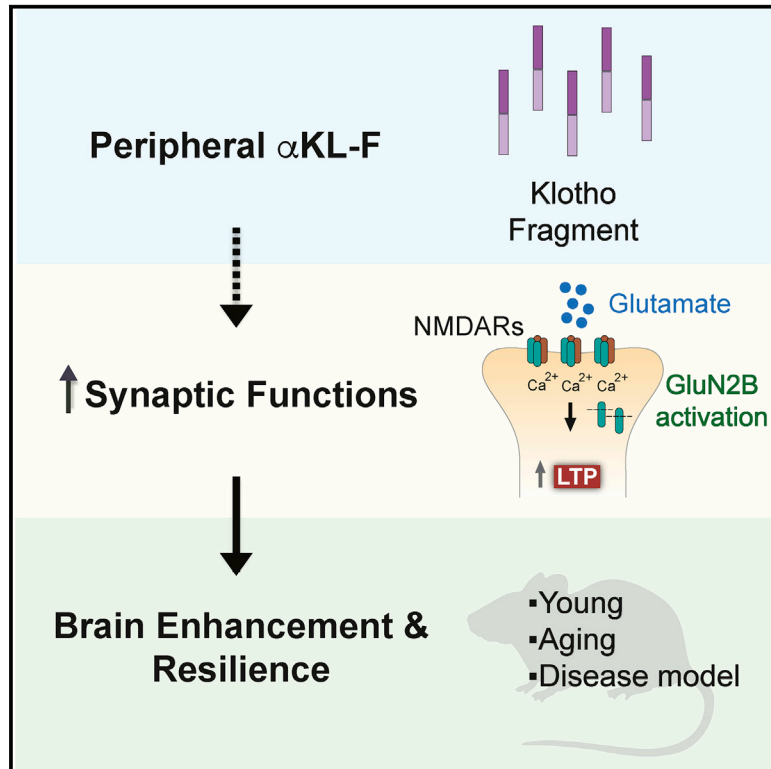


Peripheral Elevation of a Klotho Fragment Enhances Brain Function and Resilience in Young, Aging, and α -Synuclein Transgenic Mice

Graphical Abstract



Authors

Julio Leon, Arturo J. Moreno, Bayardo I. Garay, Robert J. Chalkley, Alma L. Burlingame, Dan Wang, Dena B. Dubal

Correspondence

dena.dubal@ucsf.edu

In Brief

Klotho is a longevity factor associated with cognitive enhancement when genetically and widely overexpressed over the lifetime of mice. Leon et al. show that peripheral delivery of a klotho fragment, α KL-F, acutely enhances cognition and neural resilience in young, aging, and disease model mice, establishing its therapeutic relevance and dissecting its underlying mechanisms.

Highlights

- A klotho fragment (α KL-F) enhances cognition in young and aging mice
- α KL-F counters deficits in α -synuclein mice without altering pathogenic protein levels
- α KL-F induces GluN2B cleavage and increases NMDAR-dependent synaptic plasticity
- Selective NMDAR blockade of GluN2B subunits abolishes acute α KL-F effects



Peripheral Elevation of a Klotho Fragment Enhances Brain Function and Resilience in Young, Aging, and α -Synuclein Transgenic Mice

Julio Leon,¹ Arturo J. Moreno,¹ Bayardo I. Garay,¹ Robert J. Chalkley,² Alma L. Burlingame,² Dan Wang,¹ and Dena B. Dubal^{1,3,4,*}

¹Department of Neurology, Biomedical Sciences Graduate Program, and Weill Institute for Neurosciences

²Department of Chemistry and Pharmaceutical Chemistry
University of California, San Francisco, San Francisco, CA 94158, USA

³Senior author

⁴Lead Contact

*Correspondence: dena.dubal@ucsf.edu

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SUMMARY

Cognitive dysfunction and decreased mobility from aging and neurodegenerative conditions, such as Parkinson and Alzheimer diseases, are major biomedical challenges in need of more effective therapies. Increasing brain resilience may represent a new treatment strategy. Klotho, a longevity factor, enhances cognition when genetically and broadly overexpressed in its full, wild-type form over the mouse lifespan. Whether acute klotho treatment can rapidly enhance cognitive and motor functions or induce resilience is a gap in our knowledge of its therapeutic potential. Here, we show that an α -klotho protein fragment (α KL-F), administered peripherally, surprisingly induced cognitive enhancement and neural resilience despite impermeability to the blood-brain barrier in young, aging, and transgenic α -synuclein mice. α KL-F treatment induced cleavage of the NMDAR subunit GluN2B and also enhanced NMDAR-dependent synaptic plasticity. GluN2B blockade abolished α KL-F-mediated effects. Peripheral α KL-F treatment is sufficient to induce neural enhancement and resilience in mice and may prove therapeutic in humans.

INTRODUCTION

Cognitive dysfunction and decreased mobility from aging and age-related neurodegenerative conditions such as Alzheimer disease (AD) and Parkinson disease (PD) are major biomedical challenges. Because more effective treatments are needed, and clinical trials targeting putative pathogenic proteins have failed, it is critical to develop alternate or complementary therapeutic strategies. In light of this urgent medical need for our rapidly aging populations, delaying aging itself or increasing the function and resilience of the brain (Bennett, 2017; McEwen and Morrison, 2013) may represent new treatment strategies.

α -Klotho (klotho) is a pleiotropic protein that circulates as a hormone following cleavage from its transmembrane form. It regulates insulin (Kurosu et al., 2005), Wnt (Liu et al., 2007), and fibroblast growth factor (FGF) (Urakawa et al., 2006) signaling. Overexpression of klotho extends life in organisms (Château et al., 2010; Kurosu et al., 2005), whereas lowering klotho shortens it (Kuro-o et al., 1997). Elevated klotho levels in humans, resulting from genetic variation (Arking et al., 2002; Dubal et al., 2014; Yokoyama et al., 2017), also associate with lifespan (Arking et al., 2002; Invidia et al., 2010) in some populations. In model organisms and humans, levels of klotho decline with age (Duce et al., 2008; Semba et al., 2011), chronic stress (Prather et al., 2015), cognitive aging (Shardell et al., 2016), neurodegenerative disease (Semba et al., 2014), and models of neurodegenerative disease (Dubal et al., 2015; Massó et al., 2015).

We previously discovered that life-long, genetic overexpression of klotho causally enhances normal cognition and neural resilience independent of age and when broadly expressed in the mouse body and brain (Dubal et al., 2014, 2015). It does so, at least in part, by directly or indirectly optimizing synaptic functions through NMDA receptor (NMDAR)-dependent mechanisms (Dubal et al., 2014, 2015). Importantly, genetic, lifelong, and widespread klotho elevation also contributes to neural resilience in a human amyloid precursor protein (hAPP) model of neurodegenerative disease related to AD (Dubal et al., 2015); that is, it effectively counters cognitive and synaptic deficits despite high levels of pathogenic proteins, including A β , tau, and phospho-tau.

The relevance of klotho to brain health in humans is supported by the findings that elevated serum klotho, related to *KLOTHO* variation, are associated with better measures, including cognition (Dubal et al., 2014; Yokoyama et al., 2015), structural reserve of the prefrontal cortex in normal aging (Yokoyama et al., 2015), connectivity between cortical regions (Yokoyama et al., 2017), and physical performance in aging (Shardell et al., 2015), and that diminished klotho levels are associated with worse brain measures (Prather et al., 2015; Yokoyama et al., 2015, 2017). Furthermore, *KLOTHO* variation is associated with less cognitive decline and better cortical structure in another large cohort (de Vries et al., 2017), although positive genetic associations were

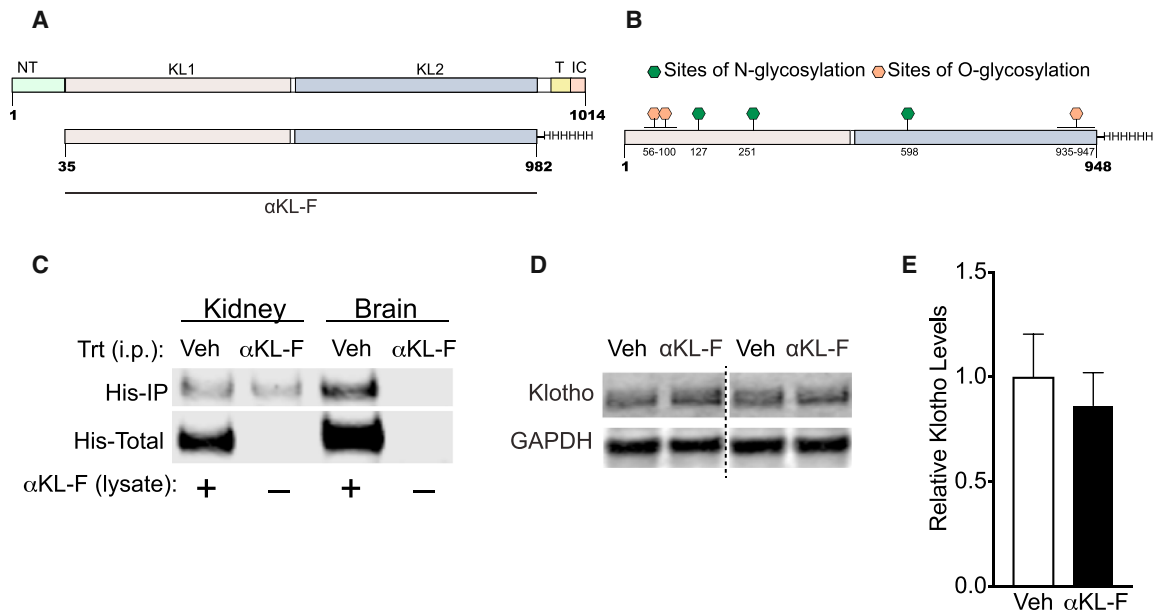


Figure 1. αKL-F Is a Recombinant Post-translationally Modified Fragment of Klotho, and Its Peripheral Administration Does Not Show BBB Crossing or Alteration of Endogenous Hippocampal Klotho Levels

(A) Amino acid sequence of the endogenous α-klotho protein (top, aa 1–1014) and the recombinant α-klotho fragment (αKL-F, bottom, aa 35–982) followed by a His tag. N-T, N terminus; TR, transmembrane region; IC, intracellular domain.
 (B) Locations of O-glycosylation and N-glycosylation sites of the recombinant protein made in Chinese hamster ovary (CHO) cells.
 (C) αKL-F was not observed to cross into the brain 4 hr following i.p. injection of Veh or αKL-F (100 μg/kg). Western blot shows immunoprecipitation against His-tagged αKL-F (His-IP) in kidney (detected) and brain (not detected) lysates. αKL-F was added to the veh-treated lysates as a positive control for detection of His-tagged protein (His-Total).
 (D) Representative western blot showing Klotho and GAPDH levels in homogenized whole hippocampus 4–5 hr following Veh or αKL-F (i.p., 10 μg/kg) treatment (n = 12/group; male; age, 4 months). Images were captured from the same gel.
 (E) Quantitation of endogenous brain klotho levels showing no differences between Veh- and αKL-F-treated mice. NTG levels are arbitrarily defined as 1.0. Data are mean ± SEM.

not observed in other populations at particularly advanced ages (Almeida et al., 2017; Mengel-From et al., 2016).

Multiple proteins contribute to the pathogenesis of neurodegenerative diseases. α-Synuclein, a membrane protein whose overexpression inhibits mechanisms of exocytosis (Logan et al., 2017), is central to PD and other parkinsonian disorders and contributes to multi-etiology dementias such as AD. Transgenic mice that overexpress wild-type human α-synuclein (hSYN) simulate key aspects of neurodegenerative disease, including cognitive and motor dysfunction (Fleming et al., 2004; Hatami and Chesselet, 2015). Therapeutic strategies that induce resilience against common pathogenic proteins such as α-synuclein involved in multiple diseases with broad phenotypes (Montine et al., 2014) could positively affect the human condition.

Whether acute klotho elevation represents a strategy that can rapidly enhance cognition, motor functions, and/or induce brain resilience is a gap in our knowledge of its therapeutic potential. Here we show that αKL-F, a fragment of the α-klotho protein similar to its secreted form, can acutely improve cognitive and motor functions following peripheral administration. It does so despite apparent impermeability to the blood-brain barrier in young, aging, and hSYN transgenic mice. Further investigation of αKL-F-mediated molecular mechanisms revealed activation of glutamatergic signaling and enhancement of synaptic plasticity.

RESULTS

αKL-F Is a Recombinant Modified Fragment of Mouse Klotho that Does Not Penetrate into the Brain or Alter Endogenous Hippocampal Klotho Levels

We first characterized the recombinant, mouse αKL-F fragment (Kurosu et al., 2005) by mass spectrometry. αKL-F is a protein representing a truncated form of the endogenous α-klotho protein but lacking its transmembrane, N-terminal signal peptide, and intracellular domains (Figure 1A). This truncated peptide (954 amino acids [aa]) includes the KL-1 and KL-2 subdomains and a C-terminal 6-His tag sequence. It resembles the extracellular portion of α-klotho (or klotho) endogenously shed from the membrane by ADAM 10 and 17 (Bloch et al., 2009; Chen et al., 2007), leading to a protein of approximately 959 aa that includes some of the transmembrane domain (Chen et al., 2015) and circulates in mouse serum, human serum, and human cerebral spinal fluid (CSF) (Imura et al., 2004).

We assessed for post-translational modifications (PTMs) of αKL-F and detected six glycosylation sites (Figure 1B). The identified N- and O-glycosylation sites map to the KL1 and KL2 domains and could potentially affect many facets of the protein, including recognition, stability, and solubility. No regulatory PTMs, such as phosphorylation, were detected. Other physical

properties of the protein, such as its aggregation, higher-form conformations, and their importance to cognition-related functions, remain to be determined.

To probe whether peripherally administered α KL-F penetrates the brain, we injected a high dose (10–40 times the doses used in our studies) of the His tag protein (100 μ g/kg, intraperitoneally [i.p.]). We assessed for its presence in the kidney and brain 4 hr later, within its estimated 7.5-hr half-life (Hu et al., 2016), by immunoprecipitation of the His tag (Figure 1C). The protein was observed in the kidney but not in the brain. Consistent with previous findings using autoradiographic methods (Hu et al., 2016), our data suggest that peripheral treatment with α KL-F does not cross the blood-brain barrier (BBB).

Because the endogenous klotho protein is also produced and circulates within the BBB, we wondered whether peripheral α KL-F treatment causes shifts in brain klotho levels. Peripheral α KL-F did not alter the levels of endogenous klotho in the hippocampus (Figures 1D and 1E). These data suggest that the central activity of peripherally administered α KL-F probably engages signals transduced from the periphery to the CNS.

A Fragment of Klotho, α KL-F, Delivered Peripherally, Acutely Enhances Cognition in Young Mice

Whether klotho, or some form of it, can acutely enhance cognition or induce resilience is a gap in our knowledge of its biologic functions and therapeutic potential. To test the therapeutic potential of mouse α KL-F, we administered it peripherally (i.p.) in young mice.

We first tested spatial learning and memory in the Morris water maze. Young mice were treated with vehicle (Veh) or α KL-F (10 μ g/kg, i.p.) daily prior to hidden platform training or a probe trial (Figure 2A). Remarkably, α KL-F treatment enhanced learning, as measured by decreased distance traveled to find the hidden platform (Figure 2B). α KL-F also enhanced spatial memory retention, as measured by increased time spent in the target quadrant (Figure 2C) and at the target site (Figure 2D) in the probe trial following hidden training. Mice treated with Veh or α KL-F showed no differences in their swim speeds (Figure 2E) or ability to find the target platform, identified by a visual cue (Figure 2F). Thus, similar to genetic, lifelong, and widespread overexpression of full-length mouse klotho (Dubal et al., 2014, 2015), peripheral delivery of mouse α KL-F was sufficient to enhance learning and memory, but acutely.

We then tested whether α KL-F improves another domain of cognition, working memory, in the small Y-maze (Figures 2H and 2I). In the same cohort and in several (over ten) independent, naïve cohorts, young mice were treated with vehicle or α KL-F (10 μ g/kg, i.p.) 4 hr prior to exploration in the small Y-maze. α KL-F acutely enhanced working memory, as measured by increased spontaneous alternations in the maze. α KL-F induced a threshold-dependent dose response on cognition, with the lowest effective dose detected at 2.5 μ g/kg (Figure S1A). In addition, α KL-F-mediated cognitive enhancement combined with cognitive training persisted for at least 2 weeks after the last treatment (Figure S1B), suggesting organizational, longer-lasting, and beneficial effects on the synapse and brain. Collectively, these data show that peripheral administration of α KL-F is sufficient to induce enhancement of a normal brain.

Of note, in our experiments, we did not detect cognitive enhancement with human α KL-F (87% homology to mouse) administration in mice following the same acute treatment paradigms in the small Y-maze or water maze (data not shown). This suggests species specificity of the biologic action and additionally shows that a protein of the same size, but with a different sequence, did not arbitrarily enhance cognition.

α KL-F Enhances Cognition in the Aging Brain

Because aging is the primary risk factor for cognitive impairment, we wondered whether α KL-F could enhance cognition in the aging brain. To test this, aging mice (18 months) were treated once with Veh or α KL-F (10 μ g/kg, i.p.) 1 day prior to training in the two-trial Y-maze (Figure 3A). This task probes both spatial and working memory by measuring exploration time in a novel compared with a familiar arm of the maze following context training (Dellu et al., 1992). During testing, Veh-treated, aged mice showed no preference for the novel arm. α KL-F induced preference for the novel arm, indicating that it enhanced spatial and working memory in aged mice (Figure 3B). Thus, a single peripheral injection of α KL-F boosted cognition in the aging brain.

α KL-F Acutely Counters Motor and Cognitive Deficits in Transgenic hSYN Mice, an α -Synuclein Model of Neurodegenerative Disease

Next, we probed whether α KL-F can acutely counter neurodegenerative disease-related deficits and pathologies in mice that overexpress wild-type hSYN mice using motor and cognitive tasks (Figure 4A). We chose this model because the majority of dementias related to both PD and AD are multi-etiology, α -synuclein is commonly shared among them, and this model enabled testing of motor deficits. hSYN mice (Rockenstein et al., 2002) accumulate α -synuclein in neurons and synapses throughout the brain (Rockenstein et al., 2002) and exhibit motor and cognitive deficits (Fleming et al., 2004, 2006; Masliah et al., 2000; Morris et al., 2011).

We first tested whether α KL-F counters motor deficits in hSYN mice. Motor dysfunction is a predominant deficit in hSYN mice and linked with clinical manifestations of α -synuclein toxicity in humans. Building on our findings that a lower dose of α KL-F enhanced function in the normal brain (Figure S1A), we injected Veh and a lower dose of α KL-F (2.5 μ g/kg instead of 10 μ g/kg, i.p.) daily for 2 days prior to rotarod testing in hSYN mice and nontransgenic (NTG) littermate controls (Figure 4A). As expected (Morris et al., 2011), all hSYN mice showed a motor deficit in training and testing, as measured by decreased time to fall off a fixed (Figure 4B) and accelerating (Figure 4C) rod. α KL-F increased motor learning in hSYN mice during training and mean motor performance during testing (Figures 4B and 4C). Thus, α KL-F improved motor learning and deficits, extending its therapeutic functions to another functional domain.

We then tested whether α KL-F counters cognitive deficits in hSYN mice. Because hSYN mice show motor deficits but move around normally, we used the two-trial Y-maze, which measures spatial and working cognition based on exploration without heavy reliance on motor strength. After rotarod testing, mice were injected with Veh and α KL-F (2.5 μ g/kg, i.p.) daily for an additional 2 days (Figure 4A). Veh-treated hSYN mice

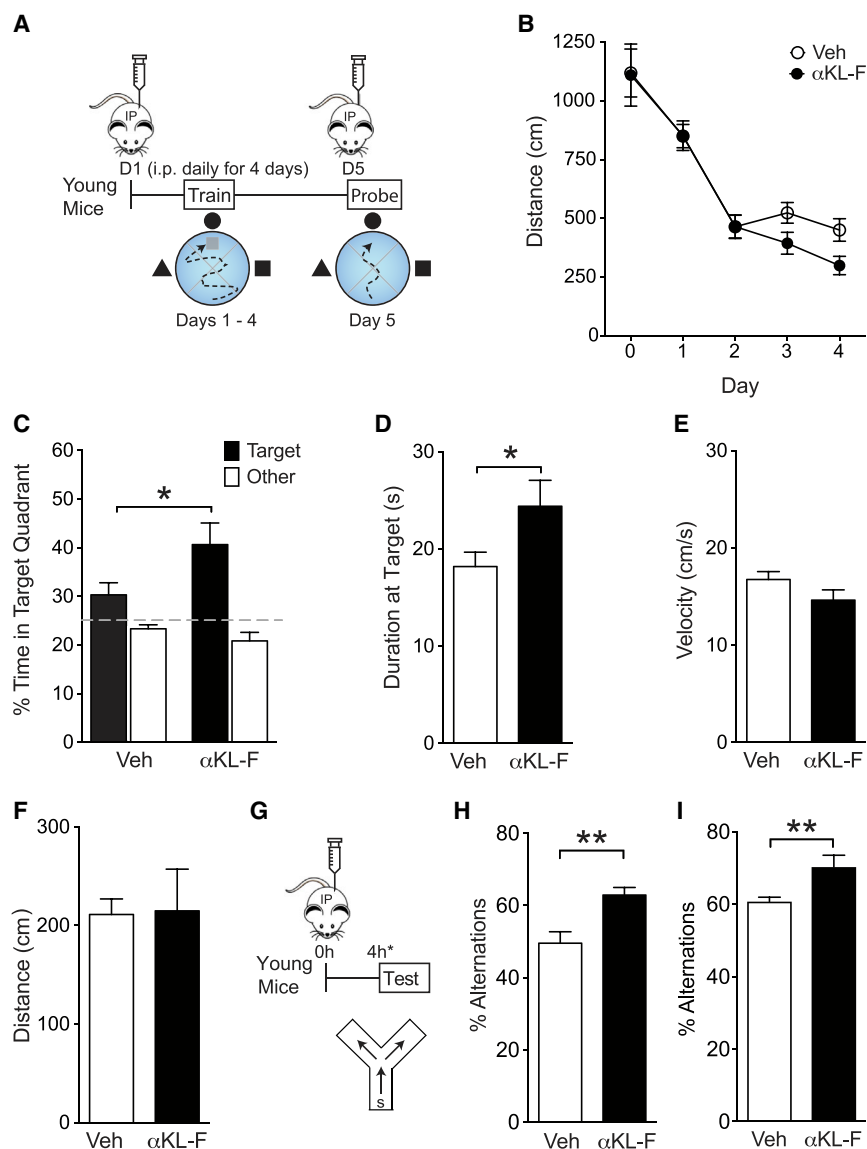


Figure 2. α KL-F, Delivered Peripherally, Acutely Enhances Cognition in Young Mice

(A) Diagram of the experimental paradigm of Veh or α KL-F injection (i.p., 10 μ g/kg) followed by spatial and working memory testing in the Morris water maze. Mice received daily treatment for 5 days, 16 or 4 hr prior to training and testing (n = 11–18/group; sex-balanced; age, 4 months).

(B) Spatial learning curves (platform hidden) in the water maze. Data represent the daily average of distance traveled to the platform. α KL-F decreased the distance, indicating enhanced learning. Rank-sum test, α KL-F effect days 2–4, p < 0.05.

(C) Probe trial with the platform removed 24 hr after completion of hidden training. α KL-F increased the percentage of time spent in the target quadrant, indicating enhanced recall of spatial memory.

(D) Probe trial at 24 hr. α KL-F increased the duration of time spent at target, indicating enhanced recall of spatial memory.

(E) Velocity in the water maze did not differ between mice treated with Veh or α KL-F.

(F) The distance traveled to find a cued, visible platform in the water maze did not differ between mice treated with Veh or α KL-F.

(G–I) Diagram of the experimental paradigm of Veh or α KL-F injection (G, i.p., 10 μ g/kg), followed by working memory testing in the small Y-maze (H and I). Mice (age, 4 months) received treatment 4 hr prior to testing.

(H) Percentages of alternations among arms during exploration of the Y-maze. α KL-F increased alternations, indicating enhanced working memory. In this cohort, mice (males, n = 12–13/group) were exposed to Veh or α KL-F 2 days before testing.

(I) Percentages of alternations among arms during exploration of the Y-maze. In a replicate independent cohort, α KL-F increased working memory. Mice (males, n = 9/group) were naive to previous treatments or testing.

*p < 0.05, **p < 0.01 (t test). Data are mean \pm SEM. See also Figure S1.

showed cognitive deficits compared with NTG controls, as measured by decreased preference for the novel arm (Figure 4D). α KL-F improved novel arm preference (by main effect) in NTG and hSYN mice (Figure 4D). α KL-F countered cognitive deficits in hSYN mice (Figure 4D) despite equivalent levels of speed and movement among all experimental groups during training (data not shown). Thus, in addition to enhancing cognition in the normal and aging brain, peripheral treatment with α KL-F acutely improved cognitive deficits in an hSYN model.

α KL-F Induces Neural Resilience in hSYN Mice without Altering Pathogenic Protein Species Related to Neurodegenerative Disease

Because deficits in hSYN mice depend on α -synuclein expression, we assessed whether α KL-F reduces levels of the pathogenic protein. Acute treatment with α KL-F, with the same exper-

imental paradigm that countered neural deficits, did not alter the steady-state levels of α -synuclein or its phosphorylated form (Figures 4E–4G) in the hippocampus or cortex. In addition, α KL-F did not alter the levels of potential co-pathogens of α -synuclein, such as tau or phospho-tau (Haggerty et al., 2011; Wills et al., 2010) in hSYN mice (Figures 4E, 4H, and 4I). Thus, acute and peripheral treatment with α KL-F induced neural resilience in hSYN mice, as defined by countering neural functions without altering the levels of pathogenic protein species.

α KL-F Acutely Increases GluN2B Cleavage without Altering Synaptic GluN2B Levels and Enriches Glutamate Receptor Signaling

To begin to explore the mechanisms underlying α KL-F-mediated cognitive enhancement and neural resilience, we focused subsequent studies on normal, young mice because effects of

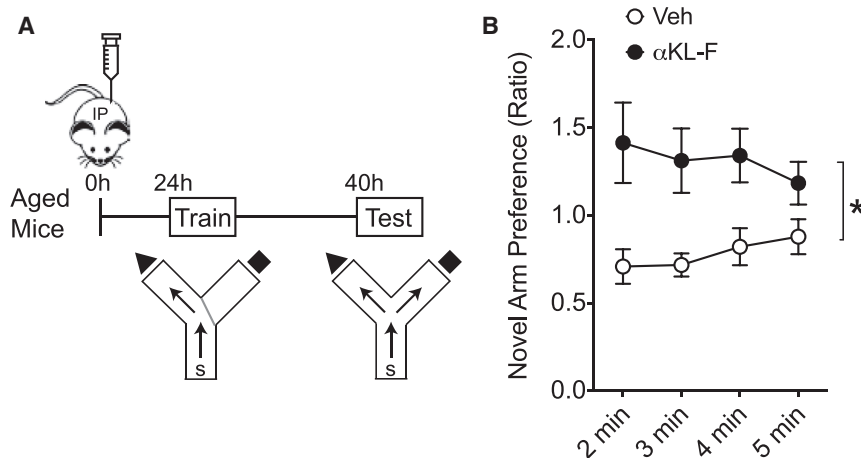


Figure 3. α KL-F, Delivered Peripherally, Acutely Enhances Cognition in Aged Mice

(A) Diagram of the experimental paradigm of Veh or α KL-F treatment (i.p., 10 μ g/kg) and testing of aged mice in the two-trial Y-maze. (n = 8–10 mice/group; sex-balanced groups; age, 18 months). Mice received an injection followed by training (24 hr after treatment) and then testing (40 hr after treatment).

(B) Duration of time spent in the novel compared with the familiar arm, expressed as a ratio to indicate novel arm preference. α KL-F increased spatial and working memory in aged mice over time. Two-way repeated measures ANOVA, α KL-F effect, **p < 0.01.

Data are mean \pm SEM.

α KL-F were discovered in this age group, and these mice were more available. We assessed the GluN2B subunit of NMDARs because transgenic overexpression of klotho increases cognition through GluN2B-dependent functions (Dubal et al., 2014), transgenic elevation of GluN2B enhances cognition in mice and rats (Cao et al., 2007; Tang et al., 1999; Wang et al., 2009), and dysfunction of GluN2B contributes to cognitive decline in aging and neurodegenerative diseases (Ittner et al., 2010; Pig-gott et al., 1992; Sze et al., 2001; Zhao et al., 2009). We first assessed, in parallel with the effects of lifelong, transgenic klotho overexpression (Dubal et al., 2014, 2015), whether α KL-F treatment acutely increases the levels of GluN2B in synaptic fractions of the hippocampus. Synaptic levels of GluN2B in the hippocampus did not differ between Veh- and α KL-F-treated mice (Figures 5A–5C) despite α KL-F-mediated cognitive enhancement in mice. Thus, acute effects of α KL-F on cognition did not induce or require the synaptic increase of GluN2B levels observed in transgenic, klotho-overexpressing mice.

Synaptic proteins are dynamic structures with activity-dependent modification and turnover (Alvarez-Castelao and Schuman, 2015). Following stimulation, the GluN2B subunit of NMDARs can undergo cleavage by calpain, producing a low-molecular-weight (LMW) form of the subunit (~115 kDa) (Dong et al., 2004, 2006; Simpkins et al., 2003). We noticed that, in whole hippocampal homogenates, an LMW form of the GluN2B protein not detected in synaptic, post-synaptic density (PSD)-enriched fractions, differed between experimental groups upon western blotting (Figures 6A–6E). α KL-F treatment elevated LMW GluN2B by approximately 4 hr following treatment (Figures 6A and 6B), and this correlated with better cognitive ability in the small Y-maze (Figure 6C). α KL-F increased LMW GluN2B in multiple replicate cohorts (Figure 6D; data not shown) and tended to do so in the absence of a cognitive task following several days of treatment (albeit less intensely) (Figure 6E). Thus, acute treatment with α KL-F led to engagement of the GluN2B subunit of NMDARs, causing its increased cleavage.

To verify the specificity of the LMW band, we generated a GluN2B blocking peptide harboring the GluN2B antibody epitope sequence. We then immunoprecipitated GluN2B proteins from mouse hippocampal homogenates and preincubated

them with and without the GluN2B-blocking peptide. The blocking peptide abrogated detection of the high-molecular-weight (HMW) and LMW bands, but not other non-specific bands, on western blotting (Figure 6F). Of note, the LMW form was not detected in synaptic fractions and surfaced clearly on western blotting of total hippocampal lysates only after increasing stringency with addition of 1% SDS to the homogenization buffer. Thus, GluN2B also exists in an LMW form, as reported previously (Dong et al., 2004, 2006; Simpkins et al., 2003), and α KL-F acutely increased its levels.

Because the klotho-derived fragment α KL-F probably engages multiple downstream pathways to enhance cognition, we probed broadly for possible targets with an independent and unbiased proteomic analysis of whole hippocampal homogenates from mice treated with Veh and α KL-F (10 μ g/kg, i.p.) 4 hr prior to exploration in the small Y-maze. α KL-F induced glutamate receptor signaling as the top enriched canonical pathway by ingenuity pathway analysis, which identifies biologic patterns of molecular changes in a large dataset (Figure S2).

Taken together, two experimental approaches, hypothesis- and non-hypothesis-driven, independently converged on evidence for glutamatergic signaling as an important downstream target pathway of α KL-F-mediated functions. α KL-F modestly changed GluN2B in proteomic mass spectrometry studies (Figure S2); it tended to increase many, but not all, GluN2B peptides following trypsinization (Figure S2). This suggests that, instead of increasing overall protein levels, α KL-F may alter GluN2B cleavage, degradation, PTM, or other baseline cellular processes. This possibility is supported by our findings that α KL-F significantly increased the LMW form of GluN2B, but not the HMW form, on western blotting (Figure 6) and requires further study.

Downstream main effect analysis of all proteomic changes predicted that α KL-F activates synaptic transmission (Figure S2). We directly probed and validated this functional prediction.

α KL-F Acutely Enhances NMDAR-Dependent Synaptic Plasticity in the Hippocampus

Activation of glutamate receptor signaling is critical for synaptic functions and plasticity. Long-term potentiation (LTP) is a form of synaptic plasticity dependent on NMDAR activation and is a

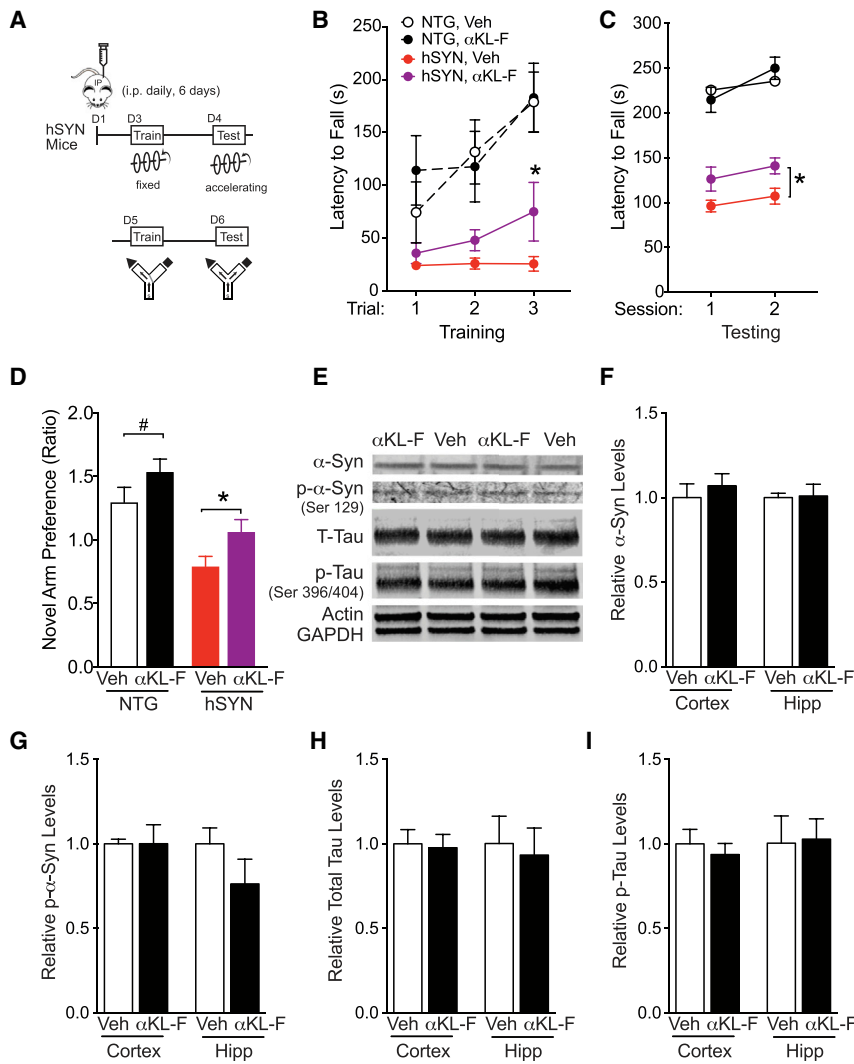


Figure 4. α KL-F, Delivered Peripherally, Induces Neural Resilience by Acutely Countering Cognitive and Motor Deficits in Transgenic hSYN Mice

(A) Diagram of the experimental paradigm of Veh or α KL-F treatment (i.p., 2.5 μ g/kg). Mice received single daily injections for 6 days. On day 4, NTG and hSYN mice were tested on the rotarod to assess motor performance. On day 7 (17 hr after the last injection), mice were tested in the two-trial Y-maze to assess spatial and working cognition.

(B) Motor learning and function measured by time on the rotarod during training. hSYN mice showed motor dysfunction compared with NTG mice. α KL-F treatment improved motor learning in hSYN mice ($n = 10\text{--}15$ mice/group; male; age, 3–6 months). Mixed model ANOVA: hSYN effect, $p < 0.0001$.

(C) Motor function measured by time on the rotarod during testing. hSYN mice showed motor dysfunction compared with NTG mice. Mixed model ANOVA: hSYN effect, $p < 0.0001$. α KL-F treatment increased time on the rotarod across sessions in hSYN mice. Two-way repeated measures ANOVA: α KL-F effect, $^*p < 0.05$ as indicated by brackets.

(D) Duration of time spent in the novel compared with the familiar arm during testing, expressed as a ratio to indicate novel arm preference at 5 min of exploration. α KL-F improved cognitive deficits in hSYN mice ($n = 14\text{--}23$ mice/group; male; age, 3–6 months). Two-way ANOVA: hSYN effect, $p < 0.0001$; α KL-F effect, $p < 0.01$.

(E–I) The hippocampus and cortex of Veh- and α KL-F-treated hSYN mice were analyzed after 3 days of daily injection ($n = 5\text{--}6$ mice/group; male; age, 3–7 months).

(E) Representative western blots showing hippocampal levels of total α -Syn, phosphorylated α -Syn (p- α -Syn, Ser-129), total mouse tau (T-Tau), phosphorylated mouse tau (p-Tau, Ser-396/404), Actin, and GAPDH in Veh- and α KL-F-treated hSYN mice.

(F) Quantitation of α -Syn levels, showing no differences between Veh- and α KL-F-treated hSYN mice.

(G) Quantitation of p- α -Syn (Ser-129) levels, showing no differences between Veh- and α KL-F-treated hSYN mice.

(H) Quantitation of T-Tau levels, showing no differences between Veh- and α KL-F-treated hSYN mice.

(I) Quantitation of p-Tau levels, showing no differences between Veh- and α KL-F-treated hSYN mice.

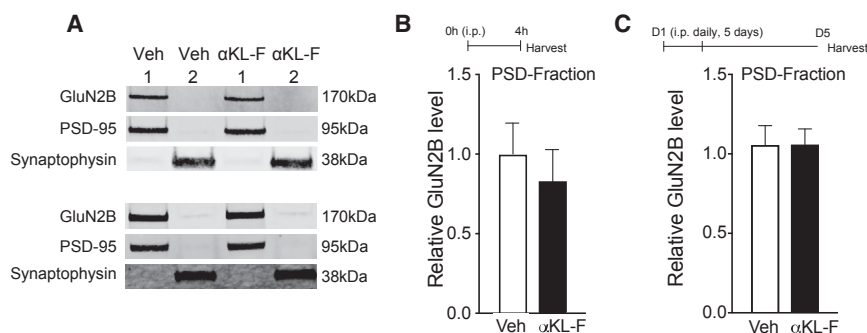
$^{\#}p = 0.10$, $^*p < 0.05$ versus trial 1 (B, paired t tests) or as indicated by brackets (C, mean effect; D, unpaired t tests) via Bonferroni-Holm test. Data are mean \pm SEM.

key cellular substrate of learning and memory (Morris et al., 1986; Nabavi et al., 2014; Nakazawa et al., 2004). To assess whether α KL-F activates synaptic transmission and enhances synaptic plasticity, we next measured LTP in acute hippocampal slices at the CA1 Schaffer collateral pathway synapse, largely mediated by NMDARs (Kauer et al., 1988). During the same time frame that it enhanced cognition and caused GluN2B cleavage (4 hr after i.p. treatment), α KL-F enhanced LTP, as determined by field excitatory postsynaptic potential (fEPSP) recordings (Figures 7A and 7B). Of note, the extent of synaptic enhancement with acute, peripheral α KL-F treatment was similar to that observed with lifetime, transgenic, full-length klotho over-expression in the body and brain, although a different hippocam-

pal region was examined in the current study (Dubal et al., 2014, 2015)

Selective Blockade of NMDAR GluN2B Subunits Abolishes α KL-F Effects on Cognition

The dynamics of NMDAR channels are governed in part by their subunit compositions (Wang et al., 2008; Yashiro and Philpot, 2008). GluN2B-containing NMDARs deactivate more slowly than those with other subunit types, transducing longer-lasting calcium-dependent signals (Wang et al., 2008). Because α KL-F acutely increased measures of GluN2B activation, including increasing its LMW form and enhancing synaptic plasticity, we tested whether blocking GluN2B-containing NMDARs



(B) Quantitation of PSD-enriched (fraction 1) GluN2B protein levels 4 hr following treatment (n = 4/group).
(C) Quantitation of PSD-enriched (fraction 1) GluN2B protein levels 5 days following daily treatment (n = 10/group).
Data are mean ± SEM.

modulates the effects of α KL-F on cognition. We specifically tested whether blockade of GluN2B in an acute, transient, and broad manner preferentially abrogates α KL-F-mediated cognitive enhancement without disrupting normal cognition.

To test this, we used a low dose of Ro 25-6981 (Ro 25), a highly specific GluN2B antagonist (Lü et al., 2017; Paoletti and Neyton, 2007) that minimally affects normal cognition (Figure S3), as described previously (Dubal et al., 2014; Mathur et al., 2009). Mice treated with a single injection of Veh or α KL-F (10 μ g/kg, i.p.) were tested in the small Y-maze 4 hr later. Ten minutes prior to Y-maze testing, they received saline or low-dose Ro 25 (5 mg/kg, i.p.) (Dubal et al., 2014; Mathur et al., 2009). As expected, in the absence of Ro 25, α KL-treated mice showed better working memory than Veh-treated controls (Figure 7C), consistent with their enhanced cognition and enhanced LTP. Ro 25 effectively and preferentially blocked α KL-F-mediated cognitive enhancement (Figures 7C and 7D). At higher doses, Ro 25 also inhibited normal cognition (Figure S3). Thus, in parallel with the effects observed in transgenic klotho-overexpressing mice (Dubal et al., 2014), a low dose of a specific GluN2B antagonist effectively and preferentially blocked the effects of peripheral, acute treatment with α KL-F.

DISCUSSION

Our cognitive, behavioral, biochemical, protein-based, electrophysiological, and pharmacological studies in mice reveal a role for the peripheral delivery of a post-translationally modified mouse klotho fragment, α KL-F, in acute enhancement of synaptic and brain functions. α KL-F enhanced normal cognition in young mice and boosted cognition in aging mice. Furthermore, α KL-F countered cognitive and motor dysfunction in hSYN mice that model aspects of neurodegenerative disease. It did so without altering the levels of α -synuclein or related co-pathogenic proteins, indicating that α KL-F increases neural resilience.

Further investigation into its mechanisms revealed that peripheral α KL-F delivery increased glutamatergic signaling. It increased the LMW form of the NMDAR subunit GluN2B on western blotting, a putative marker of its activation in the hippocampus (Dong et al., 2004, 2006; Simpkins et al., 2003) within 4 hr of delivery and cognitive testing. Furthermore, peripheral α KL-F acutely increased synaptic function, as measured by

enhanced LTP, a cellular substrate of learning and memory. Antagonism of NMDAR function through acute blockade of GluN2B preferentially abrogated α KL-F-mediated enhancement of cognition.

Taken together, our findings suggest that peripheral treatment with α KL-F or a similarly derived and/or modified klotho fragment could enhance brain function and resilience and could represent a new therapeutic strategy for dysfunction related to aging, neurodegenerative diseases like PD and AD, and other brain diseases.

Peripheral α KL-F Elevation Causes Central Cognitive Enhancement and Phenocopies Lifelong, Widespread, Genetic Klotho Overexpression

Surprisingly, a fragment of klotho, α KL-F, acutely enhanced cognition in young mice when delivered peripherally, thus phenocopying, in part, the functional effects we found with genetic, lifelong, and widespread overexpression of full-length, wild-type klotho (Dubal et al., 2014). This was unexpected because the fragment is impermeable to the BBB by prediction (Pardridge, 2005), direct radiography (Hu et al., 2016), and our immunoprecipitation studies. Because the α KL-F effects were acute, we conclude that neurodevelopmental effects of klotho, or sustained and high levels of wild-type klotho in the brain over a long period, are not required for neural enhancement. How peripheral α KL-F transmits a signal to the CNS is a fascinating question that remains to be answered. Possibilities include induction or modification of other factors that cross or interact with the BBB and direct or indirect alteration of central klotho levels, although we did not find evidence for the latter. Importantly, the ability of peripheral α KL-F to induce central effects is of therapeutic value because it bypasses the need to deliver protein directly into the CNS and, thus, evades a primary limitation of developing brain therapies. Our study does not rule out that klotho might also act with the CNS but, rather, establishes the relevance of its acute, peripheral elevation, particularly in a truncated form.

α KL-F, Lacking Several Structural Domains, Causes Better Cognitive Functions

The cognition-enhancing effects of α KL-F define an important segment of the klotho protein required for brain benefits.

Figure 5. α KL-F Does Not Alter Synaptic Levels of HMW GluN2B

(A–C) Synaptic membrane fraction 1 (PSD-enriched) and fraction 2 (non-PSD-enriched) isolated from the hippocampus of mice (age, 4 months; male) 4 hr after a single treatment followed by the small Y-maze or after 5 days of daily treatment with Veh or α KL-F (i.p., 10 μ g/kg).

(A) Representative western blots from hippocampal membrane fractions from mice 4 hr (top) following a single treatment or 5 days following daily treatments (bottom). Only the HMW GluN2B band was observed.

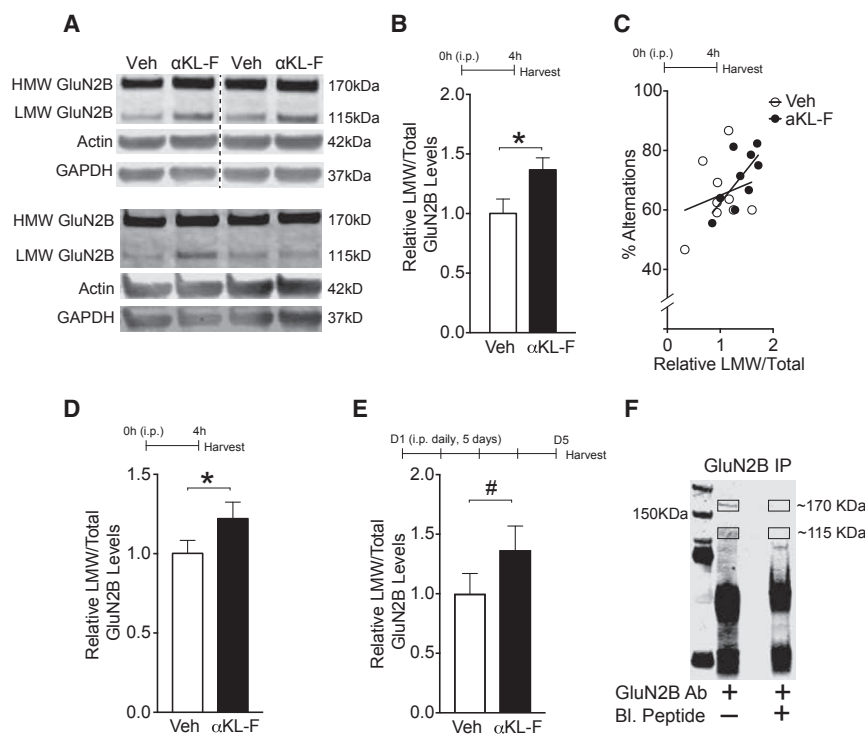


Figure 6. α KL-F Acutely Increases an LMW Form of GluN2B

(A–E) Whole hippocampal lysate isolated from mice treated with Veh or α KL-F (10 μ g/kg i.p.) after 4 hr of a single treatment followed by the small Y-maze or 5 days of daily treatment and then assessed for GluN2B levels.

(A) Representative western blots from hippocampal total lysates (with 1% SDS) from mice with Veh or α KL-F 4 hr after treatment (top, images were captured from the same gel) or 5 days following daily treatment (bottom) (age, 4 months; male). HMW and LMW GluN2B forms were observed.

(B) Quantitation of LMW/total (HMW+LMW) GluN2B protein levels from the hippocampus 4 hr after treatment (n = 10/group).

(C) Correlation of relative LMW/total GluN2B fragment levels from mice shown in (D) with percentage alternations in the small Y-maze ($R^2 = 0.5$, $p < 0.03$ with α KL-F treatment).

(D) Replicate independent cohort quantitation of LMW/total GluN2B protein levels from the hippocampus 4 hr after treatment (n = 21/group).

(E) Quantitation of LMW/total GluN2B protein levels from the hippocampus following 5 days of daily treatment in the absence of behavior studies (n = 11–12/group).

(F) Western blot showing the specificity of the HMW and LMW GluN2B band by immunoprecipitation with and without preincubation with a GluN2B-blocking peptide harboring the GluN2B antibody epitope sequence.

#p < 0.10, *p < 0.05 versus Veh as indicated by brackets. Data are mean \pm SEM. See also Figure S2.

Because α KL-F lacks the transmembrane, intracellular, and N-terminal regions, our findings identify the cognition-enhancing effects within amino acids 35–982 comprising the KL1 and KL2 domains. How this post-translationally modified protein, which exists endogenously in a similar form (sometimes called soluble klotho), and its interactions with other proteins lead to signals that converge upon glutamatergically mediated cognitive enhancement remain to be determined.

Because wild-type, full-length klotho is a pleiotropic protein that engages multiple signaling peripheral pathways, including insulin (Kurosu et al., 2005), Wnt (Liu et al., 2007), and FGF (Urakawa et al., 2006), narrowing the fragment(s) necessary for cognitive enhancement may help to further target neural functions that may be independent, or not, of klotho's putative actions on aging-related mechanisms.

The effects of peripheral α KL-F treatment on cognition largely phenocopied the effects of widespread, transgenic klotho overexpression; however, direct assessment of whether one is more robust is limited by the variability inherent to behavioral assays and the divergent nature of pharmacologic and genetic strategies. Acute pharmacologic elevation, potentially relevant for therapeutic development, may also be influenced by various factors affecting the potency and detection of neural enhancement, such as klotho aggregation and oligomerization (Imura et al., 2004) or precipitation; protein production, handling, or stability; differing influences of subtle stressors on the complex measurement of cognition (Sorge et al., 2014); or some combination of factors. In our studies, α KL-F effects were replicated in well over ten independent mouse cohorts of diverse characteristics

(young, aging, and disease model mice) with dosing of a fresh preparation. How the fragment can be optimized by dose, composition, and biologic action may increase our understanding of how to enhance the brain-targeted effects of klotho.

α KL-F Improves Cognition in the Aging Brain

Peripheral delivery of α KL-F acutely enhanced cognition in aging mice. This is important because it demonstrates that α KL-F can increase spatial and working memory in the naturally aged brain and, therefore, can effectively target vulnerable substrates of cognition in aging. The long-lasting effect in aged mice induced better cognitive functions nearly 2 days following treatment, after predicted elimination of α KL-F (Hu et al., 2016). In further studies of young mice, a long-lasting α KL-F effect combined with cognitive training was observed over 2 weeks later. Together, our data suggest that α KL-F may harbor therapeutic benefits for the young and aging brain in a long-lasting and organizational manner. Because the synapse is a target of aging and enhanced by α KL-F, we speculate that the beneficial effects occur through synaptic remodeling.

α KL-F Causes Neural Resilience against Motor and Cognitive Dysfunction without Altering Pathogenic Protein Levels

Peripheral α KL-F acutely improved motor dysfunction in an α -synuclein model of neurodegenerative diseases, extending the beneficial effects of the klotho-derived fragment from cognition to motor functions. Because transgenic klotho elevation does not modify measures of muscle function (Phelps et al.,

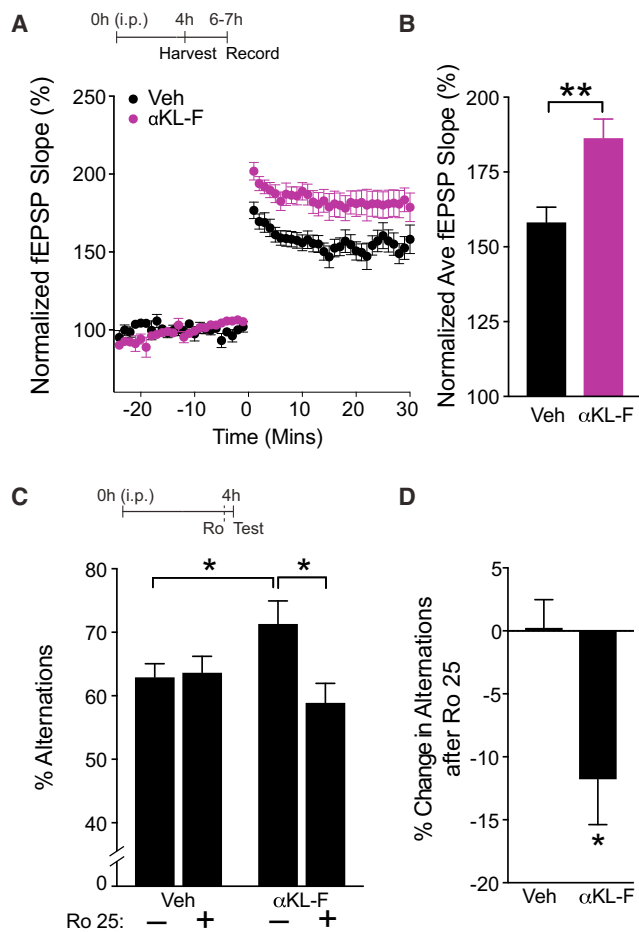


Figure 7. α KL-F Acutely Enhances NMDAR-Dependent Synaptic Plasticity in the Hippocampus, and Selective Blockade of the NMDAR Subunit GluN2B Abolishes α KL-F Effects on Cognition

(A and B) fEPSP recordings from acute hippocampal slices of 3-month-old male mice ($n = 7$ – 14 slices/group, 3 mice/group) treated with Veh or α KL-F 4 hr prior to brain slicing.

(A) LTP induction and potentiation in the CA1 region was monitored for 30 min following theta burst stimulation of the Schaffer collateral pathway.

(B) Average fEPSP slope averaged over 30 min in hippocampal slices of mice treated with Veh or α KL-F. Two-way repeated measures ANOVA: α KL-F effect, $p < 0.01$.

(C and D) Mice ($n = 15$ /group; male; age, 4 months) were treated i.p. with Veh or α KL-F (10 μ g/kg) 4 hr prior to testing, followed by i.p. saline (–) or Ro 25 (+) (5 mg/kg) 10 min prior to testing in the small Y-maze.

(C) Percentages of alternations among arms during exploration of the small Y-maze over 4 min. α KL-F increased alternations, and Ro 25 preferentially decreased α KL-F-mediated effects. Two-way ANOVA: Ro 25 by α KL-F interaction, $p < 0.05$.

(D) Percentage of decrease in alternations following Ro 25 treatment in Veh- and α KL-F-treated mice. Ro 25 preferentially decreased the percentage of alternations in mice with α KL-F treatment.

* $p < 0.05$, ** $p < 0.01$ versus Veh or as indicated by brackets by t test (B and D) or Bonferroni-Holm test (C). Data are mean \pm SEM. See also Figure S3.

2013), our findings raise the possibility that α KL-F acutely counters motor problems by facilitating cognitive motor learning or boosting motor regions of the brain, probably through substrates

of synaptic plasticity. Of note, we did not find effects of α KL-F on total movements in the open field or time spent in the open arms of an elevated plus maze (data not shown), suggesting that the protein improves motor function without causing hyperactivity or anxiety.

Peripheral α KL-F acutely counteracted cognitive deficits in a disease model with overexpression of the wild-type α -synuclein human protein, which plays a major role in α -synucleinopathies, including Parkinson disease, multiple system atrophy, and Lewy body dementia. Because dementias are multi-etiology and share common pathogenic proteins, including α -synuclein (Montine et al., 2014), the protein is also relevant to AD. Of note, the immediate beneficial cognitive effect of α KL-F described here is similar to effects of genetic, lifelong, and widespread overexpression of full-length wild-type klotho in the body and brain of hAPP mice, another disease model related to AD (Dubal et al., 2015).

α KL-F induced motor and cognitive benefit in hSYN mice without altering the levels of pathogenic or putative co-pathogenic proteins, α -synuclein, phospho- α -synuclein, tau, and phospho-tau. These data support growing lines of evidence that klotho induces neural resilience or the capacity to acutely counter or recover from cognitive and motor dysfunction. We speculate that klotho, and more specifically α KL-F, modulates common downstream targets, such as glutamatergic signaling at the synapse, in the pathogenesis of aging, AD, PD, and other diseases.

α KL-F Acutely Activates Glutamatergic Signaling to Enhance Cognition and Synaptic Plasticity

Hypothesis- and non-hypothesis-driven approaches independently converged on evidence for glutamate receptor signaling as an important downstream pathway of α KL-F-mediated functions. α KL-F acutely caused enriched levels of the NMDAR subunit GluN2B in the normal, young brain. However, instead of synaptic enrichment of HMW levels observed in transgenic mice chronically overexpressing klotho (Dubal et al., 2014), we found that acute α KL-F treatment increased LMW GluN2B in whole hippocampal homogenate within 4 hr. This probably represents calpain-mediated cleavage linked with GluN2B activation (Simpkins et al., 2003; Wu and Lynch, 2006) rather than increased transcription, translation, or trafficking of the subunit to the synapse. From a temporal perspective, it is possible that the immediate effects of klotho activate GluN2B-containing NMDARs by cleavage and that the lifelong, chronic overexpression effects of klotho cause compensatory GluN2B increases to ultimately offset klotho-mediated, ongoing cleavage and turnover.

NMDAR channel dynamics are governed by their subunit compositions (Yashiro and Philpot, 2008); GluN2B-containing NMDARs deactivate more slowly and transduce longer-lasting intracellular signals (Wang et al., 2008). Increased GluN2B-dependent NMDAR functions cause enhanced cognitive and synaptic functions (Cao et al., 2007; Tang et al., 1999; Wang et al., 2009). Thus, our findings support the hypothesis that α KL-F-mediated signals preferentially activate GluN2B-containing NMDARs. Consistent with this hypothesis, peripheral delivery of α KL-F enhanced cognition and synaptic plasticity, and selective blockade of GluN2B preferentially abrogated

α KL-F-mediated effects. Of note, a study of direct application of a klotho fragment to hippocampal slices did not observe increased hippocampal LTP (Li et al., 2017), potentially reflecting hypoactive protein, induction protocol differences, and/or a requirement for peripheral rather than central klotho delivery to elicit synaptic effects.

The direct target of α KL-F, the relevant and causal peripheral mechanisms linked to that target, and the processes by which these core peripheral events reach the brain to affect the NMDAR and other synaptic functions remain to be determined. It is interesting to speculate that immune signals, other known klotho-related mechanisms, or some combination of pathways could converge onto glutamatergic signaling to induce better synaptic functions underlying cognitive and motor enhancement and neural resilience.

Our findings highlight a role for α KL-F in promoting optimal synaptic functions in the normal brain and to boost “synaptic health” (Morrison and Baxter, 2014) in aging and disease-states. Because synaptic health may confer resilience against the effects of aging and a myriad of aging-and non-aging related neurologic and psychiatric diseases, the potential to enhance it may be relevant to the human condition. Synaptic enhancement induced by α KL-F might be non-specific or, in contrast, could directly relate to abnormal mechanisms involved in aging and disease. Future studies will need to determine whether α KL-F specifically reduces detrimental synaptic mechanisms caused by aging, α -synuclein, and other pathogenic proteins.

α KL-F-mediated enhancement of synaptic functions and brain resilience with peripheral treatment in mice is potentially promising for paths toward human therapeutics in aging and disease.

EXPERIMENTAL PROCEDURES

Mice

All studies were conducted in a blinded manner in age-matched and sex-balanced, congenic C57BL/6 mice unless indicated otherwise. Specifically, mice were randomly allocated to each group, and the experimenter was blinded to their treatment. Aged mice were obtained from the National Institute on Aging (NIA) mouse colony. Transgenic hSYN mice (Rockenstein et al., 2002) overexpress human wild-type α -synuclein under the Thy-1 promoter. Because the α -synuclein transgene is inserted on the X chromosome in this line and randomly inactivates in females, we utilized only males from this line. Mice were kept on a 12-hr light/dark cycle with ad libitum access to food (Picolab Rodent Diet 20) and water. All studies were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco and conducted in compliance with NIH guidelines. See the [Supplemental Experimental Procedures](#) for further details.

Mass Spectrometry for PTMs of the Klotho Fragment

Mouse klotho fragment peptide (R&D Systems, 1819-KL) was trypsin-digested and subjected to liquid chromatography-tandem mass spectrometry using a NanoAcquity (Waters) ultra-performance liquid chromatography (UPLC) system interfaced to a linear trap quadrupole (LTQ) Orbitrap Velos (Thermo Scientific) mass spectrometer. Data were analyzed using Protein Prospector version 5.19 (Chalkley et al., 2008). See the [Supplemental Experimental Procedures](#) for further details.

Cognition and Behavior

Mice were tested as described previously in the Morris water maze (Dubal et al., 2014), small Y-maze (Dubal et al., 2014), two-trial Y-maze (Dellu et al., 1992), and rotarod (Morris et al., 2011). Drug treatments were administered as indicated. See the [Supplemental Experimental Procedures](#) for further details.

Synaptic Membrane Fractionation

Synaptic membrane fractions were separated for protein analysis as described (Dubal et al., 2014; Dubal et al., 2015).

Western Blot Analyses

Protein studies on the hippocampus and cortex were performed and quantified as described previously (Dubal et al., 2015). Additionally, α -synuclein (1:1,000, Abcam) and phosphorylated α -synuclein-Ser129 (1:1,000, Wako Pure Chemicals Industries) were measured. SDS (1%) was included in homogenates for optimal detection of the LMW GluN2B protein.

Immunoprecipitation

Immunoprecipitation (IP) of klotho was performed using modification of methods described previously (Bonifacino et al., 2001). In brief, hippocampal lysates were incubated with magnetic Dynabeads protein G (Thermo Fisher Scientific) conjugated with GluN2B antibody (2 μ g, 06600, Millipore) for specificity of bands identified by IP, GluN2B antibody (Ab) was incubated with a blocking peptide (KFNQSSNGHVYKELSSIESDV, 21 aa, generated by Watsonbio Sciences) harboring the GluN2B antibody epitope sequence prior to conjugation with Dynabeads. Beads were magnetically pulled, washed, and eluted. The precipitates were resolved on SDS-PAGE gel. See the [Supplemental Experimental Procedures](#) for further details.

Electrophysiology

Coronal brain slices of 300- μ m thickness from 3-month-old mice were obtained as described previously (Dubal et al., 2014; Dubal et al., 2015) with modifications. Recordings were performed using the Med64-Quad II multi-electrode array (Alpha MED Scientific) in the CA1 region following stimulation of the Schaffer collateral path. See the [Supplemental Experimental Procedures](#) for further details.

Statistical Analyses

Experimenters were blinded to the treatment. Statistical analyses were performed as described previously (Dubal et al., 2014, 2015) using GraphPad Prism 7 for t tests. R Studio (v 2.0) was used for rank-sum tests as described previously (Possin et al., 2016) and post hoc tests. All t tests were two-tailed unless indicated otherwise. Post hoc tests were conducted with the Bonferroni-Holm correction (R) to control for a family-wise error rate at $\alpha = 0.05$. Error bars represent SEM, and null hypotheses were rejected at or below a p value of 0.05.

See the [Supplemental Experimental Procedures](#) for further details regarding all experimental methods.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.07.024>.

AUTHOR CONTRIBUTIONS

J.L., D.W., B.I.G., R.J.C., A.L.B., A.J.M., and D.B.D. designed and conducted experiments and analyzed data. J.L. and D.B.D. wrote the manuscript. All authors edited and reviewed the manuscript.

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