Cell Reports, Volume *20*

Supplemental Information

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

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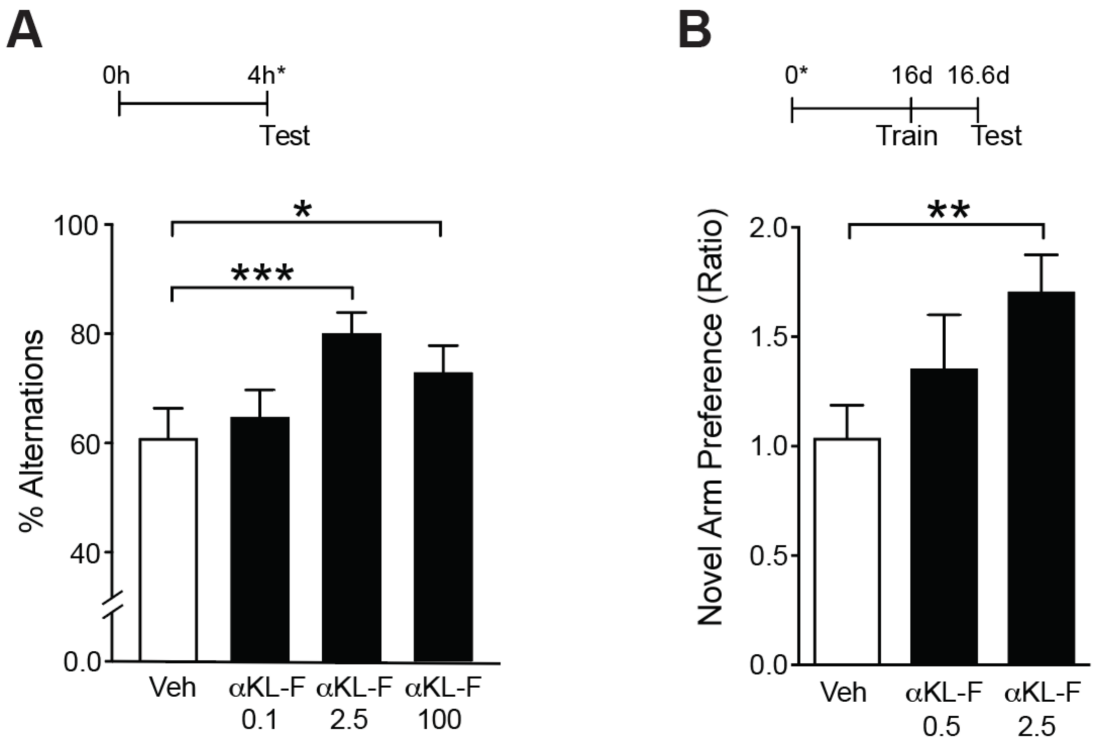
SUPPLEMENTAL RESULTS

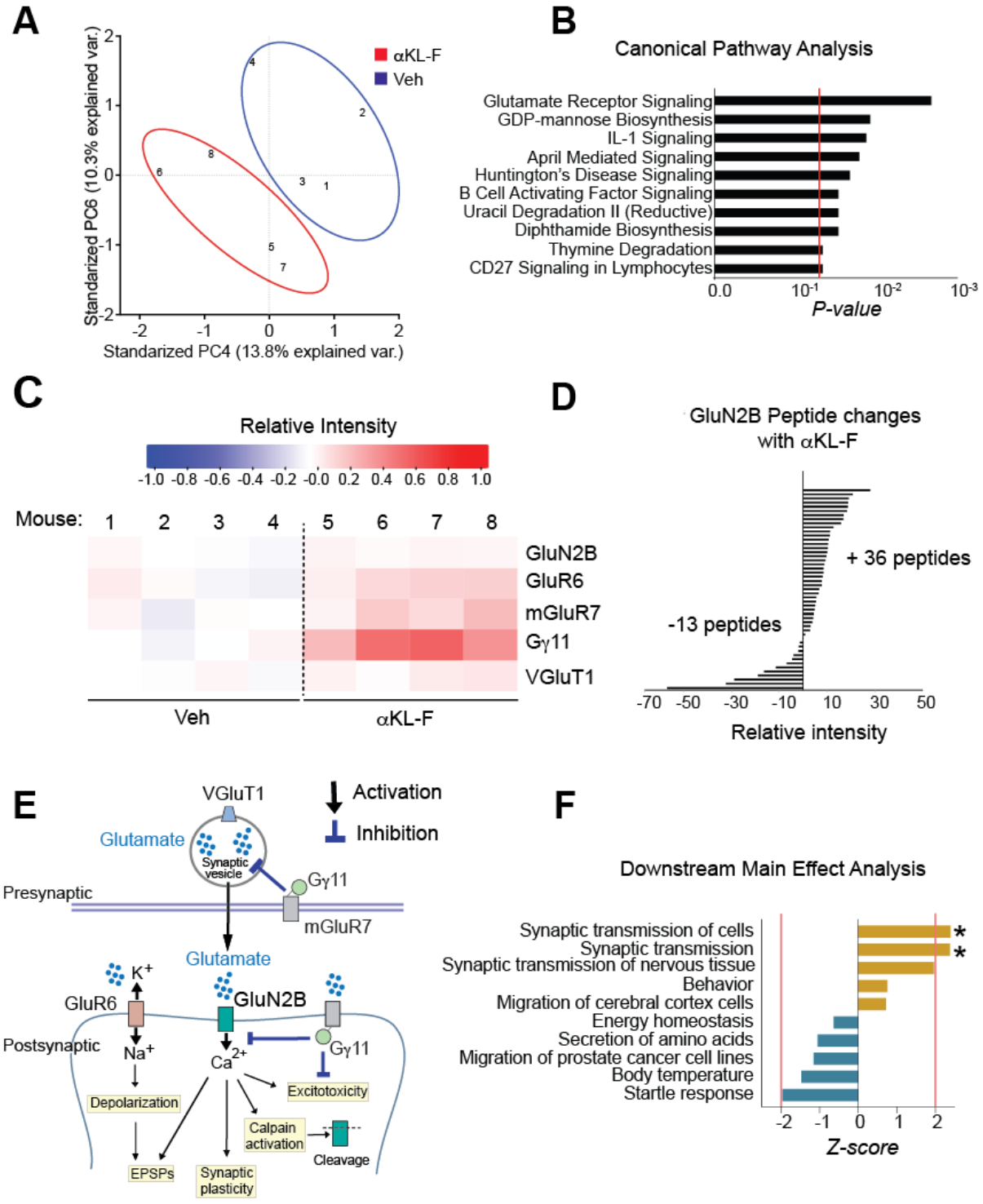


Supplemental Figure 1, α-Klotho fragment (αKL-F) delivered peripherally, induces a threshold-dependent dose response on cognition and is long lasting when combined with cognitive training, Related to Figure 2.

(A) Percent alternations among arms during exploration of the small Y-maze at two minutes. αKL-F increased alternations at 2.5 μg/kg with no further beneft at 100 μg/kg (n=9-10/group, males, age 4 months).

(B) Duration of time spent in the novel compared to the familiar arm during testing in the two-trial Y-maze as a ratio to indicate novel arm preference at 5 minutes of exploration. Mice received daily Veh or αKL-F treatment (i.p., 0.5 μg/kg and 2.5 μg/kg) for 5 days along with water maze training and testing. At 16 days following the last treatment (indicated as 0* in the diagram), mice were tested for spatial and working memory in the two-trial Y-Maze. (n=7-10 mice per group, sex-balanced groups, age 5-5.5 months). Prior peripheral αKL-F exposure combined with previous cognitive training induced a long-lasting and dose-dependent cogntive enhancement over two weeks after the last treatment.

*p<0.05, **p<0.01, ***p<0.001 (two-tailed, one sample t-tests vs Veh control level). Data are mean + SEM.



Supplemental Figure 2. α-Klotho fragment (αKL-F) acutely enriches glutamate receptor signaling as the top enriched canonical pathway, Related to Figure 6.

(A) Visualization of data for each mouse (as indicated by the mouse ID number) into a

reduced-components space defined by the principal components (PCs). From 4,172 protein groups identified in proteomic analysis, 121 proteins were recognized as changed between Veh- and αKL-F-treated samples (p≤0.05; pairwise two-tailed t-tests), and PCA analysis was performed. The distance between samples in the component space indicates dissimilarity in protein expression profiles.

(B-F) Proteomic analysis from hippocampus of mice treated with Veh or αKL-F 4h after a single i.p. injection (10 μg/kg) and small Y-maze with Ingenuity Pathway Analysis.

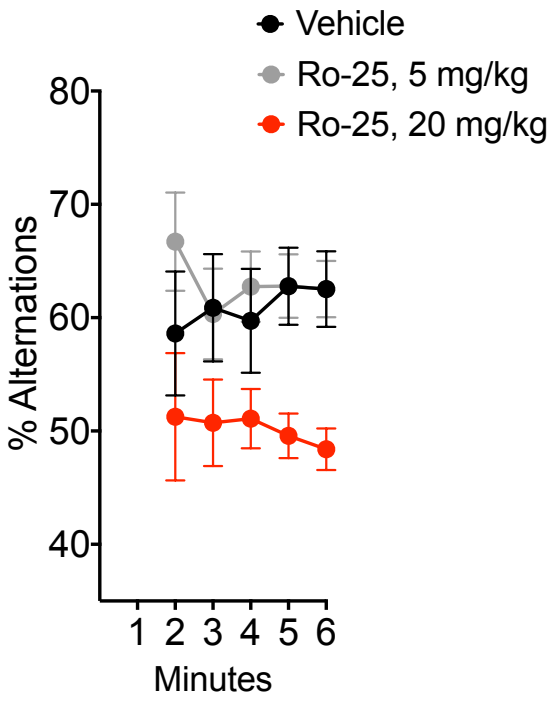
(B) Glutamate receptor signaling was the top significantly enriched canonical pathway in proteomic pathway analysis. p=0.002 (Fisher's exact test)

(C) Heat map of glutamate receptor signaling proteins significantly associated with αKL-F treatment (5 of 32 pathway molecules).

(D) αKL-F-treatment increased intensity of most, but not all, GluN2B (or GRIN2B) peptide fragments following trypsin digestion, suggesting it induces protein changes such as cleavage of GluN2B.

(E) Representation of glutamate receptor signaling pathways following acute αKL-F treatment showing activation of molecules that predict increased synaptic plasticity and inhibition of exessive signaling. Modified from IPA content analysis.

(F) Downstream main effect analysis of αKL-F treatment significantly predicted activation of synaptic transmission of cells (Z-score >2). Z-score > 2 indicates significant activation and Z-score < –2 indicates significant inhibition of biological functions (significance=red lines).



Supplemental Figure 3. Mice treated with the GluN2B antagonist Ro 25, without αKL-F treatment, show decreased alternations with a higher dose of the antagonist, Related to Figure 7. With a higher (20 mg/kg), but not lower (5 mg/kg), dose of Ro 25, mice show decreased alternations in the small Y-maze (age 4 months, males, n=11-12 per group). Mixed Model ANOVA: Ro 25 effect $p<0.05$; $p<0.05$ Ro 25, 20 mg/kg vs other groups (Bonferroni-Holm). Data are mean ± SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice and drug treatments. Studies were conducted in a blinded manner in age-matched and sex-balanced, congenic C57BL/6 mice, unless indicated otherwise. Aged mice were obtained from the NIA mouse colony. Transgenic hSYN mice (Rockenstein et al., 2002) overexpress wildtype human α-synuclein under the Thy-1 promoter. Since 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 12h 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.

Mouse α-Klotho fragment (R&D, 1819-KL) was diluted in PBS (pH 7.5) and administered i.p. at a volume of 10 μl/gram (adjusted to weight of mouse) before behavioral or synaptic plasticity testing of mice at protein doses (0.1-100 μg/kg) and timing indicated for each test. Klotho fragment was used within one week of thawing from -80º C stock solutions and stored at 4º C; all protein was received frozen and underwent freeze-thaw only once prior to use. Ro 25-6981 (Tocris) was dissolved in normal saline at 0.5 mg/ml and administered i.p. at a volume of 10 μl/gram (adjusted to weight of mouse), 10 min prior to behavioral testing of mice at doses (5-20 mg/kg) as indicated and described (Dubal et al., 2015).

To ensure rigor and reduced bias in behavior studies (Hirst et al., 2014) mice were randomly allocated to each group and the experimenter was blinded to their treatment. We retrospectively assessed our process of blinding and determined there was not a way that the experimenter could have become un-blinded. Briefly, another individual that was not the experimenter prepared the treatment tubes and logged the code in a in a secret location. The tubes appeared identical including clearness and color of solution. Mice were randomly assigned a treatment code with similar numbers of treatment in each experimental group. The experimenter then weighed each mouse prior to injection, injected the treatment and placed the mice back in their cage. No mice

experienced inflammation at the i.p. site or showed differences in behaviors such as hyperactivity or anxiety-like behaviors when systematically assessed in the open field or elevated plus maze tasks. Further, mice were weighed before and after behavior experiments and there were no differences in weight.

Mass Spectrometry for post-translational modifications of αKL-F. Mouse klotho fragment peptide (R&D, 1819-KL) was digested with trypsin then subjected to LC-MS/MS analysis using a NanoAcquity (Waters, Milford, MA) and a LTQ-Orbitrap Velos (Thermo, San Jose, CA). Chromatography was performed using an EASY-Spray Nano-LC source. Solvent A was 0.1% formic acid; Solvent B was 0.1% formic acid in acetonitrile. The flow rate was 400 nl/min. Recombinant klotho fragment samples were injected directly on-column, a 40 min linear gradient from 2–25% solvent B was employed, then organic content was increased to 50% over 5 min before returning to starting conditions. Data were acquired for 48 min. Mass measurements (m/z 350–1,400) were performed in the Orbitrap, and the six most abundant multiply charged ions were selected for HCD fragmentation analysis. Dynamic exclusion for precursor ion selection was enabled for 45 s. The trigger intensity was 2,000. The automatic gain control (AGC) settings were 2×10^6 for the mass measurements, and 9×10^4 for the HCD acquisitions

Immunoprecipitation (IP). IP of klotho was performed using modification of methods described (Bonifacino et al., 2001). In brief, hippocampal lysates (350 μg) were incubated with magnetic Dynabeads protein G (50 µl) (Thermofisher) conjugated with GluN2B antibody (2µg, 06600, Millipore) for 30 min at room temperature followed by rotation at 4 °C overnight. For specificity of bands identified by IP, the GluN2B Ab was incubated with a blocking peptide (KFNGSSNGHVYEKLSSIESDV, 21 aa, generated by WATSONBI) harboring the GluN2B antibody epitope sequence for 15 min at room temperature, prior to conjugation with Dynabeads. Beads were magnetically pulled and washed 3 times with washing buffer (150 mM NaCl, 20mM Tris-HCl pH 7.4, 0.01 % Tween) and eluted by heating for 10 minutes at 70°C in 65 µl of LDS NuPage loading buffer (Invitrogen). The precipitates were resolved on SDS/PAGE gel and run by Western blot.

Electrophysiology. Coronal brain slices of 300 μm thickness from 3 month old mice were obtained as described with some modifications (Dubal et al., 2014; Dubal et al., 2015) including that measurements were obtained from the CA1 region following stimulation of the Schaffer Collateral path. Briefly, after 4h of an i.p. injection of Veh or αKL-F, mice were anesthetized with pentobarbital. The brain was harvested and immediately placed in ice-cold artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 2.8 KCl, 2 $MgSO_4$, 1.25 NaH_2PO_4, 10 Glucose, 26 $NaHCO_3$, 3.6 $CaCl_2$, 1.3 Ascorbic acid and sliced on a vibratome (Leica). Slices were incubated at 32^oC for 30 minutes, then recovered at RT for 1 hour prior to testing. Slices were transferred to an interface chamber with circulating oxygenated (95% O_2 and 5% CO_2) aCSF at 30^oC and left to recover for 10-15 minutes prior to any stimulation.

For field potential recordings, acute hippocampal slices were placed on a Med64-Quad II multielectrode array (Alpha MED Scientific), which enables recording of 4 slices simultaneously. Field Excitatory Post Synaptic Potentials (fEPSP) were elicited and recorded via planar electrodes of the Quad II 2x8 Probe AL-MED-PG501A by aligning the electrodes and the stratum radiatum region of hippocampal slices. An input-output curve was performed at the beginning of each recording to determine the appropriate stimulation intensity. Test stimuli at 30-40% of maximal intensity were delivered at 0.05 Hz and a stable baseline of fEPSP of 15-20 mins was established before LTP induction. LTP was induced using a theta-burst protocol comprised of 3 trains delivered every 20 seconds, each train containing 10 bursts at 5 Hz, each burst containing four pulses at 100 Hz. LTP was induced at 10μA above test intensity to ensure robust LTP induction. Recordings and analysis were performed using Med64 Mobius Software (Alpha MED Scientific).

Proteomic methods and analysis. Hippocampal tissues were obtained from 4 mice treated with vehicle and 4 mice treated with αKL-F for 4 hours (i.p., 10 μg/kg) immediately following small Y-maze. Samples were derived from mice in each treatment group that showed overlapping measures of working memory, collectively represented a group mean for working memory, and were individually within one standard deviation

above or below the group mean. Protein samples were processed for and analyzed by mass spectrometry at Biognosys, Zurich, Switzerland in a manner blinded to treatment and as described (Bruderer et al., 2016). Briefly, proteins were urea-denatured, alkylated with iodoacetamide, digested with trypsin, and then purified. For spectral library generation, peptide pools for all samples were fractionated using high-pH reversed phase into 5 fractions each. Peptides were injected to an in-house packed C18 column on a Thermo Scientific Easy nLC nano-liquid chromatography system for all mass spectrometric analyses using a non-linear chromatographic gradient as described (Bruderer et al., 2016). Mass spectrometric analyses were carried out on a Thermo Scientific Q Exactive mass spectrometer equipped with a standard nano-electrospray source as described (Bruderer et al., 2016). Shotgun LC-MS/MS datasets were searched using the MaxQuant software package v 1.5.6.5 against a UniProt MOUSE database. Data was analyzed with Spectronaut v 10 software (Biognosys) using a spectral library generated from MaxQuant searches of shotgun runs. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD007150.

Proteomic dissociation patterns were investigated using principal component analysis, using the prcomp function in R. From 4,172 protein groups identified in proteomic analysis of hippocampus, 121 proteins were recognized as changed between Vehicle- and αKL-F-treated samples (pairwise, two-tailed t-tests $p \leq 0.05$). To investigate functions of these proteins and their roles in biological and physiological processes, a bioinformatics approach was applied with Ingenuity Pathways Analysis (IPA, Qiagen). This type of analysis represents a strategy to query proteins that change together. Since cellular processes largely affect sets of proteins acting in concert, even modest changes in members of a specific pathway may hold biological relevance. For IPA enrichment analysis, the gene set derived from the 121 changed proteins that mapped to the IPA Knowledge Database was included. The unfiltered proteomic data set was used as the background set. IPA analysis was performed for canonical pathway enrichment using Fisher's exact test. This approach probes the likelihood that the association between

the set of changed proteins and a given pathway is due to random chance; the lower the p-value, the less chance of a random association. Downstream main effect analysis (Kramer et al., 2014) was performed to predict the biological impact of protein changes using a Z-score calculation. This approach provides a statistical measure of non-randomness of the directionality of protein change within the data set. A Z score >2 or <-2 is considered significant. The significant prediction of the downstream main analysis was validated with functional studies.

Statistical Analyses for Behavior, Western blot, and Electrophysiology studies. Experimenters were blinded to the treatment. Statistical analyses for all behavior tasks, Western blot analyses, and electrophysiology studies were performed as described (Dubal et al., 2014; Dubal et al., 2015). Briefly, GraphPad Prism 7 was used for t-tests, R studio (v 2.0) was used for Rank Summary testing as described (Possin et al., 2016) and post-hoc tests. Exclusion criteria (greater than 2 standard deviations above or below the mean) were defined *a priori* to implement unbiased exclusion of outliers. All t-tests were two-tailed except for a replicate experiment, Figure 5G (one-tailed). Post-hoc tests were conducted with the Bonferroni-Holm correction (R) to control for a family-wise error rate at α=0.05 as indicated. Error bars represent SEM and null hypotheses were rejected at or below a p-value of 0.05.

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