SUPPLEMENTARY INFORMATION

Life extension factor klotho enhances cognition

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SUPPLEMENTAL RESULTS

Supplemental Table 1, Related to Experimental Procedures. Human and mouse cohorts analyzed in different experiments.

	Fig. Panel	Humans or Mice per Group	Total Number	From *Cohort	Age at Analysis
Humans: GENETICS and COGNITION					
Cohort 1	1A,2A S1	Non-carriers 179 KL-VS carriers 41	220 humans	A	52–85 years
Cohort 2	1B,2B S1	Non-carriers 331 KL-VS carriers 135	466 humans	B	55–85 years
Cohort 3	1C,2C S1	Non-carriers 20 KL-VS carriers 12	32 humans	C	52–78 years
Humans: SERUM ENZYME-LINKED IMMUNOASSAY					
Cohort 1	3A	Non-carriers 118 KL-VS carriers 38	156 humans	A	55–85 years
Mice: LONGEVITY					
	3C	NTG 29, KL 22	51 mice	D	3 wks–36 mos
Mice: COGNITION AND BEHAVIOR					
Water maze	3D,E, S2	NTG 9, KL 8	17 mice	E	10–12 mos
Water maze	3F,G, S2	NTG 19, KL 17	36 mice	F	4–7 mos
Fear conditioning	4B, S3	NTG 7, KL 6	13 mice	G	6 mos
Y-maze	4A	NTG 10, KL 8	18 mice	H	3–4 mos
Open field, elev plus	4C,D	NTG 14–15, KL 13–14	27–29 mice	I	3 mos
Water maze	6A,B	NTG 7, KL 7	14 mice	J	3–4 mos
Fear conditioning + Ifen	7E	NTG: Veh 19, Ifen 15 KL: Veh 17, Ifen 8	59 mice	K	5–7 mos
Y-maze + Ifen	7F	NTG: Veh 9, Ifen 11 KL: Veh 10, Ifen 10	40 mice	L	10–12 mos
Y-maze + Ro 25-6981	7G,H	NTG: Veh 19, Ro25 16 KL: Veh 18, Ro25 14	67 mice	M	3–5 mos
Fear conditioning	S6C	NTG: Veh 9, Ifen 9	18 mice	N	3.5–4.5 mos
Mice: BIOCHEMISTRY & HISTOLOGY					
Western	3B	NTG 18, KL 19	37 mice	O	3–4 mos
Western	S4A–E	NTG 13, KL 14–15	27–28 mice	O	3–4 mos
Western	5B–E,G S6A,B	NTG 15–17, KL 17–18	32–35 mice	P	3–4 mos
Immunohistochemistry	6A	NTG 7, KL 7	14 mice	J	3–4 mos
qPCR	S4F	NTG 17, KL 18	35 mice	P	3–4 mos
Mice: ELECTROPHYSIOLOGY					
fEPSP (LTP)	6C	NTG 4 slices/4 mice KL 8 slices/6 mice	12 slices, 10 mice	Q	3.5–4.5 mos
fEPSP (I/O curves)	6D	NTG 5 slices/3 mice KL 9 slices/5 mice	14 slices, 8 mice	Q	3.5–4.5 mos
Evoked EPSC and decay	6F,G	NTG 7–10 slices/3 mice KL 5–10 slices/3 mice	12–20 slices, 6 mice	R	3–4 mos
Evoked EPSC and decay	7B–D	NTG 6–7 slices, 3 mice KL 5 slices,3 mice	11–12 slices, 6 mice	R	3–4 mos
sEPSC	S5	NTG 15 cells/2 mice KL 17 cells/2 mice	32 cells, 4 mice	S	3–4 mos

*Cohorts are independent groups of humans or mice, some or all of which were analyzed at one or more time points/ages in one or more paradigms.

Supplemental Table 2, Related to Figure 1. Selection criteria for each population of aging individuals without dementia that was studied. For analyses, individuals between 52–85 years with MMSE scores of 28 or greater were drawn from Cohorts 2 and 3 (replication cohorts) to parallel cognitive profiles and demographics of Cohort 1 (the discovery cohort).

	Inclusion Criteria	Exclusion Criteria
Cohort 1 (Discovery cohort)		
	Stable medical condition for three months	Memory complaints
	Fluent in English	Diagnosed memory condition
	Age 40 years or above	Neoplastic disease
	Able to complete assessment	Parkinson's disease
	Medical history, physical exam, neurologic exam, and clinical tests completed without meeting any exclusion criteria.	Multiple sclerosis, untreated
		Sleep apnea
		Stroke
		Current or past psychiatric disorder by DSM-IV criteria
		Abnormal brain MRI
		Abnormal neurologic exam
Cohort 2 (Replication cohort)		
	Older individuals without known dementia	None (medical co-morbidities allowed)
	Annual detailed clinical evaluation and blood donation	
	Agree to organ donation at death	
Cohort 3 (Replication cohort)		
	Willingness to participate in studies on memory and aging	Baseline dementia or mild cognitive impairment
		Medical or psychiatric illnesses
		Use of medications that might affect cognition (ie, sedatives)
		Use of non-steroidal anti-inflammatory drugs
		Inability to undergo MRI because of pacemakers or hip replacement implants

Supplemental Table 3, Related to Figure 1. Demographics for each population of aging individuals without dementia studied. All individuals, except for three in Cohort 3, were Caucasian.

Study	**Demographics**	**Non-carrier (n=530)** Mean (SD)	**KL-VS carrier (n=188)** Mean(SD)
Cohort 1			
	Genotype	179	41
	Age (yrs)	67.53(7.38)	68.56(7.76)
	Education (yrs)	17.25(2.02)	17.40(2.42)
	Male/Female	71/112	23/20
	ApoE4 Carriers	41	8
	CDR total	0	0
	MMSE	29.54(0.66)	29.44(0.71)
Cohort 2			
	Genotype	331	135
	Age (yrs)	77.84(5.34)	78.12 (5.52)
	Education (yrs)	15.04 (2.96)	17.89(2.15)
	Male/Female	82/249	39/96
	[#]ApoE4 Carriers	79	30
	MMSE	29.02(0.78)	29.07(0.76)
Cohort 3			
	Genotype	20	12
	Age (yrs)	62.85(7.51)	64.50(7.83)
	Education (yrs)	16.40 (2.68)	18.00 (3.41)
	Male/Female	17/5	4/8
	[#]ApoE4 Carriers	7	2
	MMSE	29.35(0.75)	29.83(0.39)

SD=standard deviation; MMSE=Mini Mental State Exam; CDR=Clinical Dementia Rating; [#]Some *APOE* ε4 genotypes were unknown: Cohort 2 (12 KL-VS non-carriers and 9 KL-VS carriers); Cohort 3 (1 KL-VS non-carrier).

Supplemental Table 4, Related to Figure 1. Cognitive functions and domains represented in neuropsychological tests analyzed in Cohorts 1–3.

***Broad Cognitive Domains**	***Specific Cognitive Functions**	**Cohort 1 Tests** Refs (Kramer et al., 2003; Pa et al., 2010)	**Cohort 2 Tests** Refs (Bennett et al., 2012; Wilson et al., 2002)	**Cohort 3 Tests** Refs (Small et al., 2006; Small et al., 2012)
Language	Semantic/Phonemic Generation	Category & Phonemic Fluency	Category Fluency	Category & Phonemic Fluency
Executive	Working Memory, Attention/Inhibition, Processing Speed, Set Shifting	Digit Span Backwards, Modified Trails, Stroop Color Naming	Digit Span Backwards, Digit Ordering, Stroop Color Naming	Digit Span Total, Trails A+B, Stroop Interference
Visuospatial	Spatial Memory, Spatial Processing	Benson Figure Delay	Line orientation	Benson Figure Delay
Learning & Memory	Auditory/Verbal Episodic Learning & Memory	CVLT II Short Recall	Delayed Word List Recognition, Delayed Story Recall	Slope Logical Memory

*Though distinctly categorized, tests often reflect multiple cognitive functions and domains. For example, semantic and phonemic generation are influenced by verbal, language, and executive abilities.

Supplemental Table 5, Related to Figures 1 and 2. KL-VS is associated with better cognition in a meta-analysis of cohorts and in each of the three independent human cohorts included in the meta-analysis. The linear statistical model provides an estimate of change in global composite Z-score and includes age, sex, education, and KL-VS genotype, with or without *APOE* ε4 carrier status, as predictors for cognitive performance. Inclusion of *APOE* ε4 in the model did not contribute significant variance (p=0.64, meta-analysis) or change results associating KL-VS with enhanced cognition.

	Estimate	Std Error	t value	p value	Significance
Meta-analysis of Cohorts 1–3					
Age	–0.02	0.00	–5.57	3.54×10^{-8}	***
Sex (F)	0.24	0.08	3.10	2.07×10^{-3}	**
Education	0.07	0.01	5.38	1.00×10^{-7}	***
Genotype, KL-VS	0.33	0.08	4.10	4.41×10^{-5}	***
Meta-analysis of Cohorts 1–3 (*APOE* ε4 added to model)					
Age	–0.02	0.00	–5.42	8.16×10^{-8}	***
Sex (F)	0.24	0.08	3.01	2.73×10^{-3}	***
Education	0.07	0.01	5.34	1.29×10^{-7}	***
APOE ε4	–0.03	0.08	–0.46	0.64	–
Genotype, KL-VS	0.32	0.08	3.95	8.61×10^{-5}	***
Cohort 1					
Age	–0.05	0.01	–5.62	5.87×10^{-8}	***
Sex (F)	0.05	0.13	0.41	0.68	–
Education	0.11	0.03	3.59	4.17×10^{-4}	***
Genotype, KL-VS	0.39	0.16	2.45	0.01	*
Cohort 1 (*APOE* ε4 added to model)					
Age	–0.05	0.01	–5.59	6.76×10^{-8}	***
Sex (F)	0.07	0.13	0.53	0.60	–
Education	0.11	0.03	3.62	3.62×10^{-4}	***
APOE ε4	0.14	0.14	1.03	0.30	–
Genotype, KL-VS	0.40	0.16	2.51	0.01	*
Cohort 2					
Age	–0.05	0.01	–6.38	4.44×10^{-10}	***
Sex (F)	0.25	0.10	2.52	0.01	*
Education	0.09	0.01	6.20	1.25×10^{-9}	***
Genotype, KL-VS	0.25	0.09	2.64	8.61×10^{-3}	**
Cohort 2 (*APOE* ε4 added to model)					
Age	–0.05	0.01	–6.07	2.77×10^{-9}	***
Sex (F)	0.25	0.10	2.55	0.01	*
Education	0.09	0.02	6.20	1.34×10^{-9}	***
APOE ε4	–0.10	0.09	–1.11	0.27	–
Genotype, KL-VS	0.23	0.09	2.47	0.01	*
Cohort 3					
Age	–0.07	0.03	–2.89	0.01	*
Sex(F)	0.04	0.40	0.11	0.91	–
Education	0.04	0.06	0.77	0.45	–
Genotype, KL-VS	0.63	0.32	1.95	0.06	#
Cohort 3 (*APOE* ε4 added to model)					
Age	–0.07	0.03	–2.80	0.01	*
Sex (F)	–0.04	0.43	–0.08	0.94	–
Education	0.04	0.06	0.70	0.49	–
APOE ε4	–0.20	0.32	–0.64	0.53	–
Genotype, KL-VS	0.59	0.35	1.69	0.10	#

= near significance

Supplemental Table 6, Related to Figures 1 and 2. Probing for a sex by KL-VS interaction and an age by KL-VS interaction on cognition. A linear statistical model was used to probe for a Sex:KL-VS interaction and an Age:KL-VS interaction on cognition in the meta-analysis of three cohorts. The model provides an estimate of change in global composite Z-score and includes age, sex, education, and KL-VS genotype as predictors for each interaction. For the Sex:KL-VS analysis, no significant interaction effect was identified. A power analysis with the current sample size and observed variation revealed that the effect would need to result in a change (or estimate) of –0.48 in the cognitive score (compared to the current estimate of –0.25) to be detected with 80% power at the α = 0.05 significance level. Inclusion of *APOE* ε4 in the linear statistical model did not contribute significant variance (p=0.61) or change results for a Sex:KL-VS interaction on cognition. For the Age:KL-VS analysis, the interaction reached near significance for demonstrating that KL-VS–associated cognitive enhancement decreases with increasing age. A power analysis with the current sample size and observed variation revealed that the effect would need to result in a change (or estimate) of –0.028 in the cognitive score (compared to the current estimate of –0.02) to be detected with 80% power at the α = 0.05 significance level. Inclusion of *APOE* ε4 in the linear statistical model did not contribute significant variance (p=0.72) or significantly change results of the effects of Age:KL-VS interaction on cognition.

		Estimate	Std Error	t value	p value	Significance
Sex:KL-VS Analyses	**Meta-analysis of three cohorts**					
	Age	–0.02	0.00	–5.52	4.64×10^{-8}	***
	Sex (F)	0.31	0.09	3.39	7.32×10^{-4}	***
	Education	0.07	0.13	5.43	7.52×10^{-8}	***
	Genotype, KL-VS	0.74	0.30	2.50	0.01	*
	Sex (F):KL-VS	–0.25	0.17	–1.44	0.15	–
	Meta-analysis of three cohorts (*APOE* ε4 added to model)					
	Age	–0.02	0.00	–5.38	1.05×10^{-7}	***
	Sex (F)	0.31	0.09	3.35	8.45×10^{-4}	***
	Education	0.07	0.13	5.40	9.17×10^{-8}	***
	APOE ε4	–0.04	0.08	–0.51	0.61	–
	Genotype, KL-VS	0.75	0.30	2.52	0.01	*
	Sex (F):KL-VS	–0.26	0.17	–1.50	0.13	–
Age:KL-VS Analyses	**Meta-analysis of three cohorts**					
	Age	–0.02	0.01	–4.11	4.47×10^{-5}	***
	Sex (F)	0.24	0.08	3.13	1.85×10^{-3}	***
	Education	0.07	0.01	5.32	1.36×10^{-7}	***
	Genotype, KL-VS	1.56	0.76	2.06	0.04	*
	Age:KL-VS	–0.02	0.01	–1.63	0.10	#
	Meta-analysis of three cohorts (*APOE* ε4 added to model)					
	Age	–0.02	0.01	–3.87	1.18×10^{-4}	***
	Sex (F)	0.24	0.08	3.05	2.42×10^{-3}	***
	Education	0.07	0.01	5.26	1.95×10^{-7}	***
	APOE ε4	–0.03	0.08	–0.36	0.72	–
	Genotype, KL-VS	1.75	0.76	2.30	0.02	*
	Age:KL-VS	–0.02	0.01	–1.88	0.06	#

F=Female; #near significance

Supplemental Table 7, Related to Figure 3. Klotho serum levels were significantly increased in individuals with one KL-VS allele. The linear statistical model provides an estimate of change in klotho levels. The model includes age, sex, education, and KL-VS genotype as predictors for klotho levels (pg/mL). There is an age effect that reaches near significance for showing that klotho levels decrease with increasing age, an effect previously reported (Semba et al., 2011; Yamazaki et al., 2010). A power analysis with the current sample size and observed variation revealed that the age effect would need to result in a change (or estimate) of –9.50 in pg/mL of klotho levels (compared to the current estimate of –5.88) to be detected with 80% power at the α = 0.05 significance level. Inclusion of *APOE* ε4 in the linear statistical model revealed that it did not contribute significant variance (p=0.79) or change results of the effects of KL-VS genotype on klotho levels.

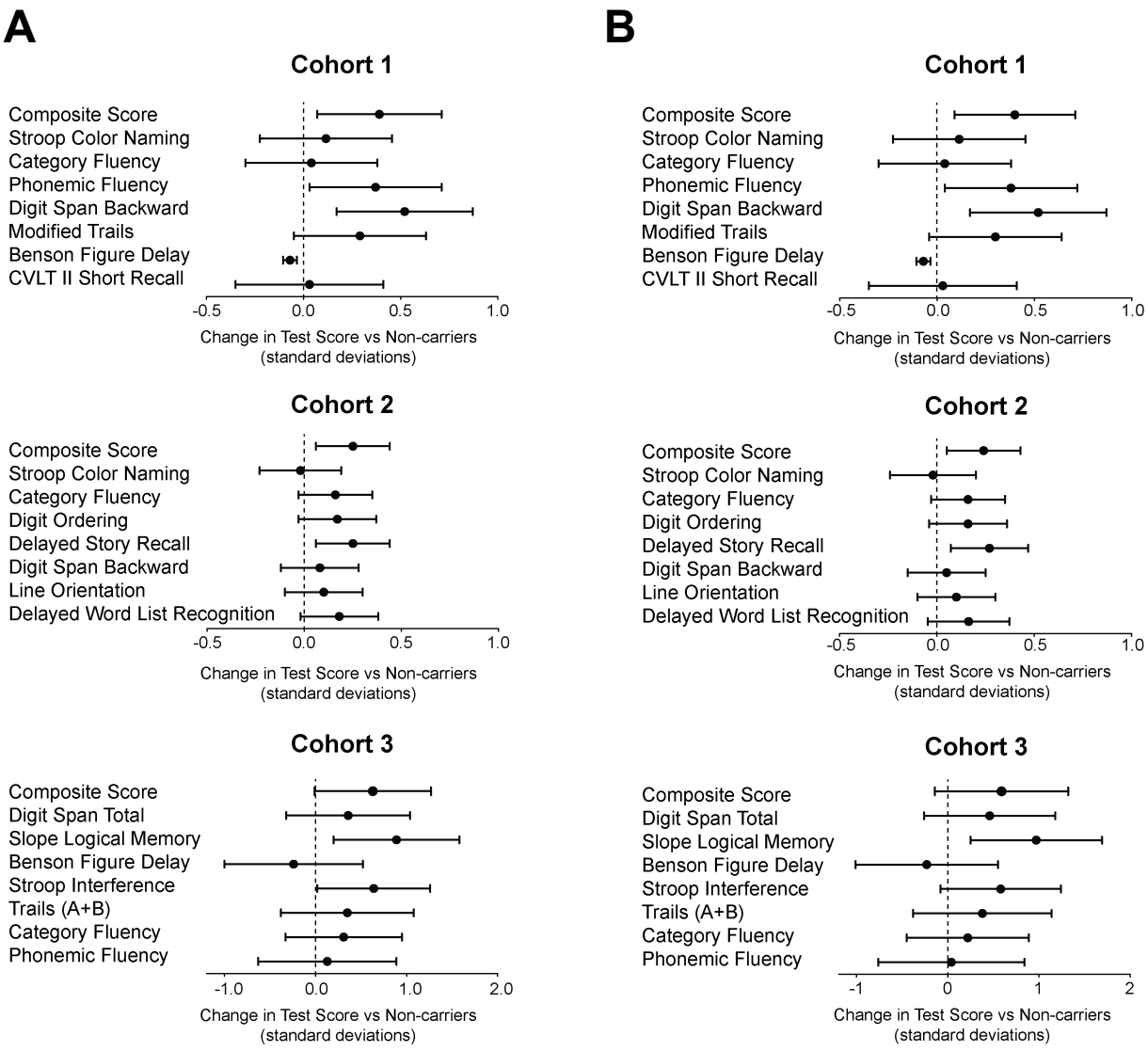
	Estimate	Std Error	t value	p value	Significance
Cohort 1					
Age	–5.88	3.41	–1.70	0.08	#
Sex (F)	33.95	41.97	0.81	0.42	–
Education	2.50	10.35	0.24	0.81	–
Genotype, KL-VS	104.27	48.40	2.15	0.03	*
Cohort 1 (*APOE* ε4 added to model)					
Age	–5.78	3.41	–1.70	0.09	#
Sex (F)	35.69	42.58	0.84	0.40	–
Education	2.50	10.39	0.24	0.81	–
APOE ε4	11.42	42.06	0.27	0.79	–
Genotype, KL-VS	105.72	48.84	2.17	0.03	*

F=Female; #near significance

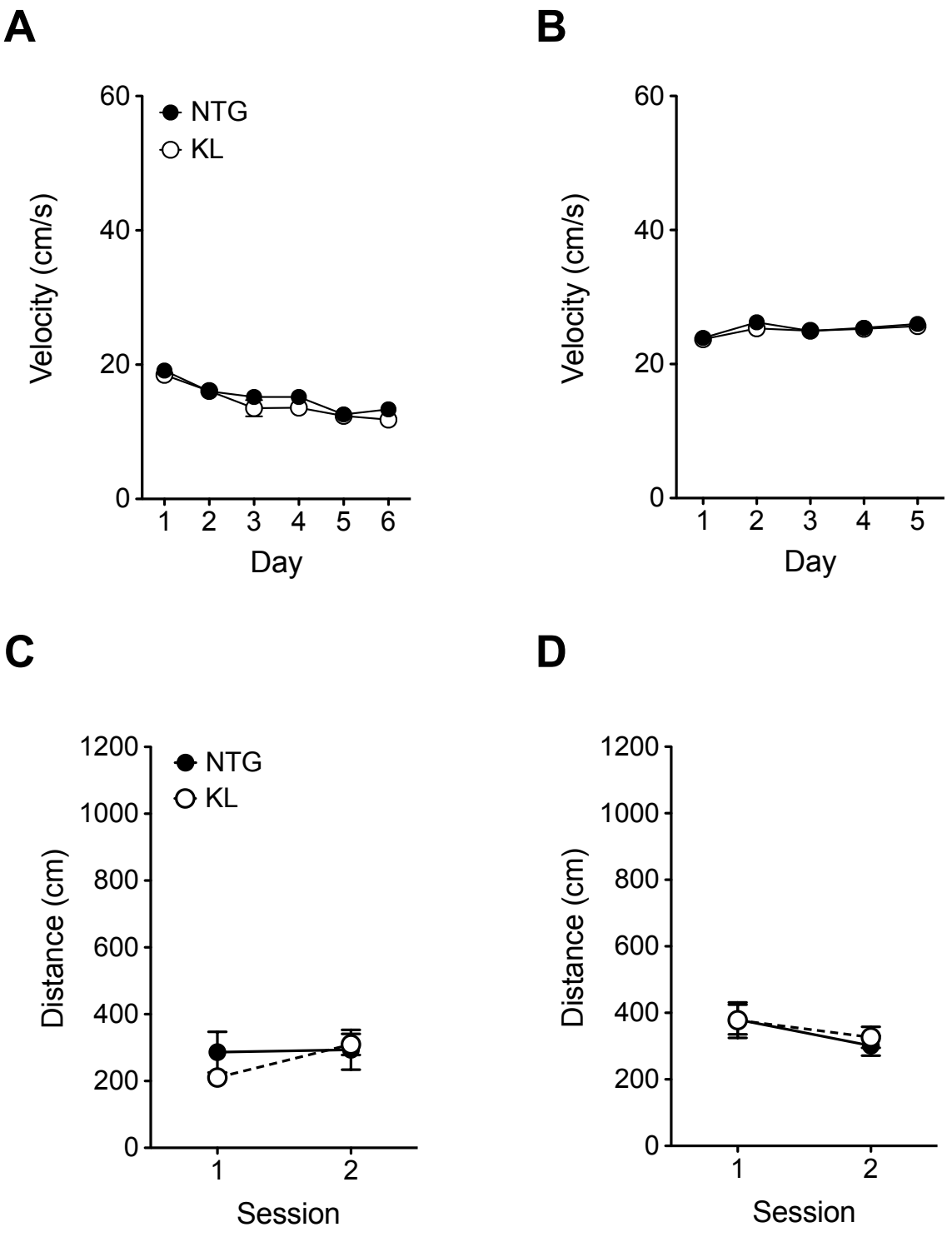
Supplemental Table 8, Related to Figure 3. Probing for a sex effect and a sex:klotho interaction on cognition in water maze testing of young and middle-aged mice. A mixed model ANOVA (factors: genotype and day) including effects of repeated measures was used as described (Young et al., 2009). No significant main effects of sex or sex:klotho interaction were identified.

	Estimate	Std Error	t value	p value	Significance
Young mice: Hidden Training, watermaze					
Sex (F)	–2.43	60.71	–0.04	0.97	–
Sex (F):klotho	–64.33	87.85	–0.73	0.95	–
Middle-age mice: Hidden Training, watermaze					
Sex (F)	61.53	77.79	0.79	0.44	–
Sex (F):klotho	–25.66	110.02	–0.23	0.82	–
Young mice: Probe Trial					
Sex (F)	–4.21	6.16	5.13	0.61	–
Sex (F):klotho	0.72	11.80	0.06	0.95	–
Middle-age mice: Probe Trial					
Sex (F)	16.48	9.59	1.72	0.11	–
Sex (F):klotho	–17.76	13.57	–1.31	0.21	–

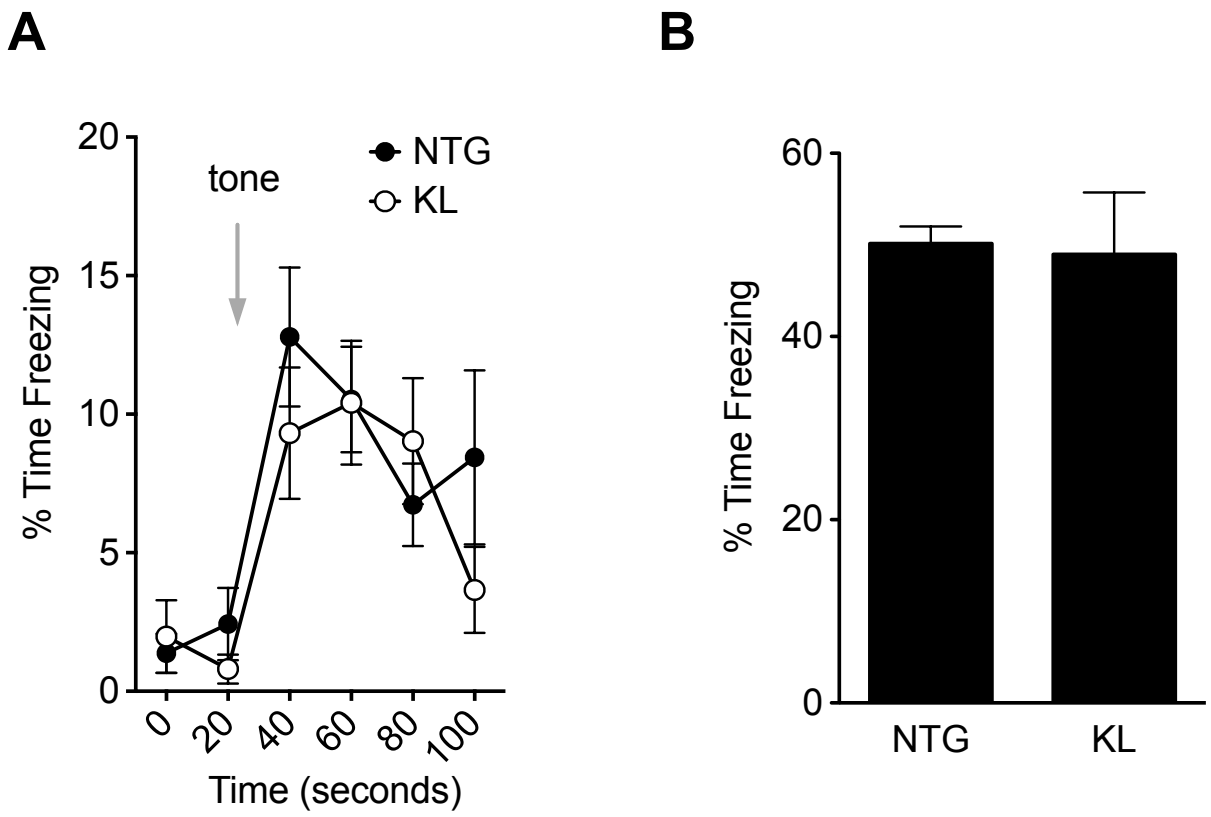
F=Female



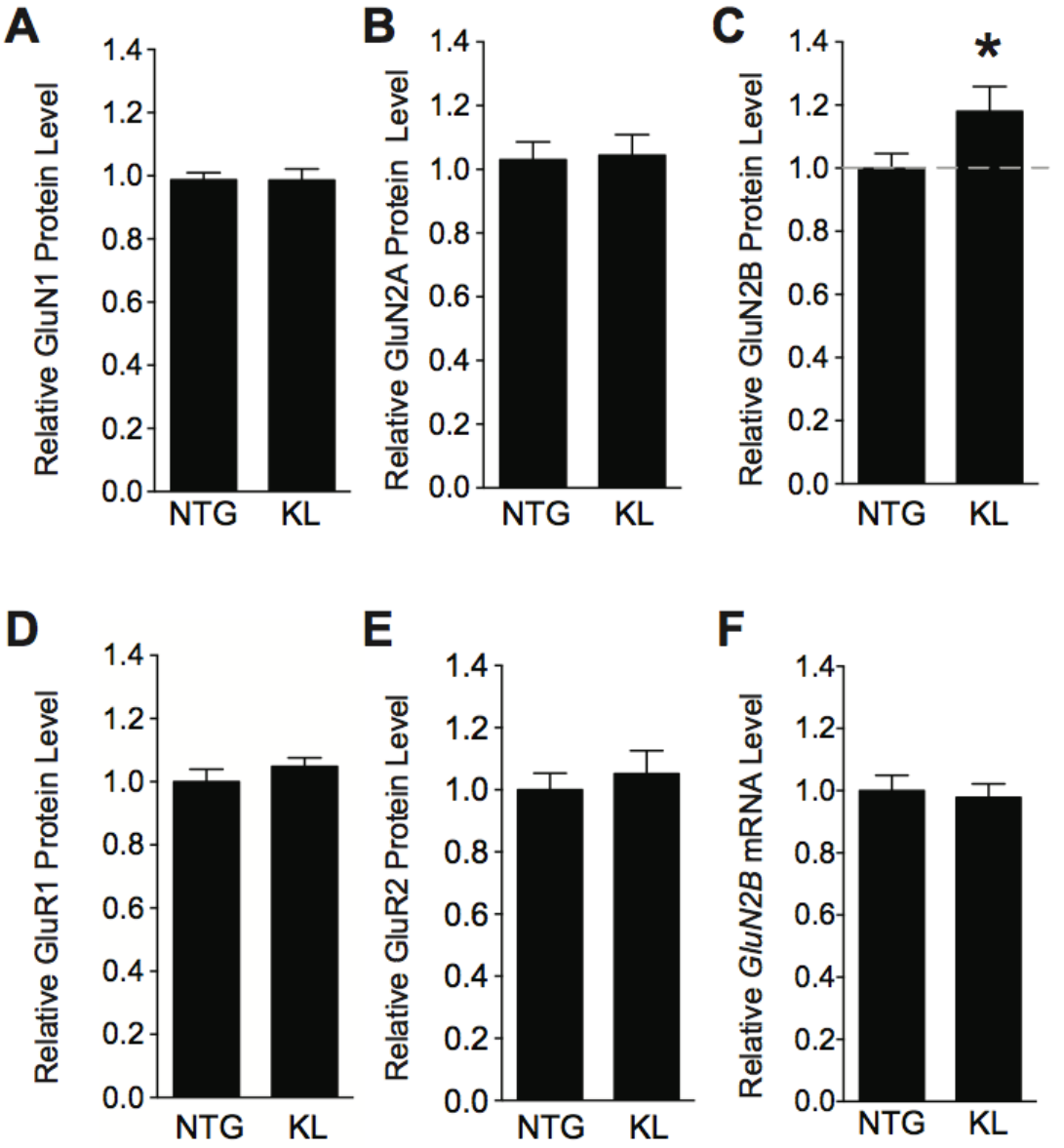
Supplemental Figure 1. Estimated changes associated with the KL-VS genotype in composite and individual neuropsychological test scores, Related to Figure 1. Each plot represents a maximum likelihood estimate of the KL-VS genotype effect on the composite score or specific test score with 95% confidence bounds after adjusting for (**A**) age, sex, and education or (**B**) age, sex, education and *APOE* ε4 carrier status. Mean results in non-carriers were used as a reference point (dotted line). Positive changes in test scores represent better cognitive performance.



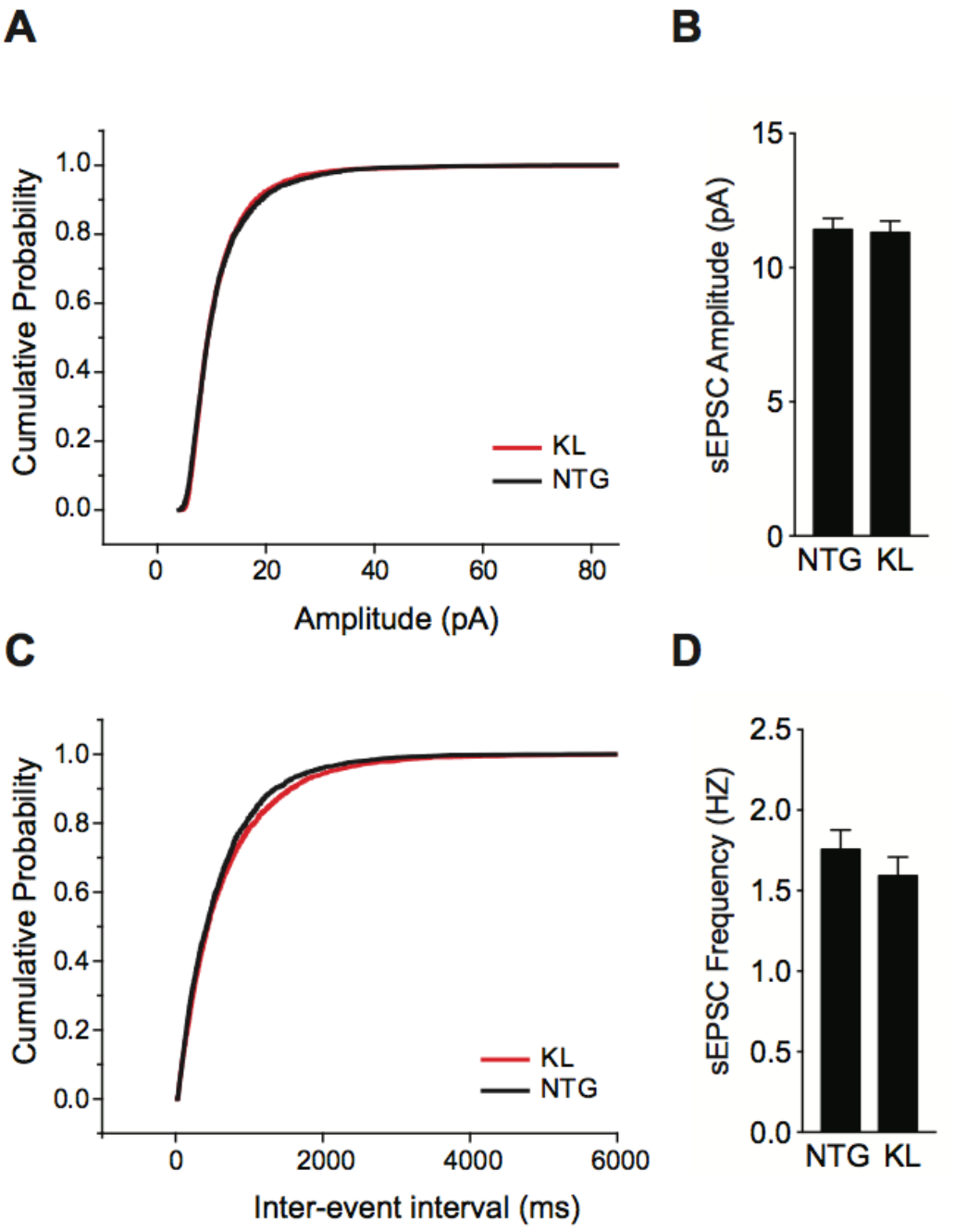
Supplemental Figure 2. NTG and KL mice showed no differences in swim speeds or distances traveled to find a cued (visible) platform in the Morris water maze, Related to Figure 3. (A, B) Swim speeds during hidden platform training in middle aged (10–12 months; n=8–9 per genotype) (A) and young (4–7 months; n=18–19 per genotype) (B) NTG and KL mice. (C, D) Distance traveled to find a cued (visible) platform in the water maze by middle-aged (C) and young (D) NTG and KL mice. Data are means ± SEM.



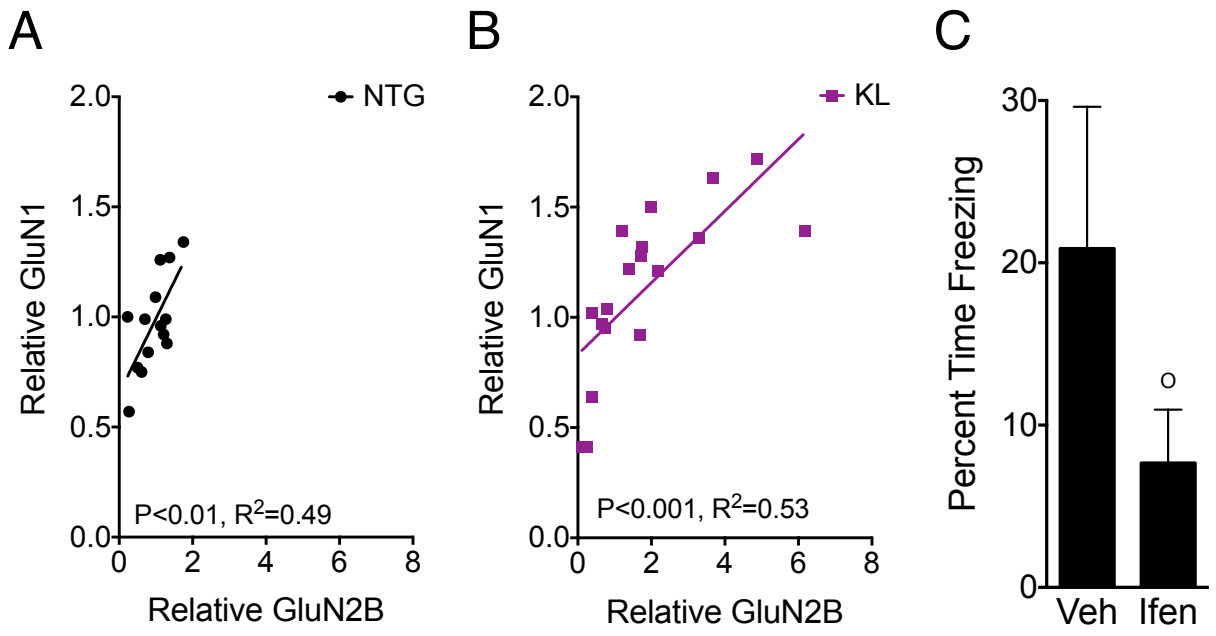
Supplemental Figure 3. NTG and KL mice showed no difference in hippocampus-independent cued recall to tone presentation in a different context, Related to Figure 4. Freezing was measured 24 h after training (n=6–7 mice per genotype, age 5–7 months). (A, B) Percent freezing following a 20-s tone and 60-s silence (A) and following 4 consecutive tone presentations averaged over the full testing period (B). Data are means ± SEM.



Supplemental Figure 4. Klotho elevation increases total hippocampal protein, but not mRNA, levels of NMDA receptor subunit GluN2B and does not alter total protein levels of GluN1 and GluN2A or AMPA receptor subunits GluR1 and GluR2, Related to Figure 5. Quantification of western blot signals from whole hippocampal homogenates relative to levels found in NTG mice (n=13–15 mice per genotype, age 3 months) for NMDA receptor subunits (A) GluN1, (B) GluN2A, (C) GluN2B, and AMPA receptor subunits (D) GluR1 and (E) GluR2. *p<0.05 vs NTG (t-test). Actin served as a loading control and did not differ between groups (not shown). Dashed grey line is level of GluN2B protein in NTG mice. (F) Quantification of *GluN2B* mRNA levels by quantitative PCR (n=17–18 mice per genotype, age 3 months). Data are means ± SEM.



Supplemental Figure 5. Spontaneous EPSCs (sEPSCs) of dentate granule cells in acute hippocampal slices are not significantly different between NTG and KL mice, Related to Figure 6. (A) Cumulative plot and (B) means of sEPSC amplitudes (p=0.86, unpaired t-test). (C) Cumulative plot of sEPSC inter-event intervals. (D) Means of sEPSC frequency (p=0.34, unpaired t-test). Number of cells/mice: NTG 15/2, KL 17/2 (age 3.5–4.5 months). Data are means ± SEM.



Supplemental Figure 6. GluN1 levels correlate with GluN2B levels in PSD fractions from NTG and KL mice; and higher dose of ifenprodil suppresses percent time freezing during context testing in NTG mice trained with lower number of shocks, Related to Figure 7. (A, B) Levels of GluN1 and GluN2B were quantified in PSD-95 enriched membrane fractions from the hippocampus of NTG (A) and KL (B) mice (age 3–4 months; 14–18 mice per group). (C) Mice (n=9 per group, age 3.5–4.5 months) received a single i.p. injection of vehicle or ifenprodil (Ifen., 7.5 mg/kg) 30 min before training in a fear conditioning paradigm consisting of 2 shocks (compared to 4 shocks used in the testing paradigm of Figure 7E). The percent of time mice spent freezing during the context testing session 24 h later was monitored. ^{o}p=0.08 (t-test). Data are means ± SEM.