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ORIGINAL ARTICLE

Secreted αKlotho isoform protects against age-dependent memory deficits

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α*Klotho* is a gene regulator of aging, increasing life expectancy when overexpressed and accelerating the development of aging phenotypes when inhibited. In mice, expression levels of the secreted isoform Klotho (s-KL) are very high in the brain, suggesting that s-KL activity may have an important role in the nervous system. Here we study the functional relevance at behavioural level of modifying s-KL levels in the aging brain. We used AAVrh10 vectors to deliver and sustained expression of s-KL in 6- and 12-month-old wild-type C57BL/6J males. This study demonstrates for we believe the first time *in vivo* that 6 months after a single injection of s-KL into the central nervous system, long-lasting and quantifiable enhancement of learning and memory capabilities are found. More importantly, cognitive improvement is also observable in 18-month-old mice treated once, at 12 months of age. These findings demonstrate the therapeutic potential of s-KL as a treatment for cognitive decline associated with aging.

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INTRODUCTION

In the late 90's, a study in mice revealed that mutation of a single gene (*αKlotho*) causes rapid aging with typical symptoms such as atherosclerosis, osteoporosis and ectopic calcification in different tissues, leading to a short average life expectancy of 2 months.[1,2] In brain, kl^{-}/kl^{-} mice have neuronal degeneration in the hippocampus;[3] hypomyelination;[4] decreased levels of related synapse proteins;[3] defective axonal transport;[5] and deficits in memory processing.[6] Consistent with these findings, a recent article by Dubal *et al.*[7] demonstrates that elevating Klotho levels have beneficial effects on synaptic and cognitive functions through a mechanism involving the GluN2B subunit of the NMDA receptor (NMDAR). Further, chronic administration of ligustilide prevents the development of Alzheimer's disease-like neuropathologies and memory impairment via *klotho* upregulation.[8] Moreover, studies in three independent human cohorts showed that human carriers of the *klotho* KL-VS allele, which increases secretion of Klotho *in vitro*, obtained better results in various cognitive tests.[9]

To date, all studies have focused on the transmembrane and the processed forms of Klotho (named m-KL and p-KL by Foster *et al.*[10]). In pioneering work, Masso *et al.*[11] have recently demonstrated that alternative splicing of Klotho (s-KL) produces a stable truncated isoform, which can be detected directly in mouse protein extracts. This work also shows a strong correlation between high expression levels of the two *klotho* transcripts in brain and healthy status while aging. Significantly, the secreted s-KL isoform is almost exclusively ($>90\%$) found in brain, while m-KL is mostly expressed in kidney and to a lesser extent in brain. This suggests s-KL may have an important role in brain.[11] More detailed study revealed that the s-KL protein could be detected in different murine brain regions involved in learning and memory processes, such as prefrontal cortex, motor cortex and hippocampus. Conceivably both isoforms may have similar roles, but as they are transcribed differently, they may have distinct functions. Here we study the role of s-KL in cognitive processes. We hypothesise it is a neuroprotective protein involved in the onset and/or progression of cognitive deficits associated with aging. To explore its effects, we modified s-KL levels in the brains of adult wild-type C57Bl/6J male mice using AAVrh10 gene therapy vectors.

MATERIALS AND METHODS

Animals

C57Bl/6J mice (Harlan Laboratories) were housed under standard laboratory conditions (food and water *ad lib*, 22 ± 2 °C, 12 h light:dark starting at 08:00 hours). This study was carried out in strict accordance with the current National regulations (Generalitat de Catalunya Decret 214/97, 30 July 1997). The Committee on the Ethics of Animal Experiments of the Universitat Autònoma de Barcelona approved all procedures described in this study (protocol number: CEEAH 2196/DMAH 7370).

Viral vector production and purification

AAVrh10 vectors (Null, s-KL, shRNA-sKL, shRNA-scrambled) were produced, purified in the biosafety level 2 facilities of the Unitat Mixta UAB-VHIR and the Vector Production Unit (VPU) (http://sct.uab.cat/upv/). Briefly, vectors were generated using the triple transfection system in HEK293 cells. After 48 h, AAV vectors were harvested, treated with benzonase, purified in an iodixanol gradient, and titred using the Picogreen system.[12] In all cases, transgene expression was driven by a CMV promoter.

In vivo administration of AAV vectors

Mice were anaesthetised by intraperitoneal injection of ketamine ($10\ mg\ kg^{-1}$; Imalgene 500; Rhone-Merieux, Barcelona, Spain) and xylazine ($1\ mg\ kg^{-1}$; Rompun; Bayer, Barcelona, Spain). Deeply anaesthetised

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animals were placed in a stereotactic frame and injected with 10E10vg of vector at a speed of 0.5 µl min^{-1} using an ultramicropump (World Precision Instruments, Sarasota, FL, USA). Injected volume was of 3 µl in a single dose for intraventicular administration, or two doses–one per hemisphere–of 1.5 µl each for hippocampal administration. AAV vectors were administered using known coordinates: intracerebroventricular (anteroposterior: − 0.22 mm; Lateral: 1.0 mm; Depth: 2.5 mm) and intra-hippocampal (anteroposterior: − 2.46 mm; Mediolateral: 1.5 mm; Depth: 2.0 mm).

Physical status: body weight and sensorimotor function

The body weight of animals was monitored once a month while sensorimotor function was measured only once, at the beginning of the behavioural battery of tests. Reflexes (visual reflex and posterior legs extension reflex tests) were measured by triplicate by holding the animal by his tail and slowly lowering it onto a black surface. The motor coordination and equilibrium were assessed by the distance covered and the latency to fall off a horizontal wooden rod (1.3 cm wide) on two consecutive 20 s trials, respectively. Prehensility and motor coordination were measured as the distance covered on the wire hang test, which consisted in allowing the animal to cling from the middle of a horizontal wire (diameter: 2 mm, length: 40 cm, divided into eight 5 cm segments) with its forepaws for two trials of 5 s and a third 60 s trial. Muscle strength was measured as the time until falling off the wire in the 60 s trial. All the apparatus was suspended 40 cm above a padded table.

Behavioural and cognition assessment

Open field test. Mice were placed in the centre of the apparatus (home-made, wooden, white, 55 × 55 × 25 cm high) and observed for 5 min. The latency of the sequence of the behavioural events (latency to leave the centre, to arrive to the periphery, to perform the first rearing) was recorded. Horizontal (locomotion) and vertical (rearings) activities were also recorded. Self-grooming behaviour (latency and number of episodes) and defecation were measured as emotional behaviours.

T-maze. The spontaneous exploratory behaviour was tested in a T-shaped maze (arms, length 25 cm) by determining the time elapsed until the animal crossed (four-paw criteria) the intersection of the three arms. The working memory paradigm consisted in two consecutive trials: one forced choice and one free choice, with a 90 s intertrial interval. In the forced choice, only one of the arms according to a random order (contrabalanced in each group) was accessible. After spending 20 s exploring the accessible arm (learning criterion), the animal was put back into the home cage-starting box. In the free choice trial, both arms were accessible. The arm chosen by the mouse and the time spent in each arm, as well as the time spent to complete the exploration of the three arms were recorded. The choice of the already visited arm in the previous trial before exploring the arm that was inaccessible was considered as an error. The olfactory trails were removed by cleaning the surface of the maze during the intertrial intervals.[13]

Morris water maze. Animals were tested in four paradigms in the Morris water maze (MWM)[14] consisting of one cue task for visual perceptual learning, 4 days of place task for spatial reference learning and memory followed by two probe trials for short-term (2 h) and long-term (24 h) memories. In the place learning task, mice were trained to locate a platform (7 cm diameter, 1.5 cm below the water surface, position indicated by a visible 5 × 8 cm striped flag) in a circular pool (Intex Recreation, Long Beach, CA, USA; 91 cm diameter, 40 cm height, 25 °C opaque water) located in a test room with distal visual cues. This required four platform trial sessions per day with trials spaced 15 min apart. In each trial, the mouse was gently released (facing the wall) from one randomly selected starting point (N, S, E or W) and allowed to swim until escaping onto the platform (always in the middle of the SE quadrant). Mice that failed to find the platform within 60 s were placed on it for 20 s, the same period as was allowed for the successful animals. Two hours after the fourth trial of place learning, the platform was removed from the maze and the mice performed a probe trial test of 60 s. Twenty-four hours after the last cued platform trial, animals were tested for *the* cue learning of a visual platform consisting of four hidden platform trials (20 min apart). The platform was hidden 1.5 cm below the water surface, with its new position (NW) indicated by a visible striped flag (5 × 8 cm), and the distal cues were removed. During each trial, the escape latency, the distance travelled and the mean speed were measured by means of a computerised tracking system (SMART, Panlab, Barcelona, Spain).

Analysis of mRNA levels by qPCR

Total RNA was extracted from the tissue samples using QIAzol Lysis Reagent (Qiagen, Madrid, Spain), then quantified on a NanoDrop spectrophotometer (Thermo Scientific, Madrid, Spain), and reverse transcribed into complimentary DNA (cDNA) with iScript cDNA Synthesis Kit (Bio-Rad, El Prat de Llobregat, Spain). Gene-specific primers used for the quantitative PCR (qPCR) analysis of messenger RNA (mRNA) s-KL levels were: s-KL Fwd: 5′-TGGCTTTCCTCCTTTACCTG-3′; s-KL Rv: 5′-GCCGACACTGGGTTTTGT-3′; m36B4 Fwd: 5′-ATGGGTACAAGCGCGTCCTG-3′; m36B4 Rv: 5′-AGCCGCAAA TGCAGATGGATC-3′; CMV Fwd: 5′-TCCCGGTGTCTTCTATGGAGG-3′; CMV Rv: 5′-CAACTCCGCCCCATTGACGCA-3′. Quantitative qPCR was performed as previously described by Masso *et al*.,[11] on a Bio-Rad CFX-384 PCR machine at the Analysis and Photodocumentation Service of the Universitat Autonoma Barcelona using iTaqTM Universal SYBR Green Supermix (Bio-Rad). The analysis of qPCR output data followed the manufacturer-suggested ΔCt method. Cycle thresholds (Ct) were measured, and the relative expression of genes was calculated by comparison of Ct values. Melt-curve analysis was used to confirm the production of a single amplicon for each gene tested. On the basis of the RT-qPCR assay efficiency, gene amplification at a level higher than 35 cycles was considered to have no expression. A 'no template control' was also included in each run.

Western blot analysis

Protein extracts (15–25 µg per sample) from tissue samples were run in denaturing acrylamide gels, and then electrotransferred to PDVF membranes (GE Healthcare, Barcelona, Spain). Membranes were blocked with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween-20) containing 5% skimmed milk, and incubated with the primary K113 antibody. Detection was performed with an appropriate horseradish peroxidase-conjugated secondary antibody (EZBiolab, Carmel, IN, USA) and enhanced chemiluminiscence reagent (GE Healthcare). The K113 antibody was used at 1/5000 dilution; polyclonal rabbit anti-actin antibody (Sigma A2066, Madrid, Spain) at 1/1000; and secondary rabbit HRP-anti-Ig antibody (DakoCytomation, P0399, Madrid, Spain) at 1/10 000. Band pixel intensities were quantified using ImageJ (Wayne Rasband National Institutes of Health, Bethesda, MD, USA) and normalised to actin levels.

Statistical analysis

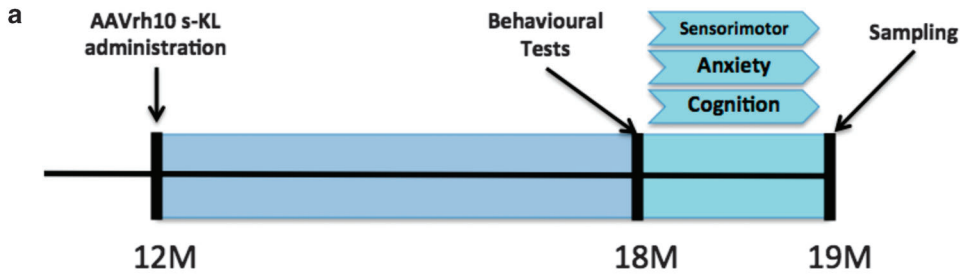
Values are presented as mean values ± s.e.m. Statistical analyses and calculations were performed using the G-Stat version 2.0 (GSK, Middlesex, UK) and Prism 5.04, La Jolla, CA, USA) programs. Statistical analysis between individual groups was performed by two-tailed unpaired Student's *t*-test or one-way of variance analysis of variance followed by Tukey *post hoc* test. In all cases, differences in means were considered statistically significant if $P < 0.05$.

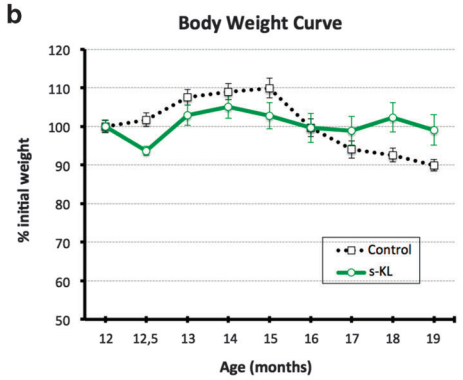
RESULTS

Experimental design in old adult mice (12–>18 months setting)

Long-term effects of *klotho* overexpression in the aging central nervous system (CNS) were evaluated in C57Bl/6 mice injected at 12 months of age (middle-aged, $N = 10$), and tested 6 months later, when they reached old age (18 months), through a battery of tests for behavioural assessment and functional analysis (Figure 1a). The study was performed using male mice because the oestrous cycle of middle-aged rodents is not disrupted like in humans. Thus, male mice provided a biological scenario free from sexual hormonal cycles (especially estrogens) known to interact with cognitive processes. In addition, Kuro-o and collaborators[15] have also found greater effects on male animals when over-expressing KL compared to female mice, suggesting that the male sex hormones may have synergistic effects with Klotho, or that female hormone has negative effects with Klotho.

The control group was injected with an AAVrh10 vector (AAV-Null) carrying a non-codifying DNA sequence and the treated group with an AAVrh10 vector encoding the secreted (s-KL) Klotho





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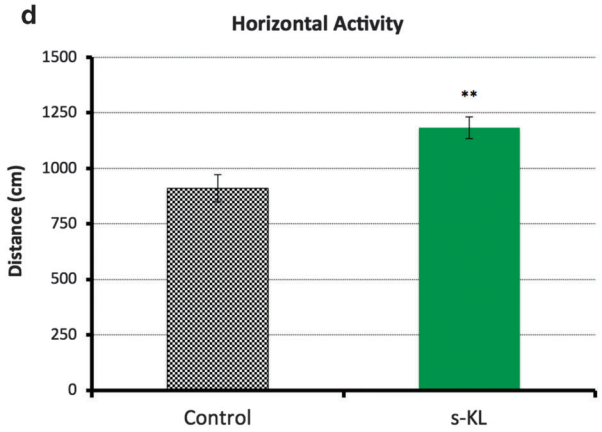
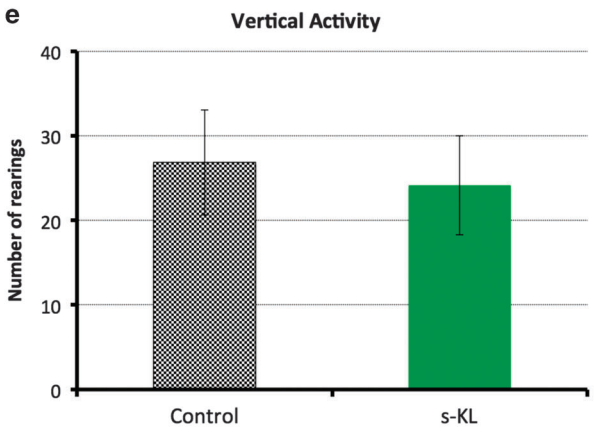
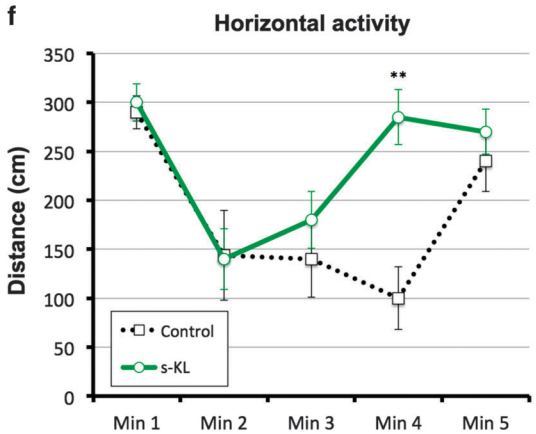
Sensorimotor Tests

	Control	s-KL
Visual reflex (correct)	100 %	100%
Wooden Bar (s)	19.0 ± 1.0	17.86 ± 1.25
Metal Bar (s)	3.5 ± 0,67	3.21 ± 1.19







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Exploratory Activity in Open Field Test

	Control	s-KL
Latency to leave the center(s)	6.75 ± 1.79	3.98 ± 1.11
Latency to arrive to periphery (s)	9.14 ± 1.36	7,.7 ± 1.09
Latency first rearing (s)	18.42 ± 2.91	24.33 ± 3.92
Number of groomings	2.86 ± 0.10	2.29 ± 0.12 **
Latency first grooming (s)	84.17 ± 11.63	90.33 ± 24.06
Number of defecations	2.13 ± 0.49	1.94 ± 0.32

Figure 1. *In vivo* study in 12-month-old C57Bl/6J male mice. (**a**) Experimental design. Mice ($n = 10$ per group) were injected at 12 months of age with AAVrh10 vectors, and analysed 6 months later by a battery of behavioural tests. (**b**) Evolution of body weight. Represented as percentage (%) of initial weight (Control: AAVrh10/null); s-KL: AAVrh10/s-KL. (**c**) Evaluation of sensory–motor skills: visual reflex, balance and motor coordination (latencies to fall). Mean ± s.e.m. (**d**–**g**) Open field test 12–>18 months setting. Tests were performed 6 months after administration of the viral vectors. Mice ($n = 10$ per group). (**d**) Horizontal activity measured as total distance travelled (cm). (**e**) Vertical activity, measured as the number of rearings. (**f**) Horizontal activity at different time points (min). (**g**) Parameters of spontaneous exploratory activity mean ± s.e.m. (Student's *t*-test: $^{**}P < 0.01$ vs Control).

a

Forced Choice in T-maze

	Control	s-KL
T-intersection (latency, s)	15.14 ± 1.38	12.04 ± 1.11
Criteria Achievement (frequency/total n)	6 / 8	5 / 6
Exploration criteria (latency, s)	76.19 ± 3.13	102.0 ± 14.56

Memory Assay in T-maze

	Control	s-KL
Criteria Achievement (frequency/total n)	5 / 6	5 / 5
Errors (frequency/total n)	2.94 / 6	0.65 / 5 **

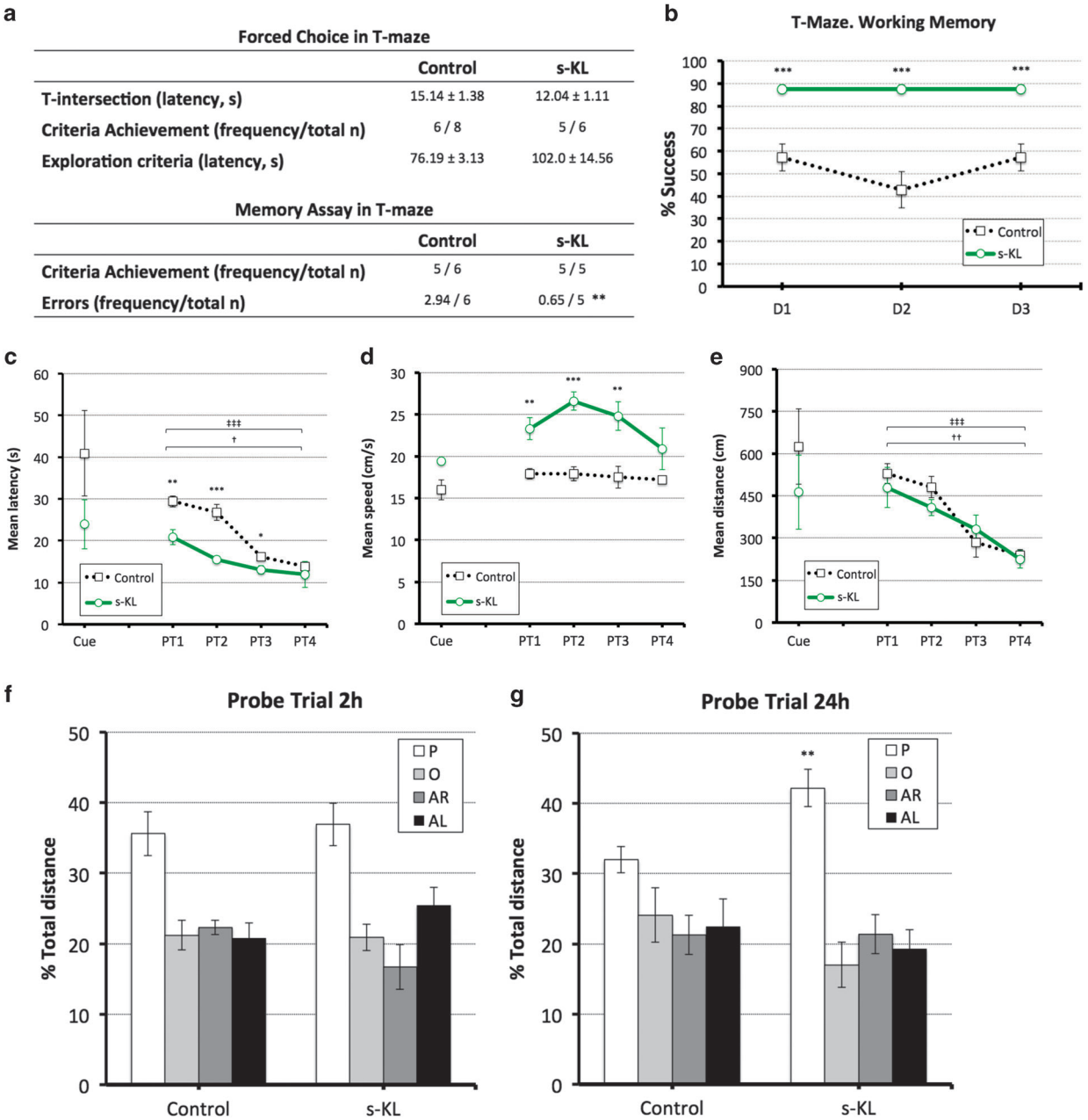


Figure 2. Quantitative analysis of working memory and learning tasks. Tests were performed 6 months after administration of the viral vectors. Mice ($n = 10$ per group). (**a**) Forced choice in T-maze. (**b**) Working memory test in T-maze. Morris water maze test: (**c**) mean latency (s), (**d**) mean speed (cm s^{-1}) and (**e**) mean distance travelled (cm) to locate the platform. Percentage of travelled distance in training quadrant of the pool, where the platform was previously located (P), and in the other three quadrants, as opposed to the platform (O), adjacent right (AR) and adjacent left (AL): (**f**) probe trial at 2 h, (**g**) probe trial at 24 h. Mean ± s.e.m. (Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, PT1 s-KL vs PT4 s-KL; $^{\ddagger\ddagger\ddagger}P < 0.001$ PT1 Control vs PT4 Control).

isoform. AAV vectors were injected intracerebroventricularly (icv) to mimic the endogenous production system, in which Klotho produced in the CNS is released into the cerebrospinal fluid (CSF) and distributed throughout the brain.

Overexpression of s-KL in the CNS does not affect body weight or sensorimotor skills in old mice

Behavioural assessment of treated animals started with the evaluation of putative effects of s-KL in physical status, namely body weight and reflexes as well as basic sensorimotor functions such as balance, coordination, prehensility, strength and resistance. These measures allow detecting possible differences between groups that could subsequently affect results depending on motor performance. As observed in Figure 1b, there are no significant differences in the mean weight between both groups of animals ($P = 0.48$). However, s-KL-treated mice had a relatively stable body weight throughout the 6 months post injection, while the null-treated group's weight progressively increased during middle age and decreased in old age. The results also show a similar sensorimotor function in s-KL-treated and control groups as measured by the visual placing and hindlimb reflexes test, the wood and metal rod tests; and the hanger tests (Figure 1c).

We therefore, assume that all animals were in similar physical conditions when analysed in subsequent behavioural tests.

s-KL overexpression in the CNS ameliorates age-related motor decline without affecting anxiety-like behaviours in old mice

Because locomotion and exploratory behaviours decrease with age,[16] we first sought to determine whether overexpression of the secreted Klotho isoform in the CNS could affect locomotor/exploratory behaviour in injected mice compared to controls using the open field test. This test was developed to study neophobia and anxiety-like behaviours, and is most often used in rodents to qualitatively and quantitatively measure general locomotor activity (horizontal and vertical activities) and willingness to explore (mostly shown by the vertical activity).[17] Open field activity, including total distance travelled, rearing exploratory behaviour, latency of behavioural events, self-grooming behaviour and defecations, were examined in order to determine whether sKL overexpression in the aged mice brain elicited changes in locomotion, exploratory activity, emotional and anxiety-like behaviours. As shown in Figure 1d, when tested at the age of 18–19 months, mice previously icv administered at 12 months with s-KL travelled a greater total distance compared to control mice ($P = 0.0054$), while no changes in vertical exploratory activity were found (Figure 1e). Interestingly, the greater locomotor activity observed in s-KL-treated mice does not appear to be related to anxiety, since differences in the distance travelled were not observed in the first 3 min of the test (Figure 1f). This is in agreement with the other parameters measured, such as the sequence of behavioural events and the number of defecations, which indicate similar levels of neophobia and emotionality in both groups of animals when confronting the open arena (Figure 1g). Only, a reduction in the number of groomings ($P = 0.0032$) in treated animals was statistically significant.

CNS s-KL overexpression improves cognitive performance in old mice

In order to study the possible long-lasting neuroprotective effect of Klotho in the CNS, animal's cognitive skills were evaluated in two learning and memory tests: T-maze and MWM. As shown in Figure 2a, in the forced choice tasks in the T-maze all groups met the criteria similarly, although not all animals were able to complete the task. Subsequently, only those animals that met the criteria were administered a second trial, the memory task. Results show that the increase in s-KL expression in the CNS in aged mice significantly improved their memory score compared to the control group. This is demonstrated by significantly less errors in choosing the maze path ($P = 0.0018$). Moreover, this ability was sustained over 3 consecutive days (Figure 2b).

Further analysis of cognitive abilities in treated animals was performed in the MWM. Notably swimming speeds were significantly higher in the group treated with s-KL (Figure 2d). This is in agreement with the increased horizontal locomotor activity observed in these same animals in the open field test. We therefore considered distance was a more accurate variable than latencies or speed to evaluate learning and memory skills in these animals. Indeed when the distance travelled to get to the platform was analysed (Figure 2e), the effect of s-KL seen in latency (Figure 2c) disappeared. Importantly, despite their age, both groups demonstrated learning over the 4 training days since there was a gradual reduction in the distance covered to solve the task (Figure 2e, $P < 0.01$ for Control; $P < 0.001$ for s-KL). Finally, the acquisition of spatial learning was similar, regardless of the group. This indicates that before the memory tests, all animals were equal when facing the task.

Two hours after the end of the last session on the fourth day, the platform was removed in order to evaluate short-term memory retention (probe trial 2 h). Given the significant differences in speed found between groups (Control: 20.64 ± 0.83 cm s^{-1}; s-KL: 28 ± 1.68 cm s^{-1}) the distance covered was analysed as the most accurate measure. As illustrated in Figure 2f, both groups showed preference for the training quadrant, while distance covered in other quadrants was equivalent to random (~25%). Finally, 24 h after the last session of acquisition (probe trial 24 h), aged control mice had a poorer performance in the long-term memory trial. They showed less preference for the training quadrant than they displayed in the short-term memory trial. In contrast, the s-KL-treated group had a clear preference for the training quadrant, statistically significant from the control ($P < 0.01$; Figure 2 g). These results indicate that the cognitive effects of increased levels of s-KL in the CNS are connected with selective improvement in long-term memory.

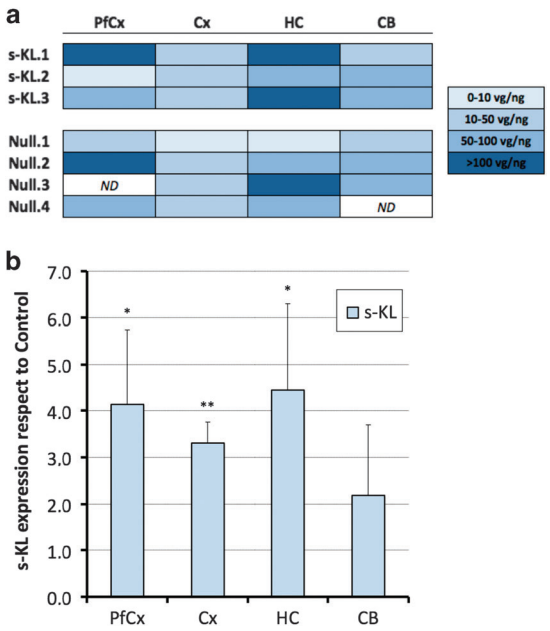
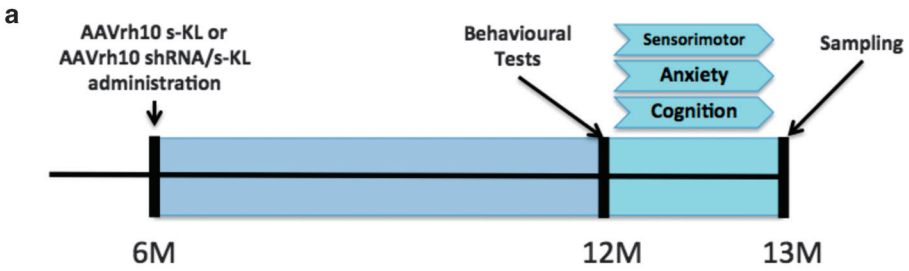
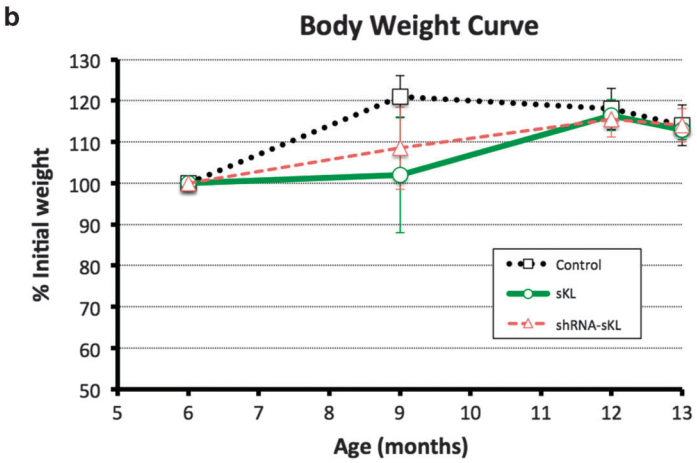


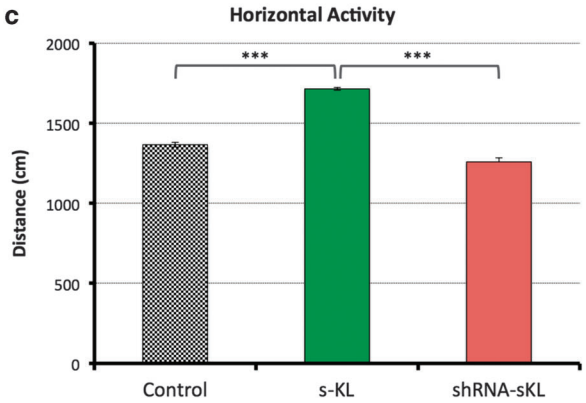
Figure 3. Analysis of s-KL in different brain areas after intraventricular injection of AAVrh10 vectors. (**a**) Quantification of viral genomes per ng tissue. (**b**) Quantification of s-KL mRNA levels. Mean ± s.e.m. (Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$ vs Control). CB, cerebellum; CX, cortex; HC, hippocampus; mRNA, messenger RNA; PfCx, prefrontal cortex.

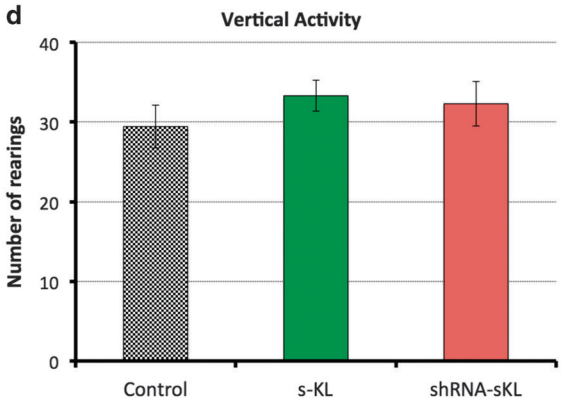
Quantification of viral genomes in the CNS of injected mice

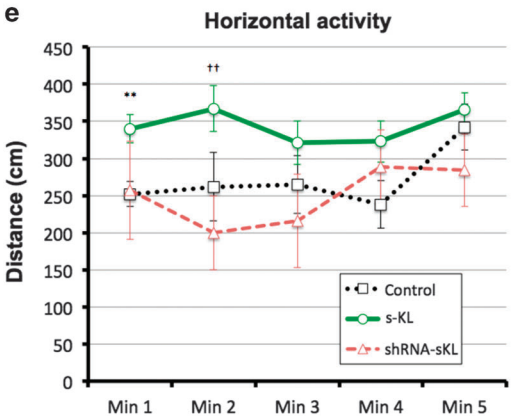
Following the behavioural assessment, specific neuroanatomical regions involved in cognitive processes (prefrontal cortex, cerebral cortex, hippocampus and cerebellum) were dissected from mouse brains, and viral genomes were quantified. Figure 3a shows AAV genomes were detected by qPCR in all injected animals, AAV-s-KL distributed similarly to AAV-Null control. Thus 6 months after intraventricular administration, AAVrh10 vector was still present in the CNS of 18-month-old mice. However, there was certain variability in virus distribution between the different brain regions studied, probably due to the route of administration, since the release of the vector in the CSF permits a wide, random distribution of viral particles through the brain parenchyma. More importantly, s-KL expression was increased in all the brain areas analysed (by qPCR on s-KL mRNA), ranging from two times higher in cerebellum to four times in prefrontal cortex and hippocampus (Figure 3b).











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Exploratory Activity in Open Field Test

	Control	s-KL	shRNA/sKL
Latency to leave the center(s)	5.18 ± 1.60	3.00 ± 0.97 ***	8.00 ± 0.78
Latency to arrive to periphery (s)	9.12 ± 1.57	7.33 ± 1.22	10.34 ± 1.09
Latency first rearing (s)	27.63 ± 3.96	26.92 ± 4.08	23.80 ± 2.74
Number of groomings	1.81 ± 0.42	1.42 ± 0.32	1.81 ± 0.31
Latency first grooming (s)	115.54 ± 25.49	164.92 ± 23.16	112.36 ± 18.43
Number of defecations	2.33 ± 0.47	1.96 ± 0.35	2.4 ± 0.40

Figure 4. *In vivo* study in 6-month-old C57Bl/6J male mice. (**a**) Experimental design. Mice were injected at 6 months of age with AAVrh10 vectors, and analysed 6 months later by a battery of behavioural tests. Control: AAVrh10/scrambled and AAVrh10/shRNA-sKL groups with $n = 11$; AAVrh10/s-KL group with $n = 14$. (**b**) Evolution of body weight. Represented as percentage (%) of initial weight. Mean ± s.e.m. (**c**–**f**) Open field test in 6–>12 months setting. Tests were performed 6 months after administration of the viral vectors. Control: AAVrh10/scrambled and AAVrh10/shRNA-sKL groups with $n = 11$; AAVrh10/s-KL group with $n = 14$. (**c**) Horizontal activity measured as total distance travelled (cm). (**d**) Vertical activity, measured as the number of rearings. (**e**) Horizontal activity at different time points (min). (**f**) Parameters of spontaneous exploratory activity Mean ± s.e.m. (Student's *t*-test: $^{**}P < 0.01$ vs Control; $^{***}P < 0.001$ vs. Control; $^{\dagger\dagger}P < 0.01$ vs shRNA-sKL).

Experimental design in middle-aged adult mice (6–>12 months setting)

In order to study whether long-term expression of s-KL could improve the physical, non-cognitive and cognitive status of middle-aged animals, a second set of mice ($n = 11$–14) was treated at 6 months of age. They were then assessed at 12 months by the same battery of tests (Figure 4a). There is a much lower efficiency of AAV transduction in older compared to younger brains (R Castro, personal communication). By testing a group of relatively younger adult animals, we could avoid this limitation. In addition, for this experiment AAV was specifically injected into the hippocampus. The main reasons were (i) the *klotho* gene is abundantly expressed in hippocampus; (ii) hippocampus is involved in learning and memory processes; and (iii) in mice, the

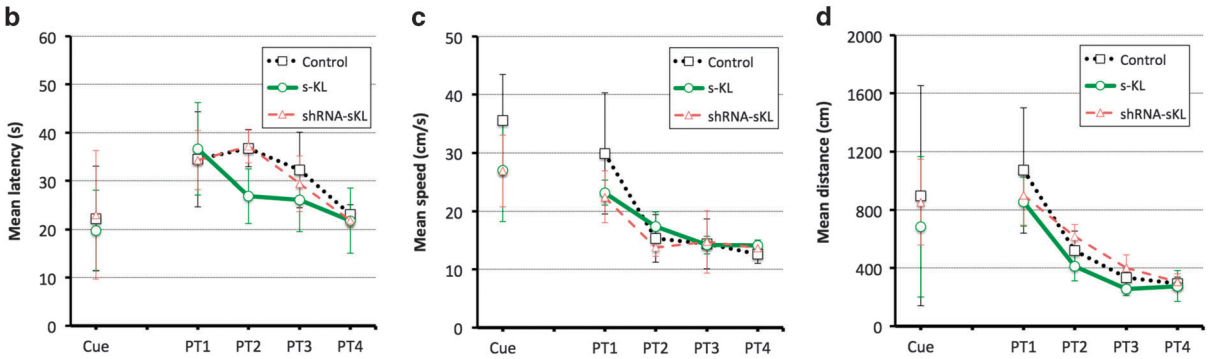
a

Forced Choice in T-maze

	Control	s-KL	shRNA/sKL
T-intersection (latency, s)	8.48 ± 1.77	5.29 ± 1.29 **	10.28 ± 1.11
Criteria Achievement (frequency/total n)	11/11	14/14	10/11
Exploration criteria (latency, s)	72.15 ± 5.41	50.47 ± 4.38	57.27 ± 5.24

Memory Assay in T-maze

	Control	s-KL	shRNA/sKL
Criteria Achievement (frequency/total n)	11/11	14/14	10/11
Errors (frequency/total n)	4 / 11	3 / 14	4 / 10



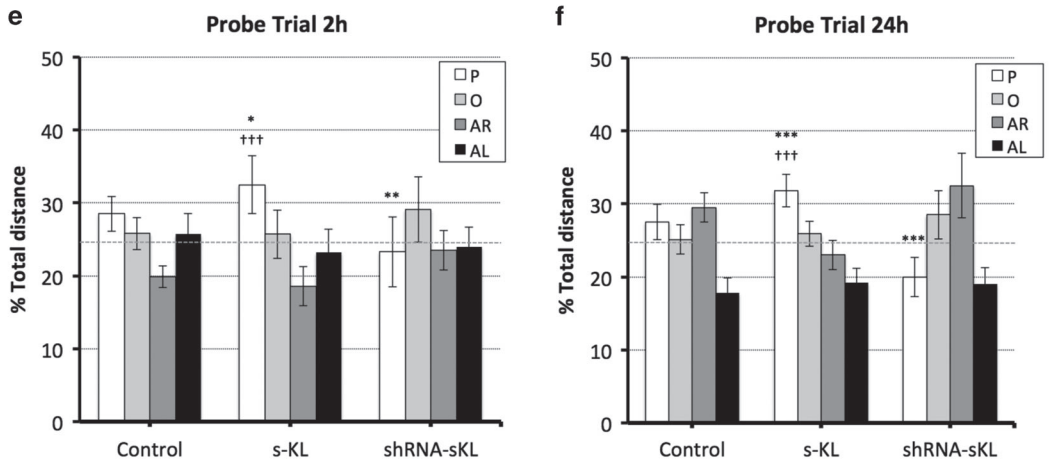


Figure 5. Quantitative analysis of working memory and learning tasks in 6–>12 months setting. Tests were performed 6 months after administration of the viral vectors. Control: AAVrh10/scrambled and AAVrh10/shRNA-sKL groups with $n = 11$; AAVrh10/s-KL group with $n = 14$. (**a**) Forced choice in T-maze. Morris water maze test: (**b**) mean latency (s), (**c**) mean speed (cm s^{-1}) and (**d**) mean distance (cm) to find the platform. Percentage of travelled distance in training quadrant of the pool, where the platform was previously located (P), and the other three quadrants, as opposed to the platform (O), adjacent right (AR) and adjacent left (AL). (**e**) Probe trial at 2 h, (**f**) Probe trial at 24 h. Mean ± s.e.m. (Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control; $^{\dagger\dagger\dagger}P < 0.001$ vs AAVrh10/shRNA-sKL).

hippocampus develops structurally until 12 months of age, later undergoing age-dependent functional decline. Actually, there are other brain regions where *klotho* is also highly expressed (for example, choroid plexus and cerebellum). However, we were interested to see if specifically enhancing s-KL in the hippocampus during a period of plasticity would improve hippocampal-dependent learning and memory processes, and thereby reduce the future impact of functional decline.

In the same experimental design, we also compared whether s-KL inhibition worsened cognitive deficits in naive mice, with respect to control-treated and s-KL treated animals. To achieve specific inhibition of the secreted Klotho isoform, we administered AAV vectors carrying short hairpin RNA (shRNA) against s-KL. The shRNA sequence was designed against the extra sequence in the tail of s-KL not present in m-KL. An AAV carrying a shRNA-scrambled sequence was used as a control.

Each animal received a bilateral injection with a total of $5 \times 10E9$ vg per mouse. Animal body weight was measured at the beginning of the experiment (6 months) at 9 and 12 months of age. As shown in Figure 4b, from 6 to 12 months of age, sustained overexpression or inhibition of s-KL over time had no significant effect and all groups showed a steady, similar weight gain. Likewise, in the previous experiment with older animals, the reflexes and sensorimotor skills of all groups were not affected (data not shown).

Hippocampal s-KL overexpression results in an increase in horizontal activity and has a mild anxiolytic effect in middle-aged adult mice

Consistent with our previous results in aged mice, modification of s-KL levels by gene therapy approaches was able to change horizontal locomotor activity (Figure 4c), but not the vertical activity (Figure 4d) of mice in the open field test. Thus, when s-KL cDNA was overexpressed in hippocampus, the total distance travelled by mice was significantly higher than in the other two groups ($P < 0.0001$) (Control: 1365 ± 14 cm; s-KL: 1734 ± 8 cm; shRNA/s-KL: 1259 ± 27 cm). The higher locomotor activity in s-KL-treated mice was observed thoroughout the experiment (Figure 4e) suggesting mild hyperactive behaviour in these animals. On the other hand, during the first minute of the test, some control animals froze for a while, which is a direct measure of increased anxiety. In the case of mice overexpressing s-KL, a shorter latency to leave the central area was recorded although this difference was only statistically significant with respect to shRNA-sKL-injected mice ($P = 0.0008$) (Figure 4f). No differences were detected between the groups in the other variables analysed.

Hippocampal s-KL overexpression improves cognitive performance in middle-aged adult mice, while s-KL inhibition impairs it

Working memory in animals with modified brain s-KL levels was assessed by the T-maze test. Results are presented as the mean values obtained for each of the 3 test days. First, in the forced choice trial, all control animals and those overexpressing s-KL were able to meet the criteria. On the contrary, about 10% of shRNA-sKL-treated animals did not meet the criteria, and therefore were discarded. In addition, in the first trial, mice overexpressing s-KL needed less time to reach the intersection point of the maze compared to mice injected with shRNA-sKL ($P = 0.009$) (Figure 5a). Thereafter, in the free choice trial, all animals met all the established criteria, but differences between groups were observed in terms of efficiency in choosing the correct path. The control group solved the task with an error rate of 36.36%. This score was improved in the s-KL overexpression group with an error rate of 21.42%. In contrast, silencing s-KL increased the percentage of error up to 40% (Figure 5a).

Visual perceptual learning and spatial reference learning and memory were assessed in the MWM, following the same protocol as used previously. In the place learning task (PT) all groups showed the capacity to learn the task, reflected by the gradual reduction in latency to reach the platform (Figure 5b) and in distance travelled to it (Figure 5c). No significant differences between groups were detected in the swimming speed (Figure 5d). Finally, a memory probe test performed 2 and 24 h after last training session allowed us to assess the effects of s-KL in short- and long-term memory in 12 months old mice. Figure 5e shows the group overexpressing s-KL had a significantly higher preference for the training quadrant as compared to the control group ($P = 0.039$) and the shRNA-s-KL group ($P < 0.0001$). In contrast, the shRNA-sKL-treated animals travelled less distance in the platform quadrant as compared to the control group ($P = 0.009$). As seen in Figure 5f, the profile for long-term memory was similar to results at 2 h. Thus, animals overexpressing s-KL show a greater preference for the training quadrant ($P = 0.0003$ vs Control and $P < 0.0001$ vs shRNA-sKL). As before,

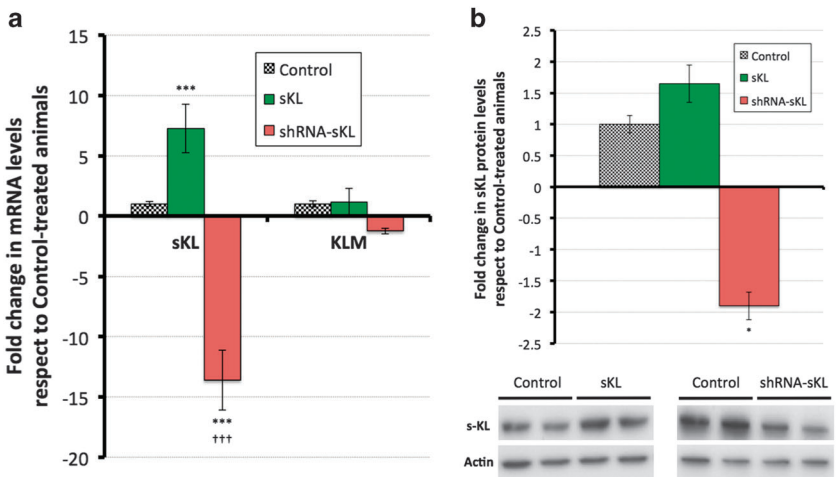


Figure 6. Analysis of s-KL levels in hippocampus after injection of AAVrh10 vectors. (**a**) Quantification of s-KL and m-KL messenger RNA (mRNA) levels. (**b**) Quantification of s-KL protein levels and western blot of representative samples. Mean ± s.e.m. (Student's *t*-test: $^{***}P < 0.001$ vs Control; $^{\dagger\dagger\dagger}P < 0.001$ vs s-KL).

shRNA-sKL-treated animals showed the lowest preference for the training quadrant and random preference for the other quadrants ($P < 0.0001$ vs control animals). We therefore conclude that, as in aged animals, elevation of s-KL levels enhances the ability to discriminate the quadrants, especially at long-term. Furthermore, silencing s-KL worsens the animals' performance, confirming it has a role in cognitive functions.

Finally, s-KL levels were quantified in the hippocampus of treated animals to determine whether the effects observed in cognition were induced by s-KL overexpression and/or s-KL inhibition. Hence, mRNA levels of s-KL in the AAV/s-KL-injected group were increased 7.25 ± 2.0 times with respect to animals injected with AAV-Control ($P = 0.035$), while in AAV/shRNA-sKL animals, s-KL expression levels were reduced by 13.6 ± 2.5 times ($P = 0.007$) (Figure 6a). These changes were specific to the secreted Klotho isoform since expression of the m-KL transmembrane isoform was not affected either by s-KL overexpression (1.15 ± 1.1 times vs control), or by specific s-KL inhibition (-1.24 ± 0.27 times vs control). Accordingly, western blot using the s-KL antibody K113 (ref. 11) showed opposite changes in hippocampal s-KL levels with a 1.65 ± 0.30-fold increase in AAV/s-KL-injected animals ($P = 0.09$), and a -1.90 ± 0.22-fold reduction in AAV/shRNA-sKL-treated animals compared to control AAV/null mice ($P = 0.013$) (Figure 6b).

DISCUSSION

Klotho is a gene regulator of aging that when overexpressed increases life expectancy in mice[15] and when inhibited, accelerates the development of aging phenotypes.[1] In brain, *klotho* is expressed abundantly in different cell types, and it appears to be a key element in the changes observed during aging in the white matter of primate brains.[18] Our group has previously reported that expression levels of the isoform s-KL in mice are very high in brain but contrary to m-KL, much lower in kidney, suggesting that s-KL activity might be particularly important in the nervous system. However, the specific function of s-KL still is unknown, and therefore new studies focused on the role of s-KL are needed.

To date, studies on the functions of Klotho have been made in two transgenic mice: mice overexpressing *klotho*,[19–21] and kl^{-}/kl^{-}mice, which are hypomorphs with very low α*klotho* expression.[15,22,23] Our approach, however, is to not alter natural Klotho levels from the birth of the animal, until key stages in the aging process are reached. Then levels are specifically modified in the brain through administration of AAVrh10 vectors with neuronal tropism. In exploratory studies, gene therapy vectors carrying the *klotho* gene have been used as delivery tools to modify Klotho levels *in vivo*. Thus, intravenous administration of an Adenovirus-Klotho vector increased removal of creatinine, decreased protein excretion in urine and improved the histopathological damage induced by angiotensin II.[24] In addition, intravenous administration of AAV2-Klotho in a model of spontaneous hypertensive rats showed an improvement in the progression of hypertension and renal damage.[25]

In this report, we first evaluated the neuroprotective effect of s-KL in old wild-type mice (12–>18 months setting). When working with old mice, several considerations must be taken into account: (a) high mortality rate between 15 and 18 months of age; (b) aging is a process that affects the whole body, and therefore it can affect many variables in addition to cognition, such as muscle coordination, strength, speed, exploratory behaviour or habituation capacity to new stimuli;[26,27] and (c) deficits shown in learning and memory tests may be due to alterations of other cognitive processes such as attention and perception.[28] Since recently Dubal *et al.*[20] reported Klotho also stimulated cognition in young and middle-aged animals, we decided to include mice in a 6–>12 months' setting in order to provide information about a younger adult group, at the same time avoiding the inevitable limitations of working with older animals. Overall, despite differences in the design of certain experiments, such as the age of the animal and the route of administration (intraventricular vs intrahipocampal), the results of the tests are consistent and robust, demonstrating that s-KL overexpression in the CNS causes significant effects in some of the measured variables, especially those related to cognition and anxiety.

Before assessing cognitive function, we examined the physical conditions of treated animals. First, we found no significant differences in the body weight between the control group and the groups treated with s-KL or shRNA-sKL. Similarly, although Klotho is associated with the decrease in tissue homoeostasis during aging,[29] we did not observe differences in the sensorimotor skills between the control animals and animals expressing s-KL or shRNA-sKL, indicating that within the same experiment, all animals were in equal physical conditions and therefore, the results of subsequent behavioural tests are not influenced by them. This is consistent with results reported by Abramovitz *et al*.,[30] who also detect no changes in body weight nor in general health parameters after daily intraperitoneal injection of recombinant Klotho protein. However, other studies suggest a close relationship between Klotho levels and body weight, especially in patients with obesity or anorexia nervosa.[31,32] Some possible hypotheses to explain such contrasting results could be: (i) the local administration in the CNS in our study, rather than systemic intravenous administration, (ii) 6 months of *klotho* overexpression is not enough time to observe general physical effects; and/or (iii) the vector dose injected is not sufficient to increase Klotho levels in plasma up to concentrations affecting the skeletal muscle.

The open field test was used to study whether s-KL is able to affect locomotion, exploratory activity, emotionality, neophobia and anxiety-like behaviours. Briefly, we found that regardless of age, the animals overexpressing s-KL show mild hyperactivity, an increased locomotor activity when compared to control animals, and therefore, that the administration of s-KL in the CNS seems to reverse, at least partially, the gradual decline in locomotor activity observed around 12 months of age.[33] In addition, the latencies of movement (an indicator of behavioural inhibition as a result of neophobia or anxiety) tended to be shorter in s-KL-treated animals, indicating a higher disinhibition, although this effect reached statistical significance only in middle-age animals (6–>12 setting), but not in old animals (12–>18 setting). Consistently, the inhibition of s-KL expression mediated by shRNA-sKL had an opposite effect. Thus, some animals of the shRNA-sKL group exhibited behavioural inhibition at the start of the test, showing freezing or petrification, and latencies in leaving the central area far superior to mice overexpressing s-KL. This concords with the hypoactivity associated to low levels of Klotho described by Kuro-o *et al*.,[1] in which 6-week-old kl^{-}/kl^{-} mice showed a very low rate of spontaneous activity in the open field test as compared to controls.

On the other hand, it has been reported that reduced klotho levels are expected to alter blood pressure,[34–36] which in turn may affect the overall health and behaviour in tests. Since blood pressure was not measured in these animals, its effects in some of the changes observed in locomotor activity cannot be entirely excluded. However, since klotho levels were increased instead of reduced, plus mild hyperactivity and/or anxiety were observed in klotho-treated vs control-treated animals, the increase in locomotor activity is unlikely to be related with long-term changes in blood pressure, but rather with long-term klotho overexpression.

Next, to evaluate the effect of s-KL overexpression on the working memory we used the T-maze test. Again, the results show that administration of s-KL, regardless of age, improves cognitive abilities since s-KL-treated mice make less errors in solving the task as compared to control animals. These results concur with the increase in s-KL levels found in the prefrontal cortex of the (12–>18 months setting) animals. Given that in mice, deficits in

working memory appear around 24 months of age,[37] Klotho seems to be acting as an enhancer of cognitive functions. Interestingly, Dubal *et al*.,[20] have reported that increased levels of Klotho improved working memory in young mice in the Y-maze test.

Finally, the MWM test was used to assess the effect of s-KL overexpression/inhibition on visual perceptual learning and memory and learning abilities. The results from both experiments showed again that s-KL-treated mice are more efficient in solving the task and they learn faster than the control animals, indicating that s-KL significantly improves long-term memory in mice. In contrast, animals injected with shRNA-sKL showed problems in learning the task and swam a greater distance to reach the platform. Moreover, these opposite effects were also observed in the final memory tests (24 h after the last training). Animals overexpressing s-KL prioritised the search in the quadrant where the platform was previously located, while shRNA-sKL-treated animals showed no particular preference for the training quadrant, suggesting memory and/or learning problems. As expected, when compared to controls, quantification of s-KL levels 6 months after treatment (both mRNA and protein) showed a very strong positive correlation with cognitive capacities, being statistically higher in AAV/s-KL-treated mice and lower in AAV/shRNA-sKL-treated mice. This pattern was observed regardless of whether animals were injected in adulthood (6−>12 months setting) or middle age (12−>18 months setting).

In summary, our study provides new evidence indicating an important role for s-KL in cognitive functions, with reduced levels in hippocampus being associated with low cognitive performance. The study also demonstrates that a single icv injection of s-KL into the CNS has great potential as a long-lasting and quantificable agent to stimulate cognitive skills, even, protecting age-dependent cognitive decline when mice were treated at old ages. To our knowledge, these are the first data obtained *in vivo*, in which the action of only the secreted Klotho protein improves the learning and memory capabilities of old animals when treated in adulthood. Furthermore, taking into account that these experiments were performed in naive aged animals, our results suggest s-KL may have therapeutic potential for dementia. This opens up a promising new field of study with evident implications for neurodegenerative disorders such as Alzheimer's disease or multiple sclerosis among others.

CONFLICT OF INTEREST

Portions of this work are the subject of a provisional patent application held by the Universitat Autonoma de Barcelona (Spain).

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