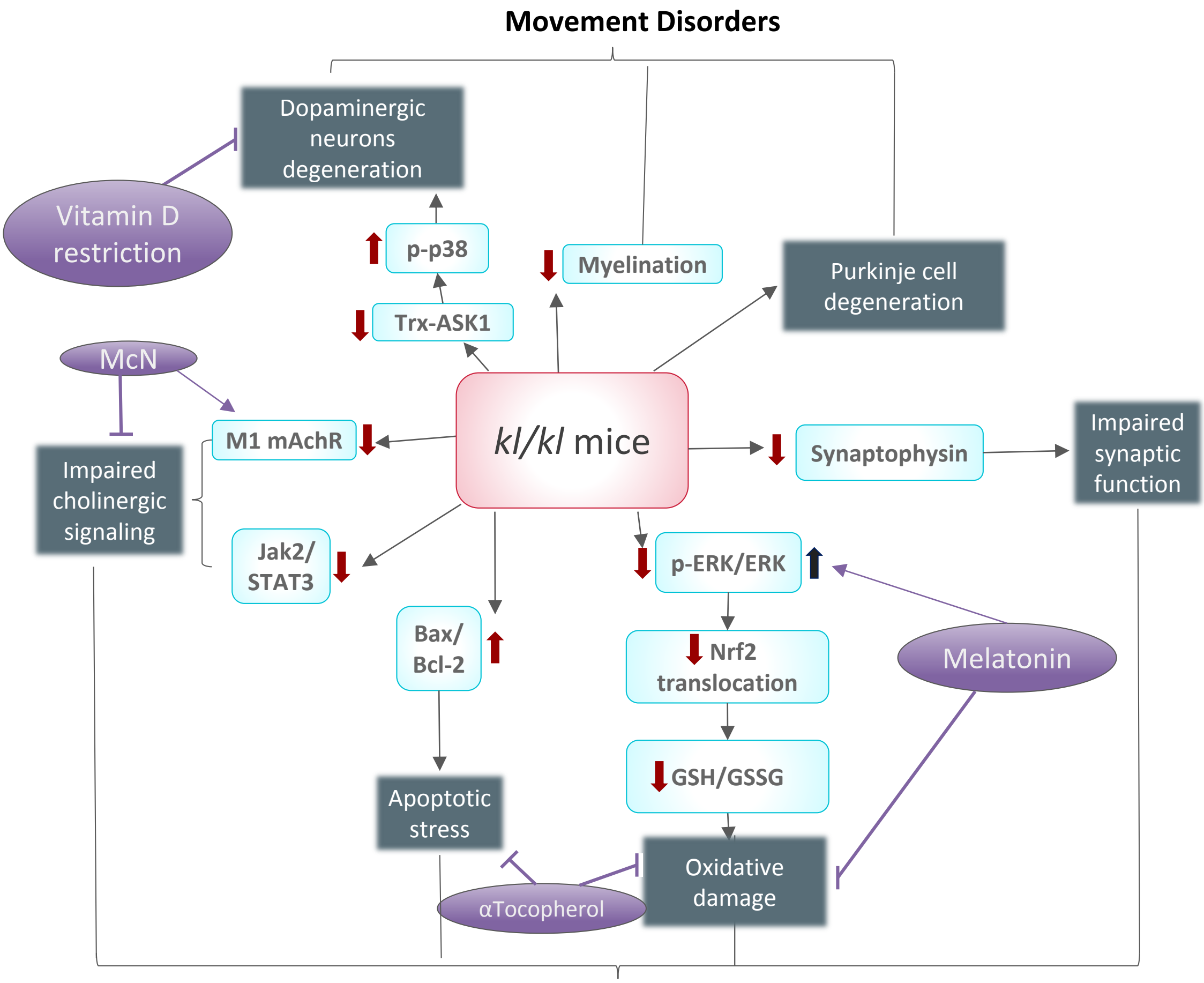
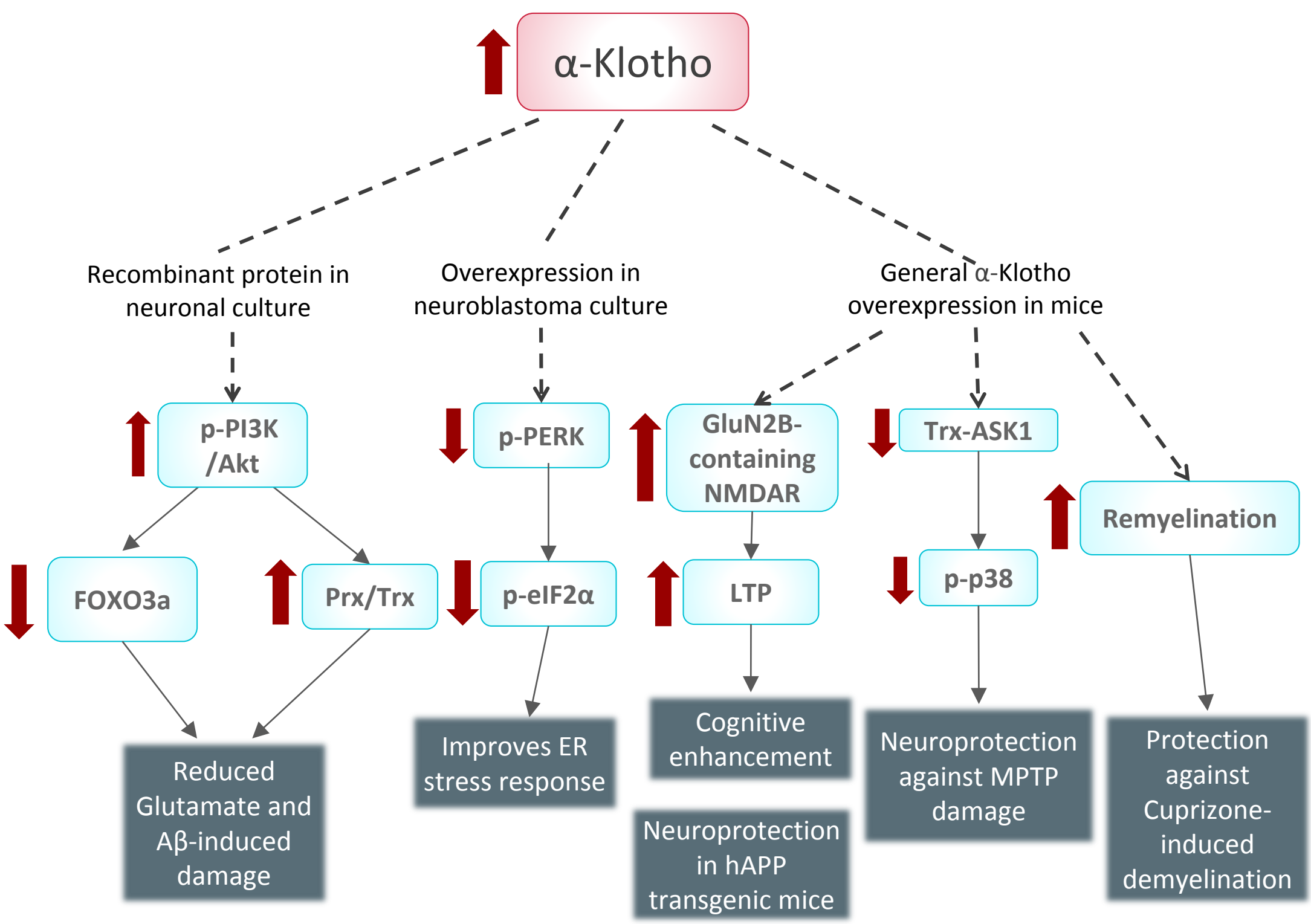


Figure 2. α-Klotho and CNS function: Lessons from klotho-/- mice. Here we summarize the main findings from studies of kl/kl mice which are directly related to impaired synaptic function, cholinergic signaling, oxidative and apoptotic stress. All of these features are well known to be related to impaired cognitive function. Depicted in purple are therapeutic interventions that have been reported to be capable of reverting these alterations and improving cognition in kl/kl mice.



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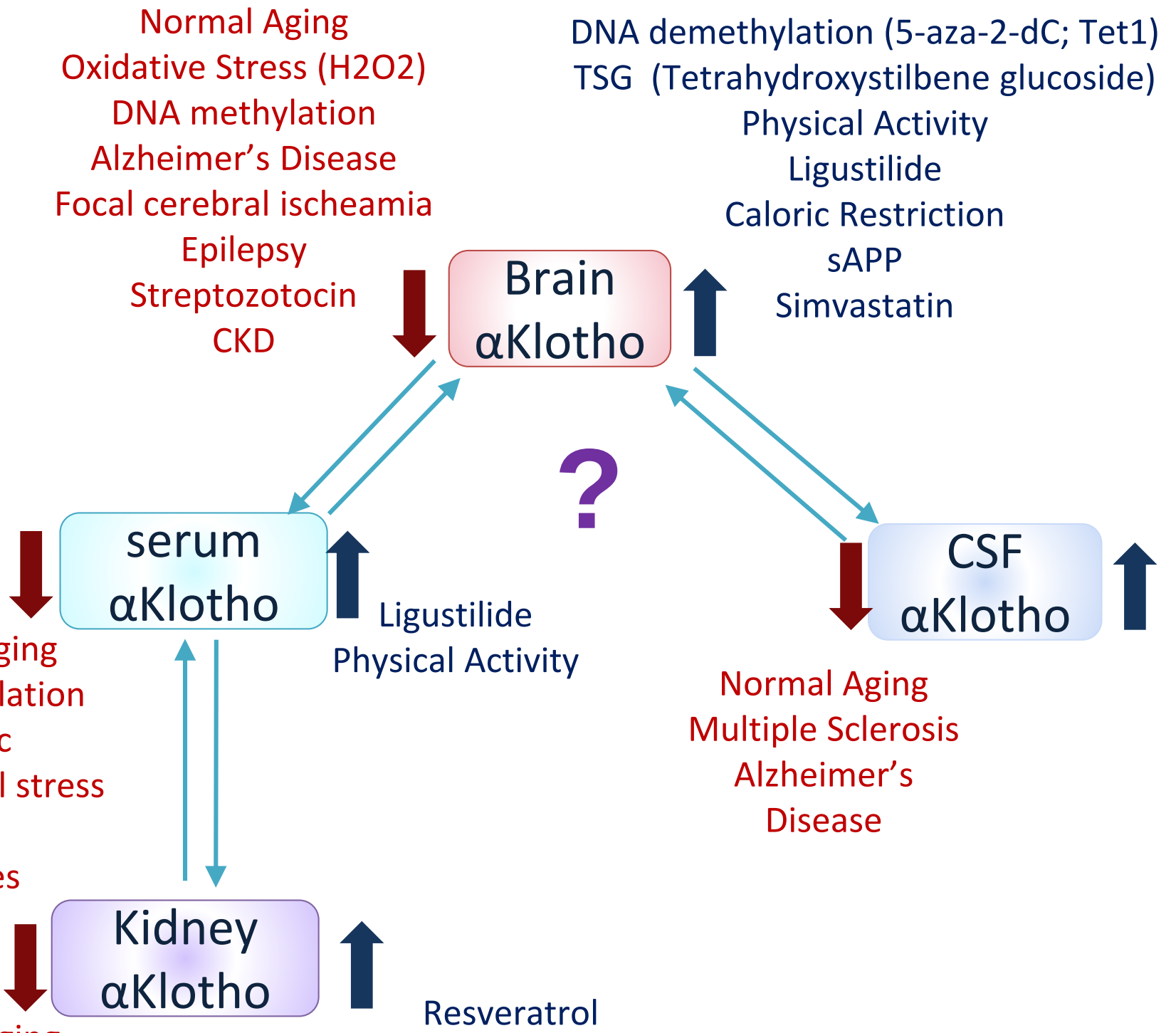

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