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(54) Title: SULFATED C19 STEROID HORMONES TO PROTECT AGAINST AGING-ASSOCIATED PROTEOTOXICITY

(57) Abstract: The present invention is encompassed within the field of medicine and provides a composition for use in a method for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, or age-related cognitive impairment in a subject in need thereof.



Sulfated C19 steroid hormones to protect against aging-associated proteotoxicity.

Technical field

The present invention is encompassed within the field of medicine and provides a compound
5 for use in the prevention of aging-associated proteotoxicity caused by protein-aggregation
diseases and/or to increase the lifespan of a eukaryotic organism.

Background Art

Animals can extend life span by activating different genetic pathways. This increase of longevity
10 is a regulated process that relay in the coordination of different tissues and environmental
signals. Hormones are key players in tissues and cell communication. Consequently, they are
involved in different pathways that regulate longevity, among those insulin and insulin-like
growth factor, TGF β or dafachronic acids which are described to affect life span at least in the
model organism *Caenorhabditis elegans*. Gonad is an endocrine tissue that produces steroids
15 hormones to regulate different physiological aspects of the organism, including longevity. In *C.*
elegans, germline ablation extends life span by non-completely understood mechanisms.
Several factors are needed for the increase in longevity, including synthesis of dafachronic acid
by the somatic gonad as well as the transcription factor encoded by *daf-16*, homologue to the
human FOXO, and the nuclear receptors encoded genes *daf-12*, *nhr-80* and *nhr-492*.

20 The classical function of steroid hormones is considered to be the activation of hormones
receptors to transcribe their target genes. Steroid hormones are not only produced in gonads
but also in other tissues. Those produced in the nervous system are known as neurosteroids.
Neurosteroids, in addition to bind to hormone receptors, modulate neurotransmission either
through direct interaction with neurotransmitter receptors or by other mechanisms. Steroid
25 hormones can be sulfated by a sulfotransferase enzyme, generating a profound change in the
chemical features of the hormone that impairs its function as hormone receptor activator. Those
sulfated hormones are considered to be an inactive reservoir of hormones that can be activated
upon removal of the sulfate moiety by the activity of hormone sulfatases. Sulfated steroid
hormones can also be active as neurosteroids, regulating neurotransmission.

30 Some sulfated steroid hormones, like dehydroepiandrosterone sulfated (DHEAS), have long
been related to aging. The level of this hormone declines with age and in age-related diseases
such as sarcopenia or Alzheimer's disease, which has generated the speculation of a causative
effect.

Here we show that inhibition of the steroid sulfatase increases the percentage of sulfated hormones and, associated with that, an increase in longevity and the improvement of the symptoms related to protein aggregation diseases. This increase in longevity is mainly dependent on the same factors described for longevity caused by germline ablation. Treatment with STX64, a specific inhibitor of the steroid sulfatase enzyme, mimics the beneficial effects in longevity and protein aggregation diseases observed in the mutant. Interestingly, treatment with STX64 also ameliorates the cognitive symptoms and plaque formation in a mammalian model of Alzheimer's disease. Finally, the observed phenotypes are recapitulated by treatment with sulfated C19 androgens steroid hormones but not with the non-sulfated forms or the sulfated C21 pregnenolone hormone, indicating that the causative beneficial effect of sul-2 inhibition is due to the increase of sulfated C19 steroid hormones rather than reduction of the non-sulfated form. This invention thus demonstrates that STX64 or specific sulfated C19 steroid hormones are a possible treatment for aging and/or aging-related diseases, more particularly specific sulfated C19 steroid hormones extend lifespan and protect against aging-associated proteotoxicity.

Brief description of the figures

Figure 1 Reduction of activity of sul-2 increases longevity and affects the levels of sulphated steroid hormones. A) sul-2(pv17) point mutation allele lives longer than wild type. B) The null allele sul-2 (gk187) also increases lifespan. C) Phylogenetic tree of mammalian sulfatases and the three *C. elegans* sulfatases. Note that SUL-2 clusters with steroid sulfatases (type C) among others, in blue. Phylogenetic relation with other *C. elegans* sulfatases is indicated in grey. D) Inhibition of steroid hormones sulfatase by STX64 (1µg/ml) increases lifespan in wild type animals, but not in sul-2 (gk187) background. Worms were cultivated in UV-killed *E. coli*. E) Deletion of sul-2 generates an increase in the percentage of steroid hormones in the sulfated stage. Data from three independent assays are shown. One-tailed Mann-Whitney t-test.

Figure 2 Genetic interactions and cellular location of sul-2 expression. Genetic analysis shows that sul-2 mimics most of the genetic interaction described for animals without germline, but do not affect fertility. a sul-2 enhances longevity in daf-2(e1370) background. b daf-16 transcription factor is required for sul-2 longevity. c, d, e. The essential factors for germline-loss longevity kri-1(ok1251), tcer-1(tm1452), and partially nhr-80(tm1011) are required for sul-2 longevity. f nhr-49(nr2041) does not suppress sul-2 increased lifespan. g,h sul-2 deletion has not significant increase of longevity in germline-less mutants glp-1(e2141) and mes-1(bn7). i Brood size of sul-2 mutants are not significant different to wild type. Mean±SEM; One-way ANOVA test; ns.

j Reproductive period of sul-2 mutants are not affected (25°C). k Dietary restriction conditions (DR) show enhanced longevity in sul-2 deletion background. l, daf-12 transcription factor is required for sul-2 longevity. m The Rieske-like oxygenase daf-36 is necessary for the increase of longevity upon inhibition of steroid hormone sulfatase, STX64 (1µg/ml). n sul-2 is transcriptional expressed in sensory neurons, mainly ADF and ASE in the head, and PHA and PHB in the tail. Scale bar 10 µm.

Figure 3 Reduction of steroid hormone sulfatase activity ameliorates the symptoms of proteotoxicity models in *C. elegans* and murine model. a NL5901 strain expressing α -synuclein in muscle cells reduce mobility with age at a slower rate in a sul-2(gk187) background (n \geq 17 per day). Statistic test vs α -synuclein control. b or under treatment with STX64 (1µg/ml) (n \geq 9 per day). Additional biological replicates assays are shown in Fig. 12 a and c. c, d Neurodegeneration of dopaminergic (DA) neurons in UA44 strain expressing human α -synuclein is reduced in sul-2(gk187) background. A representative image of each condition and quantification of survivor neurons at day 9. Scale 50 µm. Data from two biological replicates are displayed, n=37. e Q35 aggregates are reduced in sul-2 (gk187) background (n \geq 6), 8-day old worms. f or by treatment with STX64 (1µg/ml) in Q40 background (n \geq 12), 5-day old worms. Strains AM140 and AM141 respectively. g Expression of human β -amyloid in muscle provokes a thermodependent paralysis in L4-young adult stage in GMC101 strain, the paralysis phenotype is ameliorated in sul-2 deletion mutant. Data display from four independent biological replicates, n=125. h or by STX64 (1µg/ml) treatment. Data display from six independent biological replicates, n \geq 215. i, j The effect of intrahippocampal and oral administration of STX64 in the passive avoidance test in wild type mice injected with β -amyloid oligomers in the hippocampus. The number of mice in each group were $>$ 5. k Representative β -amyloid-immunoreactive images assessed in the frontal cortex and the hippocampus of APP-PS1 mice older than 15 months of age after 3-4 weeks of vehicle or STX64 intake (0.005mg/ml in drink water). l-n Quantification of percentage of β -amyloid area (c), deposition density (d) and average plaque size (e) in the frontal cortex and the hippocampus of $>$ 15 months-old APP-PS1 mice after 3-4 weeks of oral administration with STX64 or vehicle. n=4 mice per groups. o Temporal course of β -amyloid deposition in APP-PS1 mice and the effect of 3-4 weeks STX64 oral treatment on β -amyloid area in the frontal cortex (upper panel) and the hippocampus (lower panel). The number of microphotographs used was more than 3 in all the mice used. p Effect of oral administration with STX64 in more than 15-month-old APP-PS1 mice, and comparison with APP-PS1 and wild type mice older than 15 months in the passive avoidance test. The number of mice in each group were $>$ 5. In histological analysis, * represent significant differences between vehicle-and STX64 administrated APP-PS1 mice. In behavioural test, * represent significant differences between the short-term and long-term

memory sessions (STM and LTM, respectively) and the training session in the same experimental group; and, + represent significant differences between the STM and LTM sessions between each experimental group and β -amyloid group. A symbol, $p < 0.05$, two symbols, $p < 0.01$, and three symbols, $p < 0.001$.

5 **Figure 4** Sulfated C19 androgen steroid hormones mimic sul-2 inactivation. a NL5901 strain expressing α -synuclein in muscle cells reduce mobility with age. Treatment with DHEAS, TS or ES improves the mobility (1 μ g/ml). Data from three biological replicates are displayed, $n=30$. b Paralysis of GMC101 Alzheimer's disease model is ameliorate with TS and ES. Data from six biological replicates are displayed, $n \geq 200$. Additional assays in normal NGM plates are shown in Fig. 13a. c,d No effect is observed with non-sulfated steroid hormone DHEA or testosterone (T), neither with sulfated C21 steroid hormone, pregnenolone sulfate (PregS) in Parkinson's model, NL5901 strain (1 μ g/ml). Data from three biological replicates are displayed, $n=30$. e Treatment with abiraterone (Abi) (1 μ g/ml) does not affect mobility phenotype of NL5901 strain but suppresses the beneficial effect of sul-2 deletion allele. Data from three biological replicates are displayed, $n \geq 30$. f Treatment with ES (1 μ g/ml) increases life span in a wild type background but does not further increase in sul-2(gk187). g DHEAS or TS (1 μ g/ml) does not affect longevity. Additional biological replicates assays are shown in Fig. 13c and d.

Figure 5 Longevity assays of sul-2 and genetic interactions with daf-2. sul-2 mutants do not show visible phenotypes but are long-lived and enhance developmental phenotypes of daf-2 mutants. a sul-2(pv17) is long lived and fer-15(b26) does not affect their lifespan. b sul-2 mutants are long-lived at 20 °C. c and at 25 °C. d Pumping rate of sul-2 mutants are similar to wild type at adult day 1, while pv17 allele keeps higher pumping rate than wild type at adult day 6 stage. Worms were growth at 16° C until L4, then shifted to 25° C considering this Day 0 of adulthood. The pumping was counted under the Leica stereoscope at 100X magnification for 30 seconds. Data display from two independent biological assays ($n=30$ per day). Two-tailed Mann-Whitney t-test, $***p=0.0006$. e Thrashing in sul-2(gk187) is similar to wild type but is slightly lower in sul-2 (pv17) at 25° C on day 1 of adulthood. On day 6 at 25° of adulthood, the thrashing on sul-2(gk187) is lower than wild type and sul-(pv17), with no differences among these last strains. The population of sul-2(gk17) at day 6 seems to distribute into two different populations, one with almost no thrashing and other with values similar to wild type a sul-2(pv17). Data displayed from two independent biological assays ($n=20$ per day). f sul-1(gk151) and sul-3(tm6179) do not increase life span. g A small percentage of daf-2(e979) animals arrest development in dauer larvae at 16 °C, sul-2(pv17) mutant does not show any larval arrest but enhances dauer arrest of daf-2(e979). h Most daf-2(e1370) animals arrest in dauer stage when develop at 25 °C, but small percentage arrest in L1 stage. In this condition, all animals from

daf-2(e1370); sul-2(pv17) double mutants arrest at L1 stage. Similarly, more than 50% of animals arrest in L1 stage in daf-2(m577); sul-2(pv17) background, while none of the single mutants show this phenotype. i Example of sul-2(pv17) larvae, dauer larval arrest of daf-2(e1370) or L1 arrest of daf-2(e1370); sul-2 (pv17) and daf-2(e1370); sul-2(gk187) at 25 °C. Animals were grown up to L4 at 16 °C, then shifted at 25 °C, progeny were scored or imaged after 72 hours. Photographs were taken in a Leica scope.

Figure 6 Identification of pv17 allele and curated sequence of SUL-2. A. The pv17 allele is a missense mutation that changes the glycine indicated with asterisk to an aspartic acid residue. The mutation is located close to an evolutionary constrained region (ECR), indicated with the bar at the bottom and also near to the catalytic core of sulfatases (in green). B. The DNA sequence we identified in the wild type sul-2 differs to the one published in wormbase (SUL-2_wormbase) and identified as orthologue to ARSA (<http://www.wormbase.org>), this sequence misses 459 bp, also described recently by Li et al. (2015) and predicted as part of a new exon [wormbase_170818 gw3] based on RNAseq data (SUL-2_modified). The protein sequence obtained by cDNA sequencing of yk387h10 clone (SUL-2_curated) differs to the one predicted in three aminoacids (in bold). Notice that the three aminoacids identified in this sequence are present also in other species (CRE: *C. remanei*, CBR: *C. brigssae*). c Exon intron composition of wild type sul-2 and region deleted in the gk187 allele (in red). The mutant lesion is available at [wormbase\(http://www.worm-base.org/species/c_elegans/variation/WBVar00145594#02-45-3\)](http://www.worm-base.org/species/c_elegans/variation/WBVar00145594#02-45-3). This allele deletes the sequence that encodes to the catalytic core of sulfatases (in green) and generates a frame shift, conserving only the four first aminoacids of the original sequence; therefore we consider gk187 a null allele. The pv17 allele location is indicated with asterisk, the new DNA fragment identified in yellow and the new exon in purple.

Figure 7 Treatment with STX64 phenocopies longevity and genetic interaction of sul-2 mutants. a STX64 in non-UV *E. coli* increases lifespan of wild type. b, c Dosis curves of STX64 in non-UV *E. coli* show a significant effect at 1 µg/ml and 5 µg/ml. d Photographs of wild type and daf-2 (e1370) at 25 °C. DMSO (STX64 vehicle) does not affect development of wild type, neither STX64 treatment, daf-2(e1370) arrests mostly in dauer stage at 25°C, but mainly arrests in L1 when treated with STX64, similar interaction is also observed in the sul-2 mutants. Photographs were taken in a Leica scope. Arrow heads: dauers, arrows: L1s.

Figure 8 Genetic interactions of sul-2(pv17) allele. sul-2 point mutation allele pv17 shows similar phenotypes in longevity interactions assayed as sul-2 deletion, except in glp-1 background. a, b sul-2(pv17) enhances longevity of two daf-2 alleles, e1370 and m577. c, d sul-2(pv17) longevity is suppressed by two

alleles of *daf-16*, *mu86* and *m26*. e *sul-2(pv17)* longevity is suppressed by *tcer-1(tm1452)*. f *sul-2(pv17)* longevity is partially suppressed by *nhr-80(tm1011)*. g *sul-2(pv17)* longevity is not suppressed by *nhr-49(nr2041)*. h *sul-2(pv17)* increases lifespan in *glp-1* background. i *sul-2(pv17)* longevity is suppressed by *daf-12(m20)*.

5 **Figure 9** *sul-2* mutants affect DAF-16 location in intestinal cells, but they do not affect reproduction or gonad morphology. a Micrographs show representative images of Pdaf-16::gfp::daf-16 location in wild type (left panel) and *sul-2* mutants (central and right panels). Both *sul-2* mutants increase the nuclear location of DAF-16 in intestinal cells, like in germ-line less animals. Scale 20 μ m. b Quantifications of nuclear fluorescence in the anterior intestinal
10 cells. Data from two independent assays, $n \geq 34$ nuclei per condition. One-way ANOVA test. c *sul-2* mutants have similar brood size to control at 20 °C. One-way ANOVA test; ns. D. the reproductive period of *sul-2* mutants are not affected at 20 °C. e *sul-2* mutants show normal gonad morphology. Micrographs of one representative gonadal arm in late L4 for each strain are shown.

15 **Figure 10** *sul-2* is expressed in amphid and phasmid sensory neurons. Extrachromosomal transgenic worms express mCherry under *sul-2* promoter and its 3'UTR only in few sensory neurons. a Transcriptional reporter for *sul-2* is expressed in sensory neurons. Imaged in fluorescence and merged with the bright field. b Representative images of *sul-2*
20 extrachromosomal expression in amphids, most transgenic animals express *sul-2* in two pairs of amphid neurons, left panel, and a portion show expression in other neurons besides of those, right panel. $n=64$. c Collocation of *sul-2* neurons with FITC stains. Upper panel shows colocalization in the head with the most anterior pair amphids, possibly ASK, ADF or ADL neurons, but not with the most posterior pair, ASG or ASE. In the tail, bottom panel, the four neurons where *sul-2* is expressed colocalize with FITC staining in PHAs and PHBs phasmid
25 neurons. d Identification of the two main pair of amphid where *sul-2* is expressed by colocalization with the *tph-1* neuron-specific promoter for ADF, upper panel, and the posterior neurons expressed by *flp-6* promoter, ASE amphids, bottom panel. Scale bar 20 μ m. In intestine, signals are unspecific from autofluorescence at the conditions imaged (cyan arrow heads).

30 **Figure 11** *sul-2* is not expressed in gonadal tissues and is not affected in neuronal functions. a Integrated mCHERRY reporter of the *sul-2* transcriptional unit is only expressed in sensory neurons, there is not expression in other tissues. Image in fluorescence and merged with the bright field image. b Inset of integrated worms, i) In heads, *sul-2* is expressed in few amphid
35 neurons (white arrows). In intestine, signals are unspecific from autofluorescence at the conditions imaged (cyan arrowheads). ii) There is not specific signals in vulva, embryos or

proliferative germline zone. iii) There is not specific signals in gonad or mature oocytes. iv) In the tail, there is not significant signals in phasmids. Scale bar 50 μm . c Apart from been expressed in the Cl^- and Na^+ sensing neuron (ASE), *sul-2* mutants respond to Cl^- similarly to wild type. Data from three independent replicates. d *sul-2* mutants respond to Na^+ similarly to wild type. *tax-4(p678)* is a negative control. Data from three independent replicates. In all graphs Mean \pm SEM are displayed. e *sul-2* deletion enhances longevity of the long-lived *daf-10(m79)* mutant, which is affected in sensory neurons.

Figure 12 Neurodegeneration phenotypes are ameliorated during aging when steroid sulfatase function is reduced, and α -synuclein aggregation decreases. a *sul-2* has a beneficial effect during adulthood in muscular Parkinson's disease model. Data display from two independent biological replicates, $n \geq 31$ per day and condition. b, and *sul-2* has less body bends to wild type control at same experimental conditions, $n \geq 15$ per day and condition (20°C). c The protective effect of STX64 in muscular Parkinson's disease model is present throughout aging. Data display from two independent biological replicates, $n \geq 12$ per day and condition. d, e *sul-2* reduces significantly the number of α -synuclein aggregates in muscle at 7-day old. Photographs example of animals and quantifications, respectively. $n=18$. Scale bar 25 μm . f Expression of human β -amyloid in muscle provokes paralysis with age (CL2006 strain) that is ameliorated in *sul-2* deletion mutant. g or by STX64 treatment. Log-rank (Mantel-Cox) test, for *sul-2* deletion $p=0.0027$ and STX treatment $p=0.007$.

Figure 13 Treatment with sulfated C19 steroid hormones phenocopy *sul-2* inactivation. a Paralysis phenotype of GMC101 strain, Alzheimer's disease model, is reduced with TS and ES, but do not with DHEAS (1 $\mu\text{g}/\text{ml}$). Assays performed with normal NGM plates. Data display from three independent biological replicates, $n > 130$. b Similar to *daf-2(e1370);sul-2*, percentage of L1 arrest in *daf-2(e1370)* increase with DHEAS, TS or ES. c, d Additional biological replicates of longevity curve with sulfated C19 steroid hormones. ES (1 $\mu\text{g}/\text{ml}$) increases lifespan in wild type background but does not increase further in *sul-2(pv17)*, while DHEAS and TS do not affect (1 $\mu\text{g}/\text{ml}$).

Figure 14 Model of regulation of longevity by SUL-2. The sulfatase SUL-2 and the sulfotransferase (probably *ssu-1*) regulates the level of sulfated steroid hormones. High level of sulfated steroid hormone provokes an increase of longevity, which depend on the same factors to the longevity generated by germline reduction (*daf-16*, *daf-12*, *kri-1*, *tcer-1*). The fact that both sulfatase and sulfotransferase are expressed in sensory neurons suggests a coordination of the sulfated state of the hormones with environmental signals.

Figure 15. This figure shows that treatment with Epitestosterone sulfate (ES) increases life span: Treatment with Epitestosterone sulfate (ES) significantly increased life span $p < 0.05$ Log-Rank (Mantel-Cox). Number of nematodes per strain and condition is always more than 100.

5 **Figure 16.** 3 months treatment with STX64 in 15 months old mice improves cognition. 15 months mice still keep cognition capacities, but they lose this capacity during the following months. A: Locomotion index were similar in both group (vehicle and STX groups). B: Object recognition is improved after three months treatment. C: short term and long-term memory in passive avoidance test. * $p < 0.05$. ** $p < 0.005$. *** $p > 0.0005$. 1-way ANOVA.

10 **Figure 17.** 6 months treatment with STX64 in 15 months old mice improves cognition. 15 months mice keep cognition capacities, but they lose this capacity during the following months. Age of mice when assayed was 21 months. A: Locomotion index were similar in both groups (vehicle and STX groups). B: Object recognition is improved after three months treatment. C: short term and long-term memory in passive avoidance test. * $p < 0.05$. ** $p < 0.005$. *** $p > 0.0005$. 1-way ANOVA.

15 **Figure 18.** 1-month treatment with STX64 in 18 months old mice improves cognition. 18 months mice have impaired cognition capacities. Age of mice when assayed was 19 months. A: Locomotion index were similar in both groups (vehicle and STX groups). B: Treated animals showed significant improvement in short term and long-term memory in passive avoidance test. * $p < 0.05$. ** $p < 0.005$. *** $p > 0.0005$. 1-way ANOVA.

20 **Figure 19.** 3-month treatment with STX64 in 18 months old mice improves cognition. 18 months mice have impaired cognition capacities. Age of mice when assayed was 21 months. A: Locomotion index were similar in both groups (vehicle and STX groups). B: Object recognition is improved after three months treatment. C: short term and long-term memory in passive avoidance test. * $p < 0.05$. ** $p < 0.005$. *** $p > 0.0005$. 1-way ANOVA. B: * $p < 0.05$. ** $p < 0.005$. *** $p > 0.0005$. 1-way ANOVA.

25

Figure 20. This figure shows the results of an "Open Field" test and of a "Y maze" test. The "Open Field" is a test where the mouse must explore an unknown space for 15 minutes. In this test, both mobility and habituation (cognitive parameter that measures the individual's ability to recognize the environment) can be studied. On the other hand, the "Y maze" test is a test where
30 the mouse explores the maze for 6 minutes. This test evaluates the distance traveled as a measure of mobility and the ability to visit triplets of arms without repeating any of them as a sign of immediate memory. The results show that treatment with STX-64, E-S or both tend to improve the motor ability of 16-month-old mice. On the other hand, habituation ability and

immediate memory of aged animals are improved by treatment with STX, E-S and the combination of both

Figure 21. In this figure the evaluation of learning and memory abilities of aged mice was determined in the object recognition test and the passive avoidance test. The first, object recognition, after training with identical objects, tests the ability to identify different objects exposed together with a familiar one. When this happens, the object discrimination index is positive and higher than 0.1. In passive avoidance, the aim is to teach the mouse that its preferred place does not suit it and for them it is associated with an aversive stimulus. For this purpose, during training, the entry latency to the preferred site is established and the aversive stimulus is administered. During short- and long-term memory tests (STM and LTM) if the mouse associated the preferred place to the aversive stimulus, the input latency increases with respect to training. Vehicle-treated aged mice showed impairments in learning ability and thus memory. In both tests, mice treated with STX, E-S and the combination of both showed significantly both learning (STM) and ability to store information (memory).

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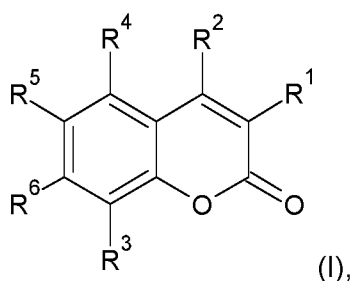
Brief description of the invention.

As shown in figure 20, treatment with STX-64, epitestosterone sulfate (ES) or both tend to improve motor ability, habituation ability and immediate memory of aged animals. In addition, and as shown in figure 21, short- and long-term memory tests (STM and LTM) improved with STX-64 and/or ES. In fact, in this figure vehicle-treated aged mice showed impairments in learning ability and thus memory. However, in both tests, mice treated with STX, ES and the combination of both showed a significant improvement in both learning (STM) and ability to store information (memory). That is, Age-related cognitive impairment in particular associated with Age-Associated Memory Impairment (AAMI) or Age-Related Cognitive Decline (ARCD) significantly improves when treated with STX-64, epitestosterone sulfate (ES) or both.

25

Therefore, an aspect of the present invention refers to a composition, preferably a pharmaceutical composition, nutraceutical composition or dietary supplement comprising epitestosterone sulfate (ES), or any salts or esters thereof, and/or compounds capable of inhibiting the steroid sulfatase of Formula (I):

30



wherein:

(a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

5 (b) at least one of R₁-R₆ is a sulfamate group, and two or more of R₁-R₆ are linked together to form an additional cyclic structure;

for use in a method for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, or age-related cognitive impairment in a subject in need thereof. In particular, the composition is for use in Age-related cognitive impairment associated with Mild Cognitive Impairment (MCI), Age-Associated Memory Impairment (AAMI), or Age-Related Cognitive Decline (ARCD). More particularly, the composition is for use in Age-related cognitive impairment associated with Age-Associated Memory Impairment (AAMI).

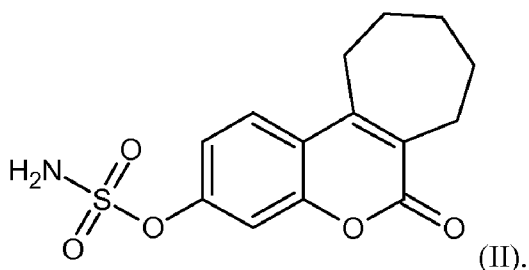
15 In an embodiment, the Age-related cognitive impairment, preferably associated with Age-Associated Memory Impairment (AAMI), is deemed to be improved when compared to the cognitive function expected in an age-matched normal subject. That is, the cognitive function might be reduced by about 5%, about 10%, about 30%, or more in the subject in need thereof, compared to cognitive function expected in an age-matched normal subject and the treatment reduces such difference, preferably in at least 0.1%, 1%, 2%, 3%, 4%, 5% or more.

20 In an embodiment, the composition is for use in a method for treating age-related cognitive impairment, preferably in a method to improve and/or treat age-related short- and/or long-term memory impairment, in a subject in need thereof.

In an embodiment, the composition is for use in a method for treating age-related cognitive impairment, preferably in a method to improve and/or treat age-related short- and/or long-term memory, in a subject in need thereof.

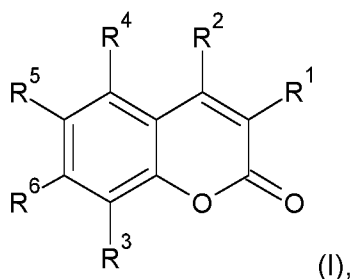
25 In an embodiment, the composition for use comprises epitestosterone sulfate (ES) or any salts or esters thereof.

In an embodiment, the composition for use comprises the sulfatase inhibitor STX64 of Formula (II):



or any salts thereof.

An additional aspect refers to a non-therapeutic method of improving age-related cognitive function, or age-related cognitive impairment in a subject in need thereof; in particular, for improving Age-related cognitive impairment associated with Mild Cognitive Impairment (MCI), Age-Associated Memory Impairment (AAMI), or Age-Related Cognitive Decline (ARCD); more particularly, for improving Age-related cognitive impairment associated with Age-Associated Memory Impairment (AAMI); the method comprising the steps of providing a composition, preferably a nutraceutical composition or dietary supplement, comprising epitestosterone sulfate (ES), or any salts or esters thereof, and/or a compound capable of inhibiting the steroid sulfatase of Formula (I):

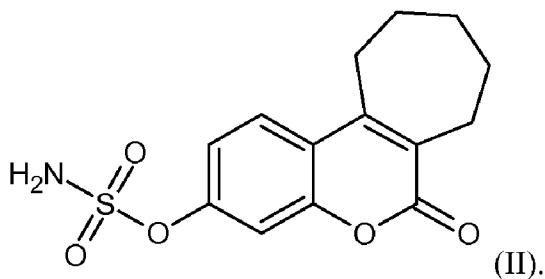


wherein:

- (a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;
- (b) at least one of R₁-R₆ is a sulfamate group; and two or more of R₁-R₆ are linked together to form an additional cyclic structure; and
- administering an effective amount of the compound to a human subject in need thereof. In particular, to a subject whose cognitive function might be reduced by about 5%, about 10%, about 30%, or more, compared to cognitive function expected in an age-matched normal subject.

In an embodiment, the method comprises the administration of epitestosterone sulfate (ES) or any salts or esters thereof.

In an embodiment, the method comprises the administration of the sulfatase inhibitor STX64 of Formula (II):



or any salts thereof.

5

Description of embodiments

Definitions

10 As used herein, "pharmaceutically acceptable carrier" or "pharmaceutically acceptable diluent" means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The term "pharmaceutically acceptable excipient" refers to any substance formulated alongside the active ingredient of a medication, included for the purpose of long-term stabilization, bulking up
15 solid formulations that contain potent active ingredients in small amounts, or to confer a therapeutic enhancement on the active ingredient in the final dosage form, such as facilitating drug absorption, reducing viscosity, or enhancing solubility. Excipients can also be useful in the manufacturing process, to aid in the handling of the active substance concerned such as by facilitating powder flowability or non-stick properties, in addition to aiding in vitro stability
20 such as prevention of denaturation or aggregation over the expected shelf life. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and, without limiting the scope of the present invention, include: additional buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine;
25 chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol,

myoinositol, myoinositol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thiocetic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulfate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone. Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington: The Science and Practice of Pharmacy 22nd edition, Pharmaceutical press (2012), ISBN-13: 9780857110626 may also be included.

“Improving” cognitive function includes promoting cognitive function and/or preserving cognitive function in a subject.

“Cognitive function” or “cognitive status” refers to any higher order intellectual brain process or brain state, respectively, involved in learning and/or memory including, but not limited to, attention, information acquisition, information processing, working memory, short-term memory, long-term memory, anterograde memory, retrograde memory, memory retrieval, discrimination learning, decision-making, inhibitory response control, attentional set-shifting, delayed reinforcement learning, reversal learning, the temporal integration of voluntary behavior, and expressing an interest in one's surroundings and self-care. In humans, cognitive function may be measured, for example and without limitation, by the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog), the clinical global impression of change scale (CGIC-plus scale), the Alzheimer's Disease Cooperative Study Activities of Daily Living Scale (ADCS-ADL), clinical global impression of change scale (CIBIC-plus scale); the Mini Mental State Exam (MMSE); the Neuropsychiatric Inventory (NPI); the Clinical Dementia Rating Scale (CDR); the Cambridge Neuropsychological Test Automated Battery (CANTAB) or the Sandoz Clinical Assessment-Geriatric (SCAG). See Folstein et al., J Psychiatric Res 12: 189-98, (1975); Robbins et al., Dementia 5: 266-81, (1994); Rey, L'examen clinique en psychologie, (1964); Kluger et al., J Geriatr Psychiatry Neurol 12:168-79, (1999). In addition, cognitive function may be measured using imaging techniques such as Positron Emission Tomography (PET), functional magnetic resonance imaging (fMRI), Single Photon Emission Computed Tomography (SPECT), or any other imaging technique that allows one to measure brain activity. In animal model systems, cognitive function may be measured in various conventional ways known in the art, including using a Morris Water Maze (MWM), Barnes circular maze, elevated radial arm maze, T maze or any other mazes in which the animals use spatial information. Other tests known in the art may also be used to assess cognitive function, such as novel object recognition and odor recognition tasks. Cognitive function may also be measured using imaging techniques such as Positron Emission Tomography (PET), functional

magnetic resonance imaging (fMRI), Single Photon Emission Computed Tomography (SPECT), or any other imaging technique that allows one to measure brain function. In animals, cognitive function may also be measured with electrophysiological techniques.

5 “Age-related cognitive impairment” or “function” refers to cognitive function in aged subjects that is not as robust as that expected in an age-matched normal subject (i.e. subjects with mean scores for a given age in a cognitive test) or as that expected in young adult subjects. In some cases, cognitive function is reduced by about 5%, about 10%, about 30%, or more, compared to cognitive function expected in an age-matched normal subject. In some cases, cognitive function is as expected in an age-matched normal subject, but reduced by about 5%,
10 about 10%, about 30%, about 50% or more, compared to cognitive function expected in a young adult subject. Age-related cognitive impairment may be associated with Mild Cognitive Impairment (MCI), Age-Associated Memory Impairment (AAMI), and Age-Related Cognitive Decline (ARCD).

15 “Mild Cognitive Impairment” or “MCI” refers to a condition characterized by isolated memory impairment unaccompanied other cognitive abnormalities and relatively normal functional abilities. One set of criteria for a clinical characterization of MCI specifies the following characteristics: (1) memory complaint (as reported by patient, informant, or physician), (2) normal activities of daily living (ADLs), (3) normal global cognitive function, (4) abnormal memory for age (defined as scoring more than 1.5 standard deviations below the mean for a given age), and (5) absence of indicators of dementia (as defined by DSM-IV guidelines; see
20 also Petersen et al., *Srch. Neurol.* 56: 303-308 (1999); Petersen, “Mild cognitive impairment: Aging to Alzheimer's Disease.” Oxford University Press, N.Y. (2003)).

25 “Age-Associated Memory Impairment (AAMI)” refers to a decline in memory due to aging. A patient may be considered to have AAMI if he or she is at least 50 years old and meets all of the following criteria: a) The patient has noticed a decline in memory performance, b) The patient performs worse on a standard test of memory compared to young adults, c) All other obvious causes of memory decline, except normal aging, have been ruled out (in other words, the memory decline cannot be attributed to other causes such as a recent heart attack or head injury, depression, adverse reactions to medication, Alzheimer's disease, etc.).

30 “Age Related Cognitive Decline (ARCD)” refers to declines in memory and cognitive abilities that are a normal consequence of aging in humans (e.g., Craik & Salthouse, 1992). This is also true in virtually all mammalian species. Age-Associated Memory Impairment refers to older persons with objective memory declines relative to their younger years, but cognitive functioning that is normal relative to their age peers (Crook et al., 1986). Age-Consistent

Memory Decline is a less pejorative label which emphasizes that these are normal developmental changes (Crook, 1993; Larrabee, 1996), are not pathophysiological (Smith et al., 1991), and rarely progress to overt dementia (Youngjohn & Crook, 1993). The DSM-IV (1994) has codified the diagnostic classification of ARCD.

5 The terms “treatment” and “therapy”, as used in the present application, refer to a set of hygienic, pharmacological, surgical and/or physical means used with the intent to cure and/or alleviate a disease and/or symptom with the goal of remediating the health problem. The terms “treatment” and “therapy” include preventive and curative methods, since both are directed to the maintenance and/or reestablishment of the health of an individual or animal. Regardless of
10 the origin of the symptoms, disease and disability, the administration of a suitable medicament to alleviate and/or cure a health problem should be interpreted as a form of treatment or therapy within the context of this application.

The term “prevention”, as used in the present application, refers to a set of hygienic, pharmacological, surgical and/or physical means used to prevent the onset and/or
15 development of a disease and/or symptoms. The term “prevention” encompasses prophylactic methods, since these are used to maintain the health of an animal or individual.

The term “sulfatase inhibitor” refers to any substance capable of reducing the activity of an enzyme of the esterase class that catalyzes the hydrolysis of sulfate esters. The substance may be a molecule that binds to any of the following elements: the gene that encodes the
20 sulfatase enzyme, transcription factors of said gene, any of the expression products of said gene, for example, without being limited thereto, the messenger RNA or the sulfatase enzyme, and decreases or inhibits the expression and the activity of the molecule to which it binds, and/or its intracellular or extracellular signaling, thereby leading to total or partial inhibition of the activity of the sulfatase enzyme. The inhibitor may be selected from the list consisting of,
25 without being limited thereto: antagonists against the sulfatase enzyme (preferably chemical), silencing RNA or specific antibody against the sulfatase enzyme (preferably, the antibody is monoclonal); in the present invention, this antibody may be defined as a neutralizing antibody against the effect of the sulfatase enzyme. Examples of chemical inhibitors of the activity of the sulfatase enzyme are, without being limited thereto, alternative substrates such as those in the
30 series 2-(hydroxyphenyl) indol sulfate, synthetic or natural steroids which present inhibitory activity against STS, such as 5-androstene-3 β , 17 β -diol-3 sulfate, competitive inhibitors such as E1-MTP or EMATE, non-oestrogenic inhibitors such as DU-14 (CAS NO: 186303-55-9), COUMATE (4-methylcoumarin-7-O-sulphamate) or STX64 (i.e., compound of Formula (II)), or others, such as KW-2581 or STX213, whose IC50 against the sulfatase enzyme has been
35 determined in different studies (Purohit & Foster, 2012, J. Endocrinol., 212(2):99-110).

The term "steroid hormone sulfatase" ("STS") refers to any sulfatase enzyme involved in the metabolism of steroids. In particular, the enzymes catalyze the conversion of sulfated steroid precursors to the free steroid. An exemplary STS found in humans has been sequenced, characterized and the data have been deposited in the UniProtKB database under the accession number P08842. The term "steroid hormone sulfatase inhibitor" refers to any substance capable of reducing the activity of a steroid hormone sulfatase. The substance may be a molecule that binds to any of the following elements: the gene that encodes the STS enzyme, transcription factors of said gene, any of the expression products of said gene, for example, without being limited thereto, the messenger RNA or the STS enzyme, and decreases or inhibits the expression and the activity of the molecule to which it binds, and/or its intracellular signaling, thereby leading to total or partial inhibition of the activity of the STS enzyme. The inhibitor may be selected from the list consisting of, without being limited thereto: antagonists against the STS enzyme (preferably chemical), silencing RNA or specific antibody against the STS enzyme (preferably, the antibody is monoclonal); in the present invention, this antibody may be defined as a neutralising antibody against the effect of the STS enzyme. Examples of chemical inhibitors of the activity of the STS enzyme are, without being limited thereto, alternative substrates such as those in the series 2-(hydroxyphenyl) indol sulfate, synthetic or natural steroids which present inhibitory activity against STS, such as 5-androstene-3 β , 17 β -diol-3 sulfate, competitive inhibitors such as E1-MTP or EMATE, non-oestrogenic inhibitors such as DU-14, COUMATE (4-methylcoumarin-7-O-sulphamate) or STX64 (i.e., compound of Formula (II)), or others, such as KW-2581 or STX213, whose IC₅₀ against the sulfatase enzyme has been determined in different studies (Purohit & Foster, 2012, J. Endocrinol., 212(2):99-110).

The term "protein-aggregation disease" refers to any disease in which certain proteins become structurally abnormal and thereby disrupt the function of cells, tissues and organs of the body. Often the proteins fail to fold into their normal configuration; in this misfolded state, the proteins can become toxic in some way or they can lose their normal function. Non-limiting examples of protein-aggregation diseases include systemic AL amyloidosis, Alzheimer's Disease, Diabetes mellitus type 2, Parkinson's disease, Transmissible spongiform encephalopathy e.g. Bovine spongiform encephalopathy, Fatal Familial Insomnia, Huntington's Disease, Medullary carcinoma of the thyroid, Cardiac arrhythmias, Atherosclerosis, Rheumatoid arthritis, Aortic medial amyloid, Prolactinomas, Familial amyloid polyneuropathy, Hereditary non-neuropathic systemic amyloidosis, Dialysis related amyloidosis, Finnish amyloidosis, Lattice corneal dystrophy, Cerebral amyloid angiopathy, Cerebral amyloid angiopathy (Icelandic type), Sporadic Inclusion Body Myositis, Amyotrophic lateral sclerosis (ALS), Prion-related or Spongiform encephalopathies, such as Creutzfeld-Jacob, Dementia with Lewy bodies,

Frontotemporal dementia with Parkinsonism, Spinocerebellar ataxias, Spinocerebellar ataxia, Spinal and bulbar muscular atrophy, Hereditary dentatorubral-pallidoluysian atrophy, Familial British dementia, Familial Danish dementia, Non- neuropathic localized diseases, such as in Type II diabetes mellitus, Medullary carcinoma of the thyroid, Atrial amyloidosis, Hereditary cerebral haemorrhage with amyloidosis, Pituitary prolactinoma, Injection-localized amyloidosis, Aortic medial amyloidosis, Hereditary lattice corneal dystrophy, Corneal amyloidosis associated with trichiasis, Cataract, Calcifying epithelial odontogenic tumors, Pulmonary alveolar proteinosis, Inclusion-body myositis, Cutaneous lichen amyloidosis, and Non-neuropathic systemic amyloidosis, such as AL amyloidosis, AA amyloidosis, Familial Mediterranean fever, Senile systemic amyloidosis, Familial amyloidotic polyneuropathy, Hemodialysis-related amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis, Finnish hereditary amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Icelandic hereditary cerebral amyloid angiopathy, familial amyloidosis, and systemic amyloidosis which occurs in multiple tissues, such as light-chain amyloidosis, and other various neurodegenerative disorders.

The term “protein aggregate” refers to any accumulation of abnormally folded proteins which cause and/or are associated with the negative progression of a protein-aggregation disease.

The term “amyloid” refers to a form of protein aggregates wherein the aggregates form unbranched fibers that bind Congo Red and then show green birefringence when viewed between crossed polarizers (for example, see Eisenberg & Jucker, 2012. Cell. 148(6):1188-203 and Sipe et al., 2012. Amyloid. 19(4):167-70).

The term “oligomer” refers to any accumulation of abnormally folded proteins which cause and/or are associated with the negative progression of a protein-aggregation disease and does not satisfy the definition of an amyloid. For example, polyglutamine oligomers cause and/or are associated with the negative progression of Huntington’s disease (see Hoffner & Dijan, 2014. Brain Sci. 4(1): 91–122).

Description

Gonad is a key tissue in the regulation of life span. Germline regulates longevity by inhibiting the production of dafachronic acid in the somatic gonads. Consistently, germline ablation or mutations that abolish the generation of germline, increase life span by activation of dafachronic acid synthesis. Gonads are also the classical tissue that produces sex steroids, although is not the only one. Our data indicates that inhibition of the sulfatase activity either by mutation or by STX64 raises the level of a very specific set of sulfated steroid hormones, which

in fact generate an increase in longevity. This increase in longevity depends on common factors involved in life span extension produced by germline loss, suggesting that both processes are in fact linked. We cannot distinguish whether the prolongevity effect of sulfated steroid hormones participates in the same pathway or acts in parallel to the germline longevity sharing some element of this pathway. The fact that sul-2 inhibition does not depend on NHR-49, or only partially depends on NHR-80, which are essentials for germline-mediated longevity, point to the second option.

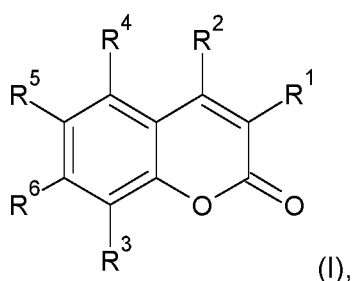
We have also studied the level of sulfated steroid hormones in the germline less glp-1 mutant, and we do not observe an increase in sulfated hormones. Those data favour the idea that gonads produce steroid hormones, which are modified by sulfation. These sulfated steroid hormones, probably altering neurotransmission, produce an increase in longevity, through common factors to germline-less animals. The fact that the enzymes involved in the sulfate modification of steroid hormones (sulfatase SUL-2 and sulfotransferase SSU-1) are expressed in sensory neurons suggests that alteration the sulfate state of hormones may act in the integration of environmental cues, such as nutrient availability, with the reproductive status, which are two-linked processes.

In *C. elegans*, cell proliferation of the somatic cells only occurs during development and in larval stages but not in the adult stage; Therefore, increasing longevity is due to the maintenance of the postmitotic cells. One of the stresses observed in *C. elegans* adult cells is the aggregation of endogenous proteins, which generates cellular dysfunction. This age-related formation of aggregates is also observed upon ectopic expression of aggregation-prone proteins, like β -amyloid or α -synuclein. Long-lived mutants such as daf-2 or glp-1 delay the aggregation toxicity through a different mechanism including chaperon expression and degradation by proteasome or autophagy. We herein show that inhibition of the sulfatase activity or treatment with some specific sulphated C19 androgen hormones impinge not only in longevity but also reduce protein aggregation and its toxic consequences in *C. elegans* models of protein aggregation diseases.

Regulation of steroid hormones by sulfation is a conserved process. In mammals, sulfotransferases and sulfatases are expressed in different tissues, including the nervous system, similar to what we observe in *C. elegans*. In humans, C19 steroid hormones have also been involved in longevity. For instance, dehydroepiandrosterone sulfate (DHEAS) declines with age and has been used as a marker of aging, raising speculations of a causative effect on sarcopenia, poor cognitive function and other aging associated diseases⁶ including Alzheimer's disease. Our data show that inhibition of the steroid sulfatase by mutation or by STX64 treatment extends lifespan in *C. elegans* and protects against aging-associated

proteotoxicity in nematodes. Interestingly, similar effects were observed upon treatment with some specific sulfated C19 steroid hormones.

Therefore, the present invention provides a method for increasing the lifespan of eukaryotic organisms comprising the steps of: providing a sulfated C19 androgen compound, or a compound capable of inhibiting the steroid sulfatase selected from the list consisting of 2-(hydroxyphenyl) indol sulfate, 5-androstene-3 β , DU-14, 17 β -diol-3 sulfate, E1-MTP, EMATE, COUMATE, STX64 (compound of formula II), KW-2581, STX213, morpholine, silencing RNA and specific antibody against the Steroid sulfatase (STS), or steryl-sulfatase (EC 3.1.6.2), formerly known as arylsulfatase C; or the sulfatase inhibitor of Formula (I):



10

wherein:

(a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

(b) at least one of R₁-R₆ is a sulfamate group; and two or more of R₁-R₆ are linked together to form an additional cyclic structure; and

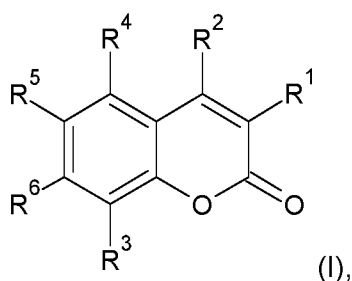
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administering an effective amount of the compound to a eukaryotic organism, such that the lifespan of the eukaryotic organism is increased.

In addition, as illustrated in figures 16 to 19, the present invention further provides methods and compositions for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, age-related cognitive impairment or the risk thereof in a subject in need thereof by administering a sulfated C19 androgen compound, or a compound capable of inhibiting the steroid sulfatase selected from the list consisting of 2-(hydroxyphenyl) indol sulfate, 5-androstene-3 β , DU-14, 17 β -diol-3 sulfate, E1-MTP, EMATE, COUMATE, STX64 (compound of formula II), KW-2581, STX213, morpholine, silencing RNA and specific antibody against the Steroid sulfatase (STS), or steryl-sulfatase (EC 3.1.6.2), formerly known as arylsulfatase C; or the sulfatase inhibitor of Formula (I):

20

25



wherein:

(a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

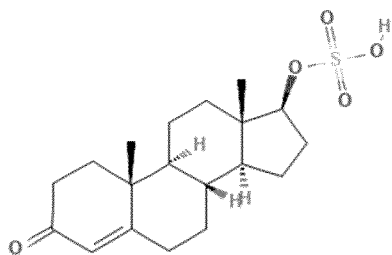
5 (b) at least one of R₁-R₆ is a sulfamate group; and two or more of R₁-R₆ are linked together to form an additional cyclic structure.

In a preferred embodiment, the compound of any of the above methods (for increasing the lifespan of the eukaryotic organism and/or for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, age-related cognitive impairment or the risk thereof) is a sulfated C₁₉ androgen selected from the list consisting of dehydroepiandrosterone sulfate (DHEAS), testosterone sulfate (TS), epitestosterone sulfate (ES) or Androsterone sulfate (AS). In a more preferred embodiment, the sulfated C₁₉ androgen is selected from the group consisting of testosterone sulfate (TS), epitestosterone sulfate (ES) or Androsterone sulfate (AS).

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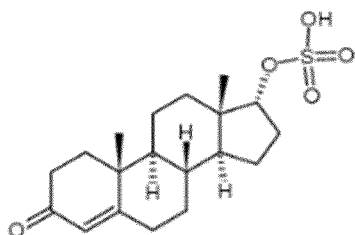
Testosterone sulfate (TS) (<https://pubchem.ncbi.nlm.nih.gov/compound/Testosterone-sulfate>) is an endogenous, naturally occurring steroid and minor urinary metabolite of testosterone, of chemical name [(8R,9S,10R,13S,14S,17S)-10,13-dimethyl-3-oxo-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-17-yl] hydrogen sulfate, and formula:

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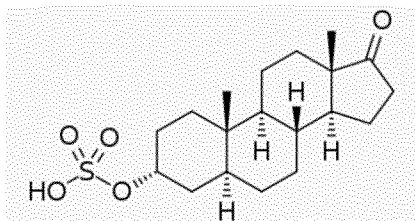


Other names. Testosterone 17β-sulfate; Testosterone 17β-sulfuric acid; 17β-(Sulfooxy)androst-4-en-3-one.

Epitestosterone structurally differs from testosterone only in the configuration at the hydroxy-bearing carbon, C17. Epitestosterone sulfate (ES) also known as Testosterone 17 α -sulfate has the following chemical name N,N-diethylethanamine;[(8R,9S,10R,13S,14S,17R)-10,13-dimethyl-3-oxo-1,2,6,7,8,9,11,12,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-17-yl] hydrogen sulfate, and formula:

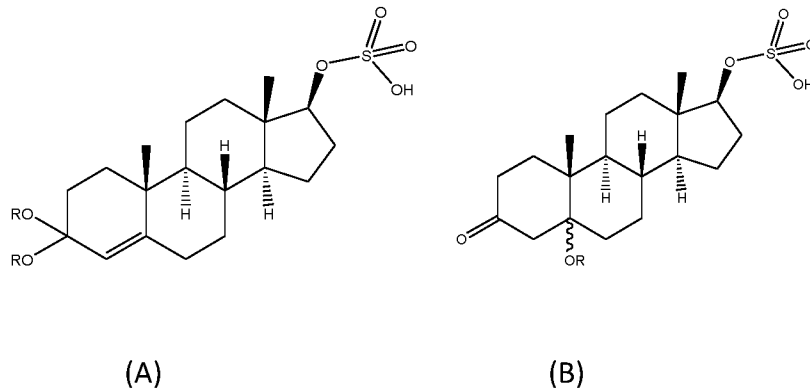


Androsterone sulfate (AS) also known as 3 α -hydroxy-5 α -androstan-17-one 3 α -sulfate, is an endogenous, naturally occurring steroid and one of the major urinary metabolites of androgens. It is a steroid sulfate which is formed from sulfation of androsterone by the steroid sulfotransferase SULT2A1 and can be desulfated back into androsterone by steroid sulfatase, of chemical name [(3R,5S,8R,9S,10S,13S,14S)-10,13-Dimethyl-17-oxo-1,2,3,4,5,6,7,8,9,11,12,14,15,16-tetradecahydrocyclopenta[a]phenanthren-3-yl] hydrogen sulfate, and formula



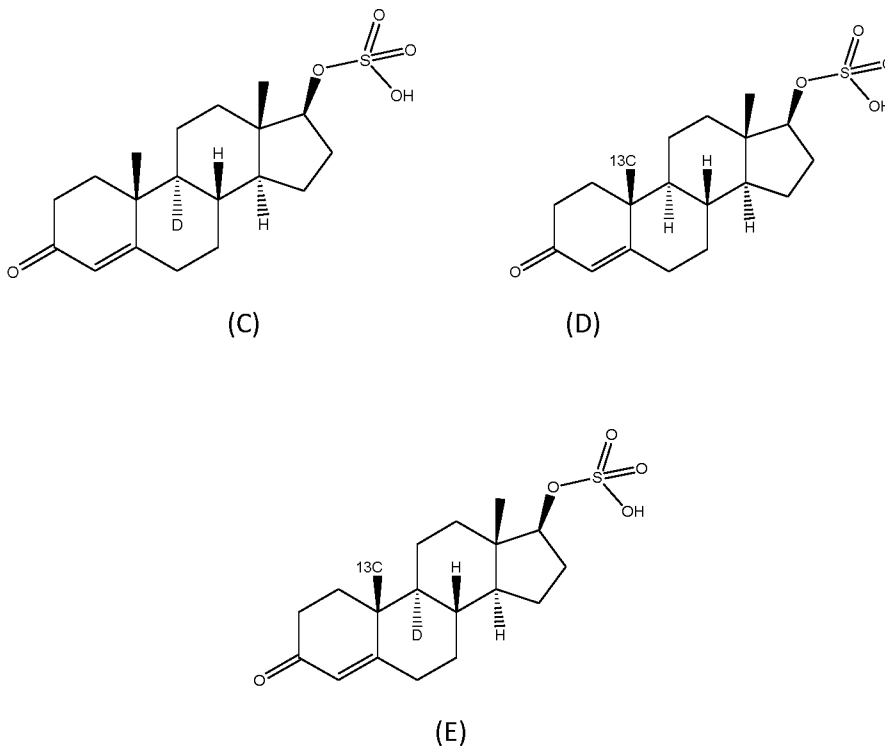
It is herein noted that any of the above mentioned sulfated C19 androgens includes the corresponding salts and esters thereof. Preferably any of the above mentioned sulfated C19 androgens includes the corresponding pharmaceutically acceptable salts, pharmaceutically acceptable solvates, isotopic variants (preferably comprising deuterium atoms and/or one or more carbon atoms with ^{13}C), different crystalline forms such as polymorphs, pharmaceutically acceptable esters, stereoisomers, tautomers, analogs and derivatives thereof.

Solvates such as (A) or (B), where R = H, alkyl or aryl and are formed by addition of water or an alcohol to the parent compound:

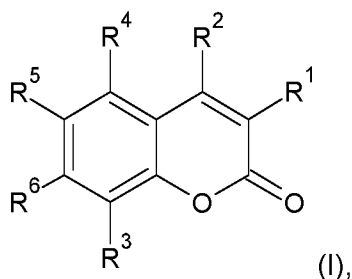


- 5 Isotopic variants for example where one or more atoms are replaced with a stable isotope of the same atom, such as replacing one or more hydrogen atoms by deuterium atoms, for example (C), or one or more carbon atoms with ^{13}C for example (D). It will be appreciated this possibility can apply to any atom within the structures, and also combinations can be considered:

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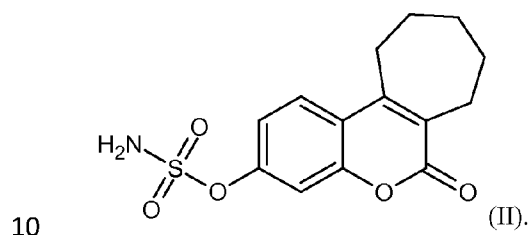
- 15 In another preferred embodiment, the compound capable of inhibiting the steroid sulfatase of any of the above methods (for increasing the lifespan of the eukaryotic organism and/or for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, age-related cognitive impairment or the risk thereof) is the sulfatase inhibitor of Formula (I):



wherein:

- (a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;
- 5 (b) at least one of R₁-R₆ is a sulfamate group; and two or more of R₁-R₆ are linked together to form an additional cyclic structure.

Preferably, the compound capable of inhibiting the steroid sulfatase is the sulfatase inhibitor STX64 of Formula (II):



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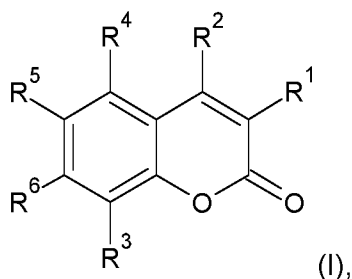
As in the case of the above mentioned sulfated C19 androgens, STX64 of Formula (II) above includes the corresponding salts and esters thereof. Preferably STX64 of Formula (II) above includes the corresponding pharmaceutically acceptable salts, pharmaceutically acceptable solvates, isotopic variants (preferably comprising deuterium atoms and/or one or more carbon atoms with ¹³C), different crystalline forms such as polymorphs, pharmaceutically acceptable esters, stereoisomers, tautomers, analogs and derivatives thereof.

15

In a preferred embodiment, the compound of any of the above methods (for increasing the lifespan of the eukaryotic organism and/or for improving age-related cognitive function and/or treating disorders involving age-related cognitive dysfunction, age-related cognitive impairment or the risk thereof) is a combination of a sulfated C19 androgen, preferably testosterone sulfate (TS), epitestosterone sulfate (ES) or Androsterone sulfate (AS), and a sulfatase inhibitor selected from the list consisting of 2-(hydroxyphenyl) indol sulfate, 5-androstene-3β, DU-14, 17β-diol-3 sulfate, E1-MTP, EMATE, COUMATE, STX64, KW-2581, STX213, morpholine, silencing RNA and specific antibody against the STS enzyme; or the sulfatase inhibitor of

20

25 Formula (I):



(I),

wherein:

(a) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

5 (b) at least one of R₁-R₆ is a sulfamate group; and

two or more of R₁-R₆ are linked together to form an additional cyclic structure.

In a preferred embodiment, the sulfatase inhibitor is STX64 and the sulfated C19 androgen is epitestosterone sulfate (ES).

10 From hereinafter, the above indicated compounds or combination of compounds, when used in a method for increasing the lifespan of a eukaryotic organism, shall be referred to as "lifespan increasing compounds".

15 A lifespan increasing compound, as identified above, is a compound that is useful for the prevention of aging-associated proteotoxicity and/or to increase the lifespan of a eukaryotic organism. Lifespan as used herein means the length of time (e.g. days or years) a cell or organism survives before dying (chronological lifespan). More particularly, a lifespan increasing compound, as identified above, is preferably a compound that prevents aging-associated proteotoxicity caused by protein-aggregation diseases and/or increases the lifespan of a eukaryotic organism.

20 Protein aggregates such as amyloids and oligomers have been associated with a number of diseases. In some cases, these protein aggregates can become toxic and can cause significant damage to cells and tissue. This toxicity is thought to be one of the contributing factors causing and/or contributing to the pathology of protein-aggregation diseases. The lifespan increasing compounds of the present invention protect against and treat protein-aggregation diseases. In a preferred embodiment, the protein-aggregation disease is selected from a list consisting of
 25 systemic AL amyloidosis, Alzheimer's Disease, Diabetes mellitus type 2, Parkinson's disease, Transmissible spongiform encephalopathy e.g. Bovine spongiform encephalopathy, Fatal Familial Insomnia, Huntington's Disease, Medullary carcinoma of the thyroid, Cardiac arrhythmias, Atherosclerosis, Rheumatoid arthritis, Aortic medial amyloid, Prolactinomas, Familial amyloid polyneuropathy, Hereditary non-neuropathic systemic amyloidosis, Dialysis

related amyloidosis, Finnish amyloidosis, Lattice corneal dystrophy, Cerebral amyloid angiopathy, Cerebral amyloid angiopathy (Icelandic type), Sporadic Inclusion Body Myositis, Amyotrophic lateral sclerosis (ALS), Prion-related or Spongiform encephalopathies, such as Creutzfeld-Jacob, Dementia with Lewy bodies, Frontotemporal dementia with Parkinsonism, Spinocerebellar ataxias, Spinocerebellar ataxia, Spinal and bulbar muscular atrophy, Hereditary dentatorubral-pallidoluysian atrophy, Familial British dementia, Familial Danish dementia, Non-neuropathic localized diseases, such as in Type II diabetes mellitus, Medullary carcinoma of the thyroid, Atrial amyloidosis, Hereditary cerebral haemorrhage with amyloidosis, Pituitary prolactinoma, Injection-localized amyloidosis, Aortic medial amyloidosis, Hereditary lattice corneal dystrophy, Corneal amyloidosis associated with trichiasis, Cataract, Calcifying epithelial odontogenic tumors, Pulmonary alveolar proteinosis, Inclusion-body myositis, Cutaneous lichen amyloidosis, and Non-neuropathic systemic amyloidosis, such as AL amyloidosis, AA amyloidosis, Familial Mediterranean fever, Senile systemic amyloidosis, Familial amyloidotic polyneuropathy, Hemodialysis-related amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis, Finnish hereditary amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Icelandic hereditary cerebral amyloid angiopathy, familial amyloidosis, and systemic amyloidosis which occurs in multiple tissues, such as light-chain amyloidosis, and other various neurodegenerative disorders. Preferably, the protein-aggregation disease is selected from a list consisting of Alzheimer's disease, Parkinson's disease and Huntington's disease. In a preferred embodiment, the protein-aggregation disease is not Alzheimer's disease and/or a type of cancer.

In a preferred embodiment, the protein-aggregation disease is selected from a list consisting of Alzheimer's disease, Parkinson's disease and Huntington's disease and the sulfated C19 androgens of the present invention are preferably selected from the list consisting of testosterone sulfate (TS), epitestosterone sulfate (ES) or Androsterone sulfate (AS). More preferably, said sulfated C19 androgens are in the form of a pharmaceutical or nutraceutical composition or in the form of Dietary Supplements.

In a preferred embodiment, the protein-aggregation disease is a central nervous system localized protein-aggregation disease. In a preferred embodiment, the protein-aggregation disease is also a neurodegenerative disease. The term "*neurodegenerative disease*" refers to any disorder characterized by the progressive loss of structure or function of neurons, including death of neurons. For example, Alzheimer's disease is an example of a protein-aggregation disease and an example of a neurodegenerative disease.

Therefore, an effective amount of a lifespan increasing compound increases the lifespan of a eukaryotic organism, preferably a human, and/or protects against aging-associated

proteotoxicity caused by a protein-aggregation disease. In one embodiment, an effective amount of a lifespan increasing compound increases the lifespan of an eukaryotic organism by a statistically significant amount compared to the lifespan of an untreated organism. The lifespan of an untreated organism may be determined in parallel or may be obtained from separately conducted studies (control). In another embodiment, an effective amount of a lifespan increasing compound increases the lifespan of an eukaryotic organism by at least 5%. In other embodiments, an effective amount of a LAC increases the lifespan of an eukaryotic organism by at least 10, 15, 20, 25, 35, 50%, or 100% over control.

Examples of eukaryotic organisms include single- and multi-cellular organisms, including higher-order organisms (such as mammals, which includes humans).

In one embodiment, the present method for increasing the lifespan of the eukaryotic organism, can be used in order to generally increase the lifespan of the cells of a eukaryotic organism and to protect its cells against stress and/or against apoptosis. In various other embodiments, the present method can be used for treating or preventing a disease or condition induced or exacerbated by cellular senescence in a subject; for extending the lifespan of a subject; for treating or preventing a disease or condition relating to lifespan; for treating or preventing a disease or condition relating to the proliferative capacity of cells; for treating or preventing a disease or condition resulting from cell damage or death.

In various embodiments, the present method may be used to prevent aging and aging-related consequences, or diseases caused by protein-aggregation diseases. In a preferred embodiment, the protein-aggregation disease is selected from a list consisting of systemic AL amyloidosis, Alzheimer's Disease, Diabetes mellitus type 2, Parkinson's disease, Transmissible spongiform encephalopathy e.g. Bovine spongiform encephalopathy, Fatal Familial Insomnia, Huntington's Disease, Medullary carcinoma of the thyroid, Cardiac arrhythmias, Atherosclerosis, Rheumatoid arthritis, Aortic medial amyloid, Prolactinomas, Familial amyloid polyneuropathy, Hereditary non-neuropathic systemic amyloidosis, Dialysis related amyloidosis, Finnish amyloidosis, Lattice corneal dystrophy, Cerebral amyloid angiopathy, Cerebral amyloid angiopathy (Icelandic type), Sporadic Inclusion Body Myositis, Amyotrophic lateral sclerosis (ALS), Prion-related or Spongiform encephalopathies, such as Creutzfeldt-Jacob, Dementia with Lewy bodies, Frontotemporal dementia with Parkinsonism, Spinocerebellar ataxias, Spinocerebellar ataxia, Spinal and bulbar muscular atrophy, Hereditary dentatorubral-pallidoluysian atrophy, Familial British dementia, Familial Danish dementia, Non- neuropathic localized diseases, such as in Type II diabetes mellitus, Medullary carcinoma of the thyroid, Atrial amyloidosis, Hereditary cerebral haemorrhage with amyloidosis, Pituitary prolactinoma, Injection-localized amyloidosis, Aortic medial amyloidosis,

Hereditary lattice corneal dystrophy, Corneal amyloidosis associated with trichiasis, Cataract, Calcifying epithelial odontogenic tumors, Pulmonary alveolar proteinosis, Inclusion-body myositis, Cutaneous lichen amyloidosis, and Non-neuropathic systemic amyloidosis, such as AL amyloidosis, AA amyloidosis, Familial Mediterranean fever, Senile systemic amyloidosis, 5 Familial amyloidotic polyneuropathy, Hemodialysis-related amyloidosis, ApoAI amyloidosis, ApoAII amyloidosis, ApoAIV amyloidosis, Finnish hereditary amyloidosis, Lysozyme amyloidosis, Fibrinogen amyloidosis, Icelandic hereditary cerebral amyloid angiopathy, familial amyloidosis, and systemic amyloidosis which occurs in multiple tissues, such as light-chain amyloidosis, and other various neurodegenerative disorders. Preferably, the protein- 10 aggregation disease is selected from a list consisting of Alzheimer's disease, Parkinson's disease and Huntington's disease. In a preferred embodiment, the protein-aggregation disease is not Alzheimer's disease and/or a type of cancer.

In a preferred embodiment, the protein-aggregation disease is selected from a list consisting of Alzheimer's disease, Parkinson's disease and Huntington's disease and the sulfated C19 15 androgens of the present invention are preferably selected from the list consisting of testosterone sulfate (TS), epitestosterone sulfate (ES) or Androsterone sulfate (AS). More preferably, said sulfated C19 androgens are in the form of a pharmaceutical or nutraceutical composition or in the form of Dietary Supplements.

As yet another example, the method of the present invention may also be applied during 20 developmental and growth phases in mammals, preferably in humans, in order to, e.g., alter, retard or accelerate the developmental and/or growth process.

It is noted that all of the compounds of the present invention, either for improving cognition or for increasing the lifespan of a eukaryotic organism, can be in the form of pharmaceutical compositions, nutraceutical compositions or dietary supplements.

25 In a preferred embodiment, the compounds are in the form of a pharmaceutical composition further comprising a pharmaceutically acceptable carrier and/or diluent. Preferably, the pharmaceutical composition may further comprise a pharmaceutically acceptable excipient.

A pharmaceutical composition as described herein may also contain other substances. These substances include, but are not limited to, cryoprotectants, lyoprotectants, surfactants, bulking 30 agents, anti-oxidants, and stabilizing agents. In some embodiments, the pharmaceutical composition may be lyophilized.

The term "*cryoprotectant*" as used herein, includes agents which provide stability to the compositions against freezing-induced stresses. Cryoprotectants may also offer protection during primary and secondary drying and long-term product storage. Non-limiting examples of

cryoprotectants include sugars, such as sucrose, glucose, trehalose, mannitol, mannose, and lactose; polymers, such as dextran, hydroxyethyl starch and polyethylene glycol; surfactants, such as polysorbates (e.g., PS-20 or PS-80); and amino acids, such as glycine, arginine, leucine, and serine. A cryoprotectant exhibiting low toxicity in biological systems is generally used.

In one embodiment, a lyoprotectant is added to a pharmaceutical composition described herein. The term "*lyoprotectant*" as used herein, includes agents that provide stability to the compositions during the freeze-drying or dehydration process (primary and secondary freeze-drying cycles. This helps to minimize product degradation during the lyophilization cycle, and improve the long-term product stability. Non-limiting examples of lyoprotectants include sugars, such as sucrose or trehalose; an amino acid, such as monosodium glutamate, non-crystalline glycine or histidine; a methylamine, such as betaine; a lyotropic salt, such as magnesium sulfate; a polyol, such as trihydric or higher sugar alcohols, e.g., glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; pluronics; and combinations thereof. The amount of lyoprotectant added to a pharmaceutical composition is generally an amount that does not lead to an unacceptable amount of degradation when the pharmaceutical composition is lyophilized.

In some embodiments, a bulking agent is included in the pharmaceutical composition. The term "*bulking agent*" as used herein, includes agents that provide the structure of the freeze-dried product without interacting directly with the pharmaceutical product. In addition to providing a pharmaceutically elegant cake, bulking agents may also impart useful qualities in regard to modifying the collapse temperature, providing freeze-thaw protection, and enhancing the stability over long-term storage. Non-limiting examples of bulking agents include mannitol, glycine, lactose, and sucrose. Bulking agents may be crystalline (such as glycine, mannitol, or sodium chloride) or amorphous (such as dextran, hydroxyethyl starch) and are generally used in formulations in an amount from 0.5% to 10%.

Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) or *Remington: The Science and Practice of Pharmacy* 22nd edition, Pharmaceutical press (2012), ISBN-13: 9780857110626 may also be included in a pharmaceutical composition described herein, provided that they do not adversely affect the desired characteristics of the pharmaceutical composition.

For solid pharmaceutical compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate,

sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For solution for injection, the pharmaceutical composition may further comprise cryoprotectants, lyoprotectants, surfactants, bulking agents, anti-oxidants, stabilizing agents and pharmaceutically acceptable carriers. For aerosol administration, the pharmaceutical compositions are generally supplied in finely divided form along with a surfactant and propellant. The surfactant must, of course, be nontoxic, and is generally soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides.

In a preferred embodiment, the compounds are prepared for oral, sublingual, buccal, intranasal, intravenous, intramuscular, intraperitoneal and/or inhalation-mediated administration.

In another preferred embodiment, the compounds of the invention are in the form of a nutraceutical composition or a dietary supplement or product.

It is expected that the compounds of the invention can be delivered to an organism using any available method and route suitable for compound delivery, including oral, parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal routes. It will be recognized by those of skill in the art that the form and character of the particular dosing regimen employed in the method of the invention will be dictated by the route of administration and other well-known variables, such as the size and age of the eukaryotic organism. Determination of such dosing regimens is within the purview of one skilled in the art. Administration of the compound could be performed in conjunction with any conventional therapies that are intended to treat a disease or disorder associated with aging including topical, oral, or injectable.

Therefore, the compounds of the invention, including pharmaceutical or nutraceutical compositions or Dietary Supplements, may be administered using any route known to the skilled person. In a preferred embodiment, the compounds of the invention are administered transdermally, sublingually, intravenously, intranasally, intracerebroventricularly, intraarterially, intracerebrally, intramuscularly, intraperitoneally, orally or via inhalation.

In a preferred embodiment, the compounds of the invention are administered transdermally, sublingually, intravenously, intraperitoneally, orally or via inhalation. Where the compound is administered via inhalation, the composition may be aerosolized and administered via, for example, an anesthesia mask.

In a preferred embodiment, the compounds of the invention are administered transdermally, sublingually, intravenously, subcutaneously, orally or via inhalation. Preferably, the composition is administered orally or sublingually.

The compounds of the invention may be administered once or more than once. A skilled person will be able to ascertain the most effective dosage regimen for the patient. For example, the most effective dosage regimen may be one where the patient is administered the composition twice daily, once a day, once every three days, once a week, once a month, once every three months, once every six months or once every year.

The following non-limiting Examples provide further description of the present invention.

EXAMPLES

Identification of sul-2 as a regulator of longevity

Unravelling new elements that govern the genetic control of aging is key to improve our understanding of this intricate biological process and improve human healthspan. To this aim, we isolated *Caenorhabditis elegans* thermotolerant mutants and identified an allele pv17 of the sul-2 gene, which encodes one of the three members of the *C. elegans* sulfatase family.

Worms carrying either the isolated (pv17) allele or the null allele (gk187) of sul-2 lived longer than wild type although the gk187 allele showed a bimodal curve with a subpopulation that had an early mortality (Fig. 1a-b, and Fig.5a). Pumping frequency and mobility during aging declined similar or slower than wild type, overall for the pv17 allele, suggesting a healthier life span (Fig.5d-e). Deletion of the other two sulfatases genes, sul-1 and sul-3, did not increase lifespan, indicating that the sulfatase sul-2 has a main role in the regulation of longevity (Fig.5f). Mutations in sul-2 also enhanced the developmental phenotypes of mutants in the insulin/insulin like growth factor (IGF) receptor daf-2 (Fig. 5g-i), which was used for gene mapping and identification. The pv17 allele introduces a single amino acid substitution (G46D) resulting in a reduction of function phenotype. The curated sequence slightly differs from the one published (Fig. 6).

sul-2 encodes a sulfatase of steroid hormones

Sulfatases are a large protein family involved in different biological processes and with a wide range of substrates. The placement of curated sul-2 in the sulfatases phylogenetic tree is uncertain, but when compared to mammalian sulfatases, sul-2 clusters closer to the

Arylsulfatases type H, F, E, D and the steroid sulfatase type C (Fig. 1c) which probably originated from a common ancestor gene. *sul-1* and *sul-3* belong to a different family of sulfatases (Fig. 1c). We hypothesized that *sul-2* may exert its activity by modifying sulfated steroid hormones. Steroid hormone sulfatases are conserved proteins that participate, among other processes, stimulating proliferation in hormone-depending cancers. Specific inhibitors for this type of enzymes have been generated, such as STX6410, which has been used to treat patients with hormone-depending cancers. We treated wild type animals with STX64 and observed an increase in longevity (Fig. 1d and Fig. 7a-c). STX64 treatment also phenocopies other *sul-2* mutant phenotypes (Fig. 7d). STX64 does not further increase the longevity of *sul-2* deletion mutants, suggesting STX64 increases longevity is by inhibiting the sulfatase activity of SUL-2 (Fig. 1d).

We measured sulfated steroid hormones levels by a high-resolution HPLC-TOF-MS in *sul-2* mutant and found a higher proportion of sulfated hormones in this strain as compared to wild type worms (Fig. 1e). All these data suggest that SUL-2 can act as a steroid hormones sulfatase and regulates longevity through the alteration of the sulfated state of one or several steroid hormones. The increased longevity of *sul-2* depends on genes mediating gonadal longevity to investigate if *sul-2* acts in a known longevity pathway, we performed genetic interaction studies with known alleles that affect longevity. Mutations in the IGF receptor *daf-2* increase lifespan through the transcription factor DAF-16/FOXO12. *sul-2* mutations further extend the lifespan of *daf-2* reduction of function mutants (Fig. 2a Fig. 8a-b), suggesting that *sul-2* acts in a different pathway to regulate longevity. However, the increased longevity of *sul-2* mutants is mainly suppressed by DAF-16 loss-of-function (Fig. 2b and Fig. 8c-d). Longevity conferred by lack of germline also requires DAF-1612, 13, which translocates to the nuclei mainly in intestinal cells. However, in insulin signalling mutants, DAF-16 localises to the nucleus of most cells. In *sul-2* mutants, DAF-16 localises predominantly to intestinal nuclei (Fig. 9a-b), suggesting a role for *sul-2* in germline mediated longevity. Other essential factors for germline-mediated longevity, such as the intestinal ankyrin-repeat protein KRI-1/KRIT-1 and the transcription elongation factor TCER-1/TCERG1 are also required for a fully increased longevity of *sul-2* mutants, although a mild effect is observed with the nuclear hormone receptor NHR-80 and no effect was observed in NHR-49, which are also required for germline-mediated longevity (Fig. 2c-f and Fig. 8e-g). Moreover, deletion of *sul-2* has little effect on the longevity of the germline-less mutant *glp-1* or *mes-1* longevity¹⁸ (Fig. 2g-h); However, we do observe an additive effect in the reduction of function allele *pv17* in a *glp-1* background (Fig. 8h), which may suggest some additional effect of the mutated protein.

All these data suggest that *sul-2* mediates signalling from the gonad to regulate longevity. Interestingly, *sul-2* mutations do not affect fertility, reproductive age or gonad morphology (Fig. 2i-j and Fig. 9c-e). Taken together, our findings suggest that *sul-2* affects a signal that regulates longevity to adjust lifespan to the reproductive status without affecting gonadal function. Similar to germline ablation, we also observed that *sul-2* mutants increase further the longevity in dietary restriction suggesting that *sul-2* is not implied in this intervention that affects longevity (Fig. 2k). In germline-less animals, the activation of the nuclear receptor DAF-12 by bile acid-like steroids called dafachronic acids (DAs) triggers an increase in longevity²⁰. We observed that *daf-12* is also needed for the increased longevity of *sul-2* mutants (Fig. 2l and Fig. 8i), indicating that *sul-2* inactivation causes the alteration of the sulfated steroid hormones pool, generating a signal upstream of DAF-12 that imitates the longevity signals of gonad depleted animals. DAF-36 converts cholesterol to 7-dehydrocholesterol in the first step of the biosynthetic pathway of Δ^7 -DA21, 22. Therefore, DAF-36 is also needed for the increased longevity of germline-less animals²³. Similarly, DAF-36 is required for the longevity conferred by the steroid sulfatase inhibitor STX64 (Fig. 2m) placing the signal generated by sulfated steroid hormones upstream of or parallel to the biosynthesis of DAs.

sul-2 is expressed in sensory neurons

We have studied the anatomical location of *sul-2* expression from an extrachromosomal array and in single-copy insertion transgenic strains. We found that *sul-2* is expressed only in a few sensory neurons, mainly in the amphids ADF and ASE, and phasmids PHA and PHB. There is no detectable expression in the germline in any transgenic strains (Fig. 2n and Fig. 10, 11a-b). ASE neurons are responsible for the attractive response of Na⁺ and Cl⁻, among others. Defects in odour sensing affect longevity. Therefore, we tested the ability of *sul-2* mutants to respond to Cl⁻ or Na⁺ and found no differences compared to wild type animals (Fig. 11c-d). Furthermore, *sul-2* mutation increases the longevity of *daf-10(m79)*, a long-lived mutant defective in sensory cilia formation²⁵ (Fig. 11e). These results show that the longevity phenotype observed in *sul-2* mutants is not due to the impaired functionality of sensory neurons. Reduction of *sul-2* improves aging associated diseases in *C. elegans*. Aging is considered the main risk factor for the onset of neurodegenerative disorders like Parkinson, Huntington, or Alzheimer's diseases. These disorders are caused by the progressive decline of proteostasis, which results in protein aggregation that compromises cellular functions and finally causes cell death. Germline-deficient *C. elegans* delay the symptoms derived from the proteotoxicity of ectopically expressed human β -amyloid (β A). We tested if *sul-2* mutations or STX64 treatment improves the symptoms of *C. elegans* models for neurodegenerative diseases. In a *C. elegans* Parkinson's disease model, in which human α -synuclein expression in muscle cells causes

age-dependent paralysis, *sul-2* mutation or treatment with STX64 significantly improved mobility (Fig. 3a-b, Fig. 12a-c).

Loss of function of *SUL-2* decreased the number of α -synuclein aggregates (Fig. 12d-e), suggesting better handling of protein aggregates in worms with reduced steroid sulfatase activity. To further assay the neuroprotective effect of reduced *sul-2* activity, we tested a strain
5 expressing α -synuclein in dopaminergic neurons. In this model, GFP-labelled dopaminergic neurons die due to α -synuclein toxicity. Consistently, *sul-2* mutants showed increased neuron survival compared with control worms, indicating a neuroprotective action of reduced steroid-hormone sulfatase activity (Fig. 3c-d). In a Huntington neurodegenerative model expressing
10 polyglutamine repeats fused to YFP, a construct that aggregates in adult worms, we found that both *sul-2* mutation and treatment with STX64 reduced the number of aggregates (Fig. 3e-f). We also tested two different strains of Alzheimer's disease (AD) in worms, where immobility is caused by expression of β A protein in muscle cells. Consistently, *sul-2* mutation and STX64 treatment delayed paralysis (Fig. 3g-h and Fig. 12fg). All these data show that inhibition of *sul-2*
15 protects the nematode against aging related proteotoxicity.

Reduction of activity of *sul-2* improves Alzheimer in a mammal model

As STX64 ameliorated neurodegeneration in *C. elegans* models, we tested the effect of this drug on cognitive alterations provoked by intrahippocampal β A oligomers infusion, an acute AD mammalian model (Fig. 3i). Previously, it has been reported that local administration of DU-
20 14, an inhibitor of steroid hormones sulfatase, could alleviate memory loss caused by intrahippocampal administration of β A oligomers in a mammalian model. We observed that both, local and systemic STX64 treatments reverted the cognitive deficiencies, measured by passive avoidance test, caused by intrahippocampal administration of β A oligomers (Fig. 3j). To evaluate the effect of STX64 oral treatment on amyloid pathology in a chronic AD mice
25 model, we assessed the effect of 3-4 weeks of STX64 oral treatment on amyloid deposition in the neocortex (the cerebral cortex and the hippocampus) of >15-month-old APP-PS1 mice (Fig. 4k). At this age corresponding to a late stage of amyloid deposition in the neocortex of the APP218 PS1 model, the analysis of β A plaque density and size revealed a significant reduction, except for plaque size in the hippocampus, in mice treated with STX64. Moreover, β A
30 immunoreactive area is reduced in both tissues (Fig. 4k-n). Interesting, when we compared β A deposition in old (>15-month-old) APP-PS1 mice treated with STX64 with the normal temporal course of amyloid deposition in un-treated APP-PS1 mice, we observed that STX64 reduces β A deposition in APP-PS1 mice older than 15 months compared to that observed in APP-PS1 mice of 10-12 months of age (Fig. 4o). All these results indicate that STX64 treatment in APP-
35 PS1 mice reduces β A deposition. We wondered if this histological improvement correlated with

amelioration of cognitive behavioural deficit. For that, we compared cognition capacities in APP-PS1 mice older than 15-month-old treated with vehicle or STX64 for 3-4 weeks. While vehicle-treated APP-PS1 mice showed a deficit in passive avoidance test (Fig. 4p), those mice treated with STX64 completely reverted cognitive deficiencies, reaching similar levels to <15-month-old wild type mice. All these results point out that the alterations in β A metabolism provoked by STX64 reduce the cognitive behaviour deficiencies induced by β A accumulation in acute and chronic AD mice models, suggesting a potential for STX64 as a pharmacological therapy against neurodegenerative diseases.

Sulfated C19 androgens hormones recapitulate the beneficial effect of reduction of sul-2 activity

In mammals, sulfated hormones have been long considered inactive forms that function mainly as reservoirs and are activated by steroid sulfatases⁶, although a direct action of sulfated hormones in the reproductive and the nervous system has been observed. In this last tissue, those hormones are named neurosteroids and their main function is the modulation of neurotransmission. In order to sort out whether the beneficial effect of sul-2 inhibition is due to the reduction of non-sulfated hormones or the increase of sulfated hormones, we tested the commercially available sulfated steroid hormones that are highly presented in the mutant (Table 1). We observed that the C19 androgens dehydroepiandrosterone sulfate (DHEAS), testosterone sulfate (TS) and epitestosterone sulfate (ES) improved the mobility in the Parkinson model of *C. elegans*, with a better result for ES (Fig. 4a). Similar results are obtained in the Alzheimer model with TS and ES but not with DHEAS (Fig. 4b and Fig. 13a). Non-sulfated DHEA or non-sulfated testosterone showed no effect, neither pregnenolone sulfate, which belongs to the C21 group of steroid hormones (Fig. 4c-d). These results indicate that at least some sulfated C19 androgens are involved in the protective effect against protein aggregation diseases and strongly suggest that the beneficial effect of sul-2 inhibition is due to the increased levels of this type of hormones. In agreement with these results, treatment with the antiandrogenic compound abiraterone (Abi)³⁶, did not affect wild type animals but suppressed the beneficial effect of sul-2 mutation (Fig. 4e).

We then tested if those hormones are also involved in the other phenotypes observed in the sul-2 mutant. Treatment with any of those sulfated hormones generated an increase of L1 arrest in a *daf-2(e1370)* background as observed in sul-2 or STX 64 treated animals (Fig. 13b). Interestingly, treatment with ES, but not TS or DHEAS, increased in longevity on a wild type background and did not further increase longevity in sul-2 mutants backgrounds (Fig. 4f-g and Fig. 13c-d), indicating that both interventions share the same molecular mechanism. Thus, addition of ES recapitulated all the phenotypes described for sul-2 inhibition and strongly

suggesting that the causative effect of sul-2 mutation is due to the increase of C19 androgen sulphated hormones related to ES.

Lifespan assays methodology:

5 Lastly, as indicated in figure 15, strains were synchronized by hypochlorite treatment of gravid adults, grown up during two generations at 16 °C. F2 L4 animals were shifted to 25°C (t=0). ES treatment started at L4 stage, control and treated plates were prepared fresh and animals transferred each three days until death. Animals lost or dead by non-physiological causes were censured. Survival curves were generated using the product-limit method of Kaplan and Meier. The log-rank (Mantel–Cox) test was used to evaluate differences in survival and p-values lower than 0.05 were considered significant. Controls of all lifespan assays were *fer-15(b26)* mutant background, to avoid progeny production. *fer-15(b26)* mutation does not have effect on life span.

Treatment with STX-64, E-S or both tend to improve motor ability, habituation ability and immediate memory of aged animals

15 Figure 20 shows the results of an "Open Field" test and of a "Y maze" test. The "Open Field" is a test where the mouse must explore an unknown space for 15 minutes. In this test, both mobility and habituation (cognitive parameter that measures the individual's ability to recognize the environment) can be studied. On the other hand, the "Y maze" test is a test where the mouse explores the maze for 6 minutes. This test evaluates the distance traveled as a measure of mobility and the ability to visit triplets of arms without repeating any of them as a sign of immediate memory. The results show that treatment with STX-64, E-S or both tend to improve the motor ability of 16-month-old mice. On the other hand, habituation ability and immediate memory of aged animals are improved by treatment with STX, E-S and the combination of both (the compounds improved Age-Associated Memory Impairment).

25

Short- and long-term memory tests (STM and LTM) is improved with STX-64 and/or E-S

Figure 21 shows the evaluation of learning and memory abilities of aged mice was determined in the object recognition test and the passive avoidance test. The first, object recognition, after training with identical objects, tests the ability to identify different objects exposed together with a familiar one. When this happens, the object discrimination index is positive and higher than 0.1. In passive avoidance, the aim is to teach the mouse that its preferred place does not suit it and for them it is associated with an aversive stimulus. For this purpose, during training, the entry latency to the preferred site is established and the aversive stimulus is administered.

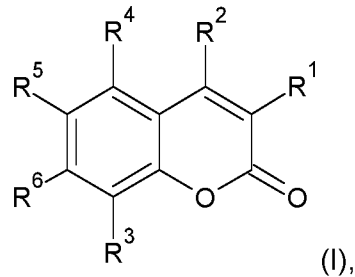
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During short- and long-term memory tests (STM and LTM) if the mouse associated the preferred place to the aversive stimulus, the input latency increases with respect to training. Vehicle-treated aged mice showed impairments in learning ability and thus memory. In both tests, mice treated with STX, ES and the combination of both showed significantly both learning
5 (STM) and ability to store information (memory) (the compounds improved Age-Associated Memory Impairment).

CLAIMS

1. A composition comprising a compound selected from the list consisting of at least one of epitestosterone sulfate (ES), or any salts or esters thereof, and compounds capable of inhibiting the steroid sulfatase of Formula (I):

5



wherein:

(c) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

10 (d) at least one of R₁-R₆ is a sulfamate group, and two or more of R₁-R₆ are linked together to form an additional cyclic structure;

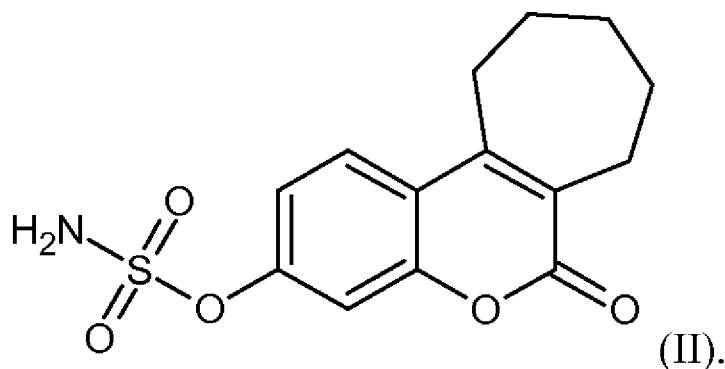
for use in a method for treating Age-related cognitive impairment in a subject in need thereof, preferably when associated with Mild Cognitive Impairment (MCI), Age-Associated Memory Impairment (AAMI), or Age-Related Cognitive Decline (ARCD).

15 2. The composition for use according to claim 1, wherein the composition is for use in treating Age-Associated Memory Impairment (AAMI) in a subject in need thereof.

3. The composition for use according to claim 1, wherein the composition comprises epitestosterone sulfate (ES) or any salts or esters thereof.

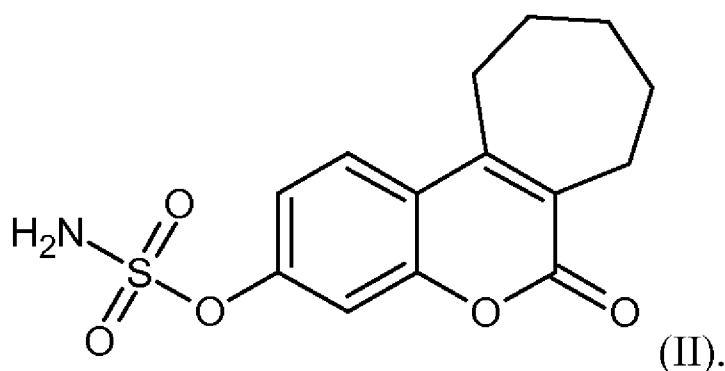
20 4. The composition for use according to claim 2, wherein the composition comprises epitestosterone sulfate (ES) or any salts or esters thereof.

5. The composition for use according to claim 1, wherein the composition comprises the sulfatase inhibitor STX64 of Formula (II):



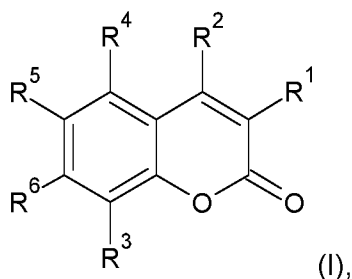
or any salts thereof.

6. The composition for use according to claim 2, wherein the composition
5 comprises the sulfatase inhibitor STX64 of Formula (II):



or any salts thereof.

7. The composition for use according to any of claims 1 to 6, wherein the
10 composition is a pharmaceutical composition, nutraceutical composition or dietary supplement.
8. A non-therapeutic method of improving Age-related cognitive impairment in a
human subject, the method comprising the steps of providing a compound
15 selected from the list consisting of at least one of epitestosterone sulfate (ES),
or any salts or esters thereof, and a compound capable of inhibiting the steroid
sulfatase of Formula (I):



wherein:

(c) R₁-R₆ are independently selected from hydrogen, halogen (fluorine, chlorine, bromine, iodine or astatine), hydroxyl, sulfamate (OSO₂NH₂), alkyl and salts thereof;

5 (d) at least one of R₁-R₆ is a sulfamate group; and two or more of R₁-R₆ are linked together to form an additional cyclic structure; and

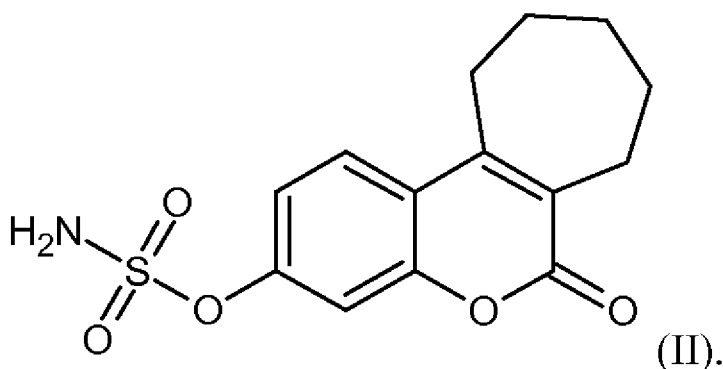
administering an effective amount of the compound to the subject.

9. The non-therapeutic method of claim 8, wherein the method is for improving Age-Associated Memory Impairment (AAMI).

10 10. The composition for use according to claim 8, wherein the composition comprises epitestosterone sulfate (ES) or any salts or esters thereof.

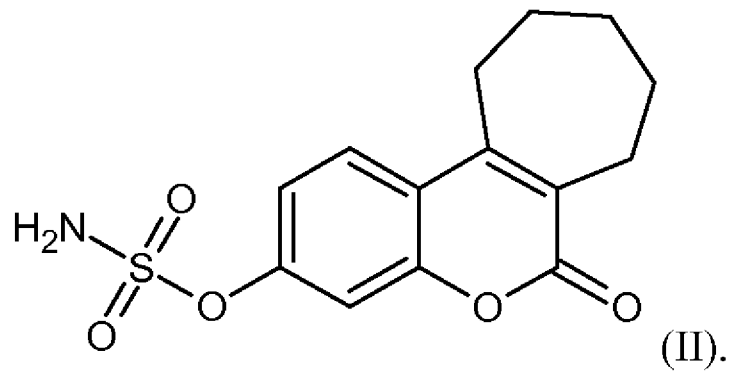
11. The composition for use according to claim 9, wherein the composition comprises epitestosterone sulfate (ES) or any salts or esters thereof.

12. The composition for use according to claim 8, wherein the composition
15 comprises the sulfatase inhibitor STX64 of Formula (II):



or any salts thereof.

20 13. The composition for use according to claim 9, wherein the composition comprises the sulfatase inhibitor STX64 of Formula (II):



or any salts thereof.

- 5 14. The composition for use according to any of claims 8 to 13, wherein the composition is a nutraceutical composition or dietary supplement.

FIGURES

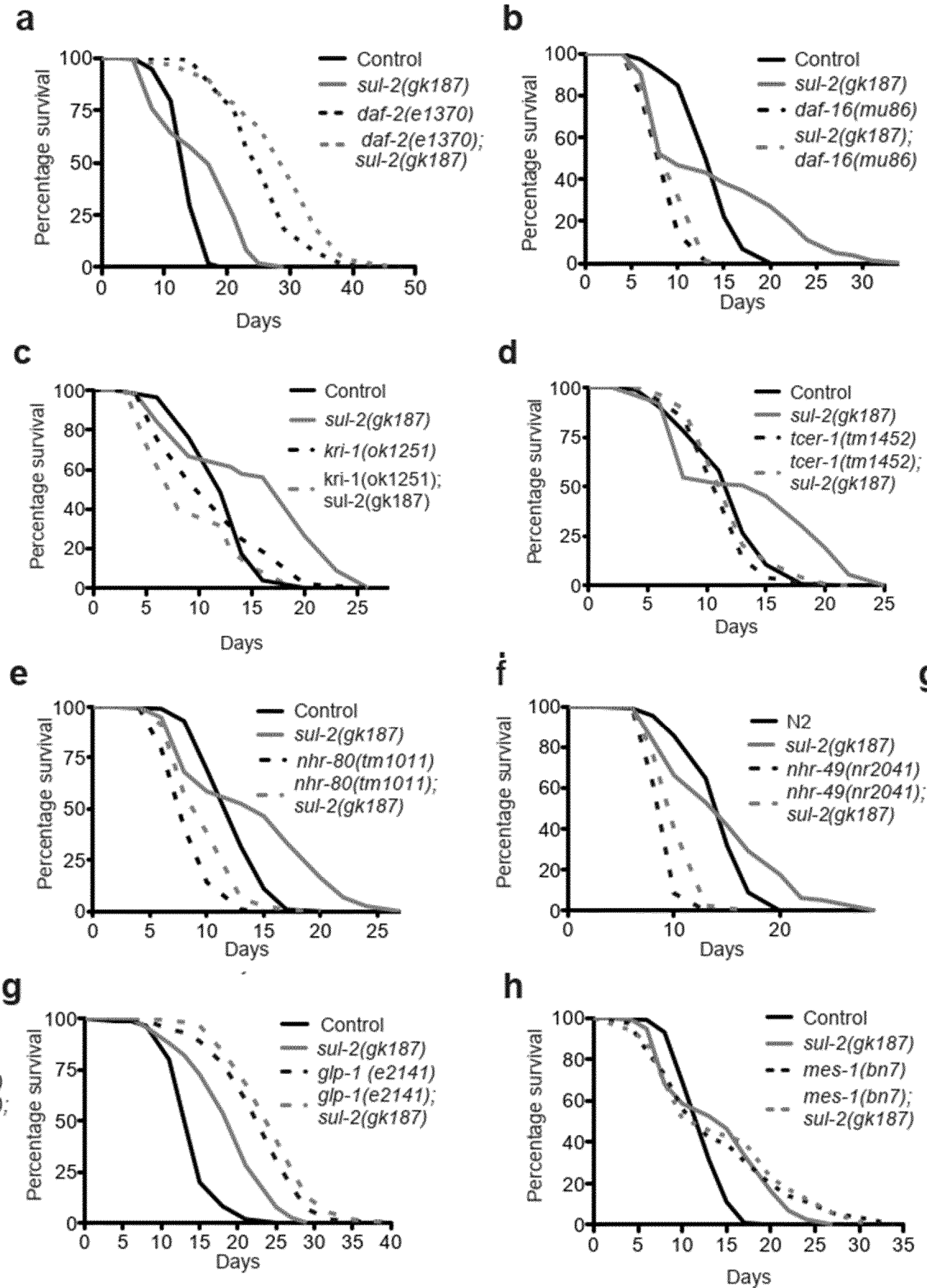


Fig. 2

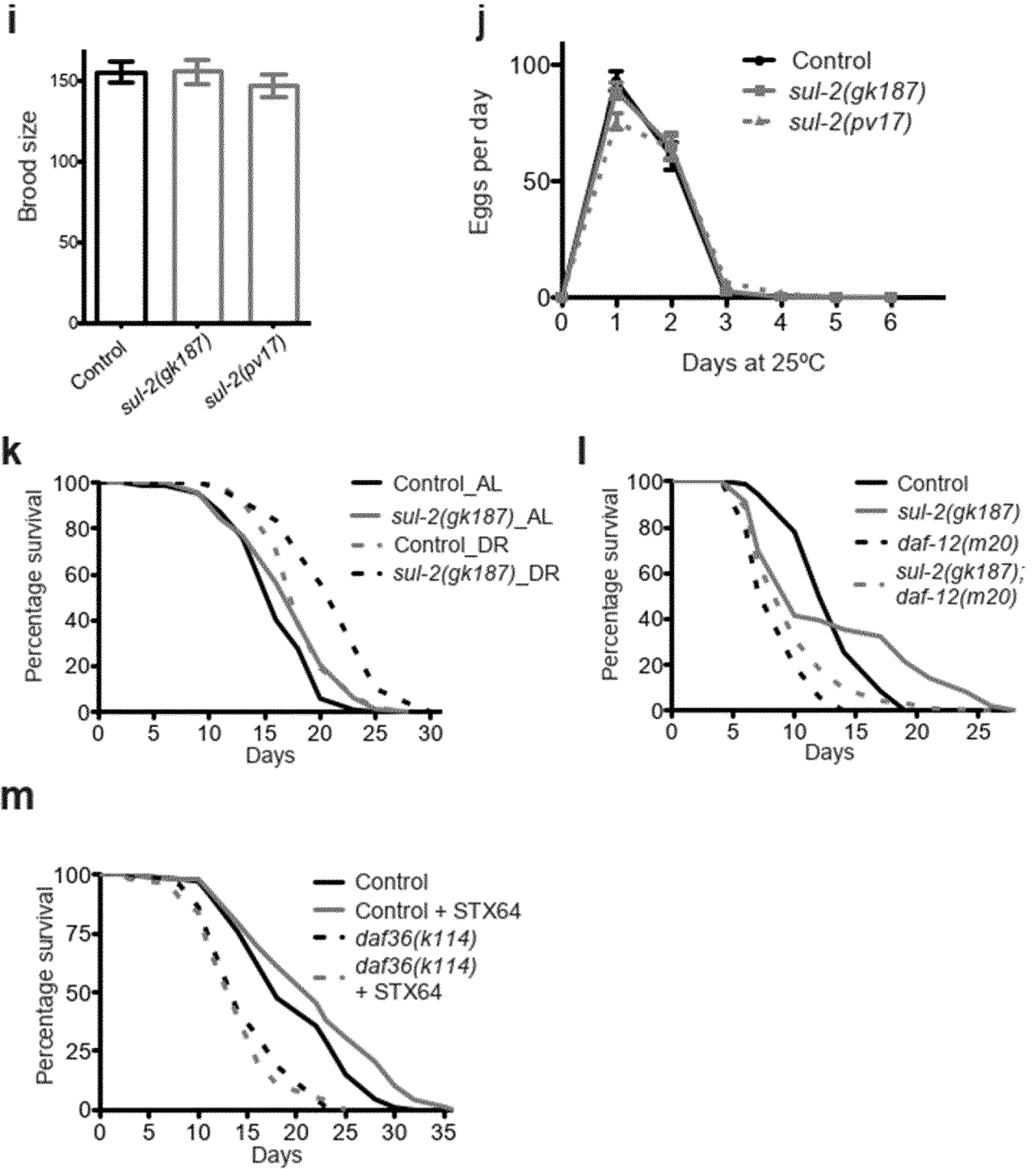
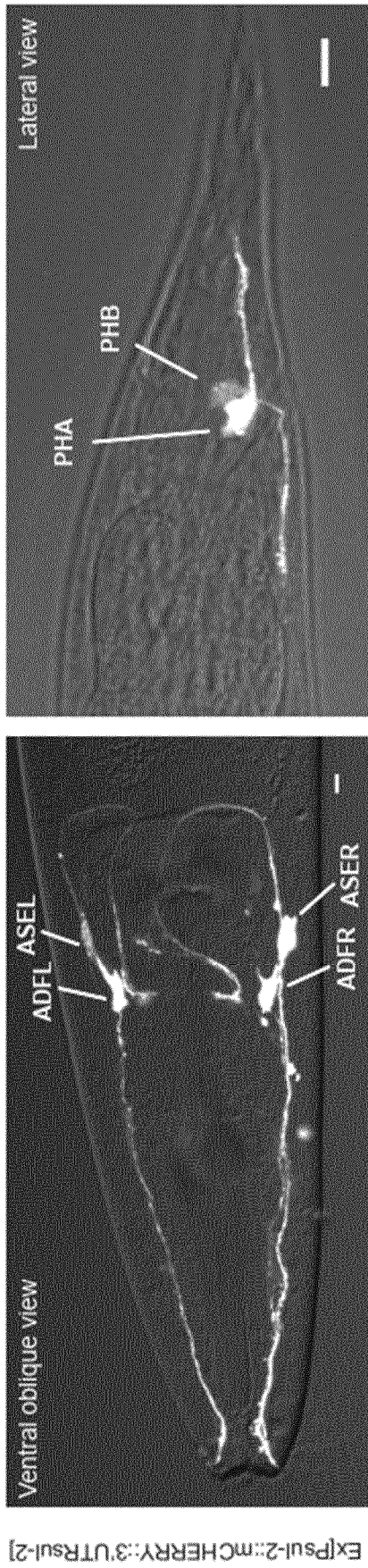


Fig. 2 (cont.)



U

Fig. 2 (cont.)

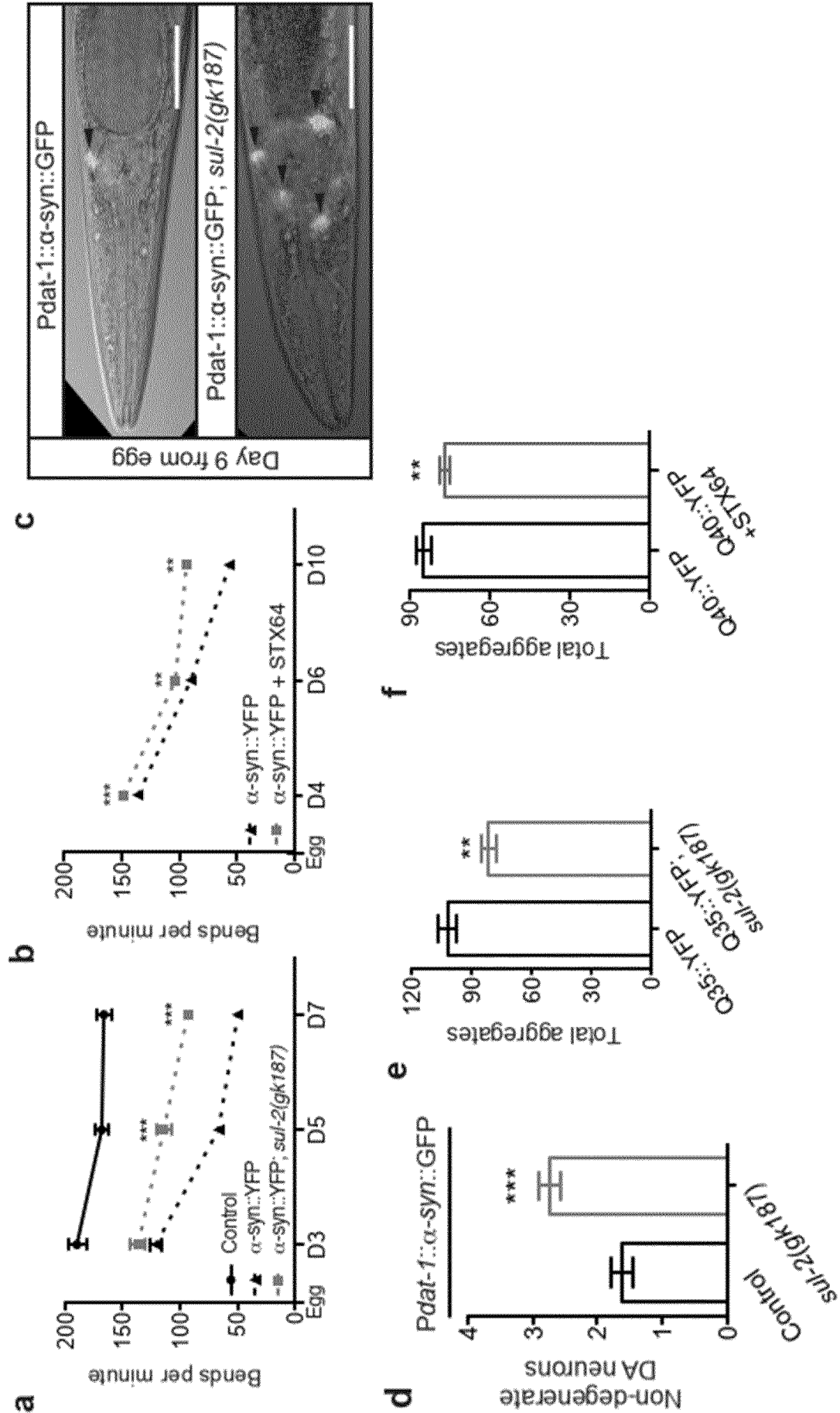


Fig. 3

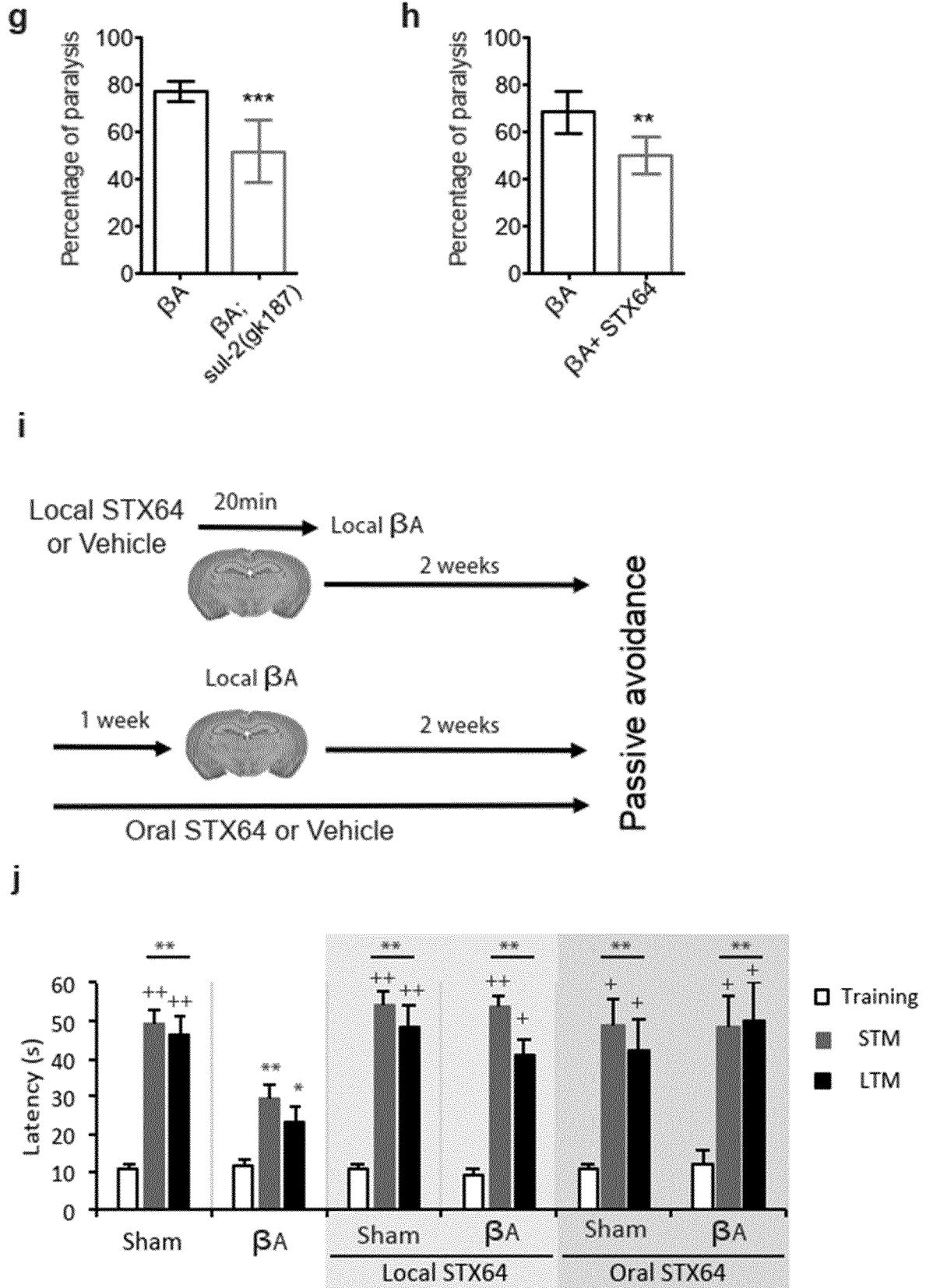


Fig. 3 (cont.)

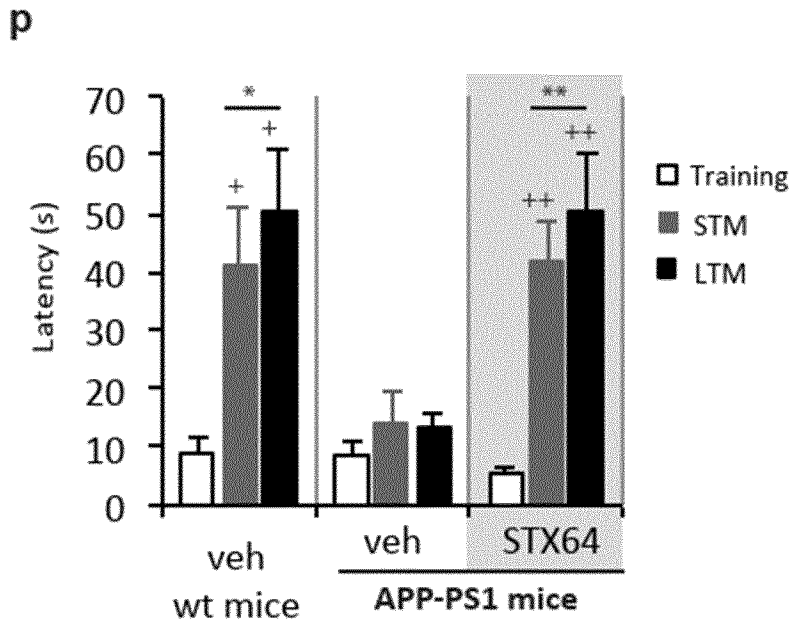
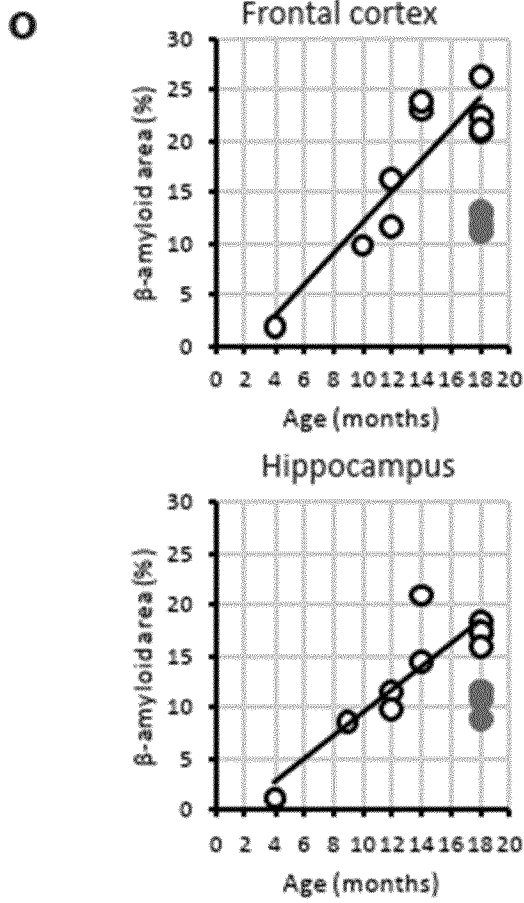


Fig. 3 (cont.)

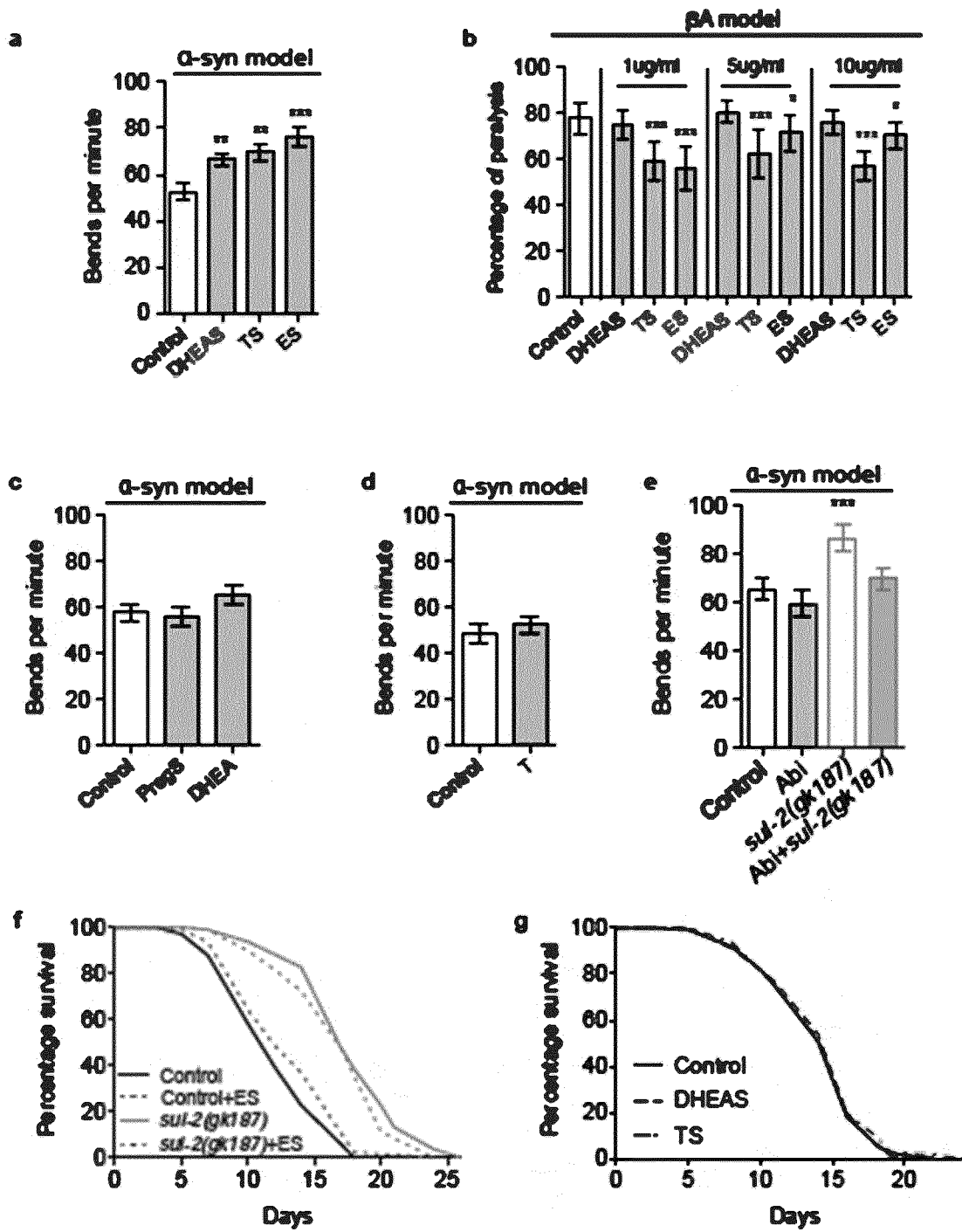


Fig. 4

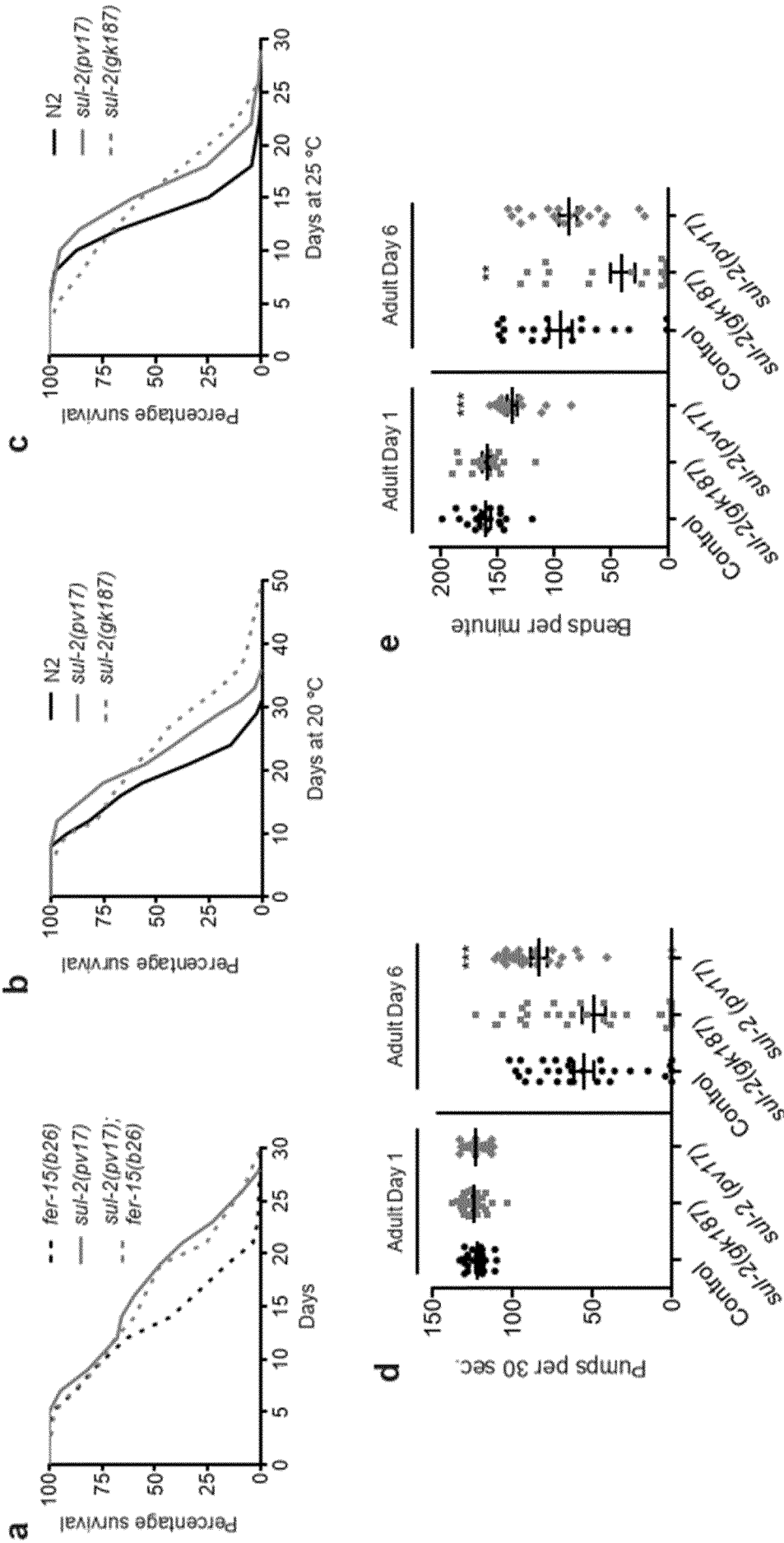


Fig. 5

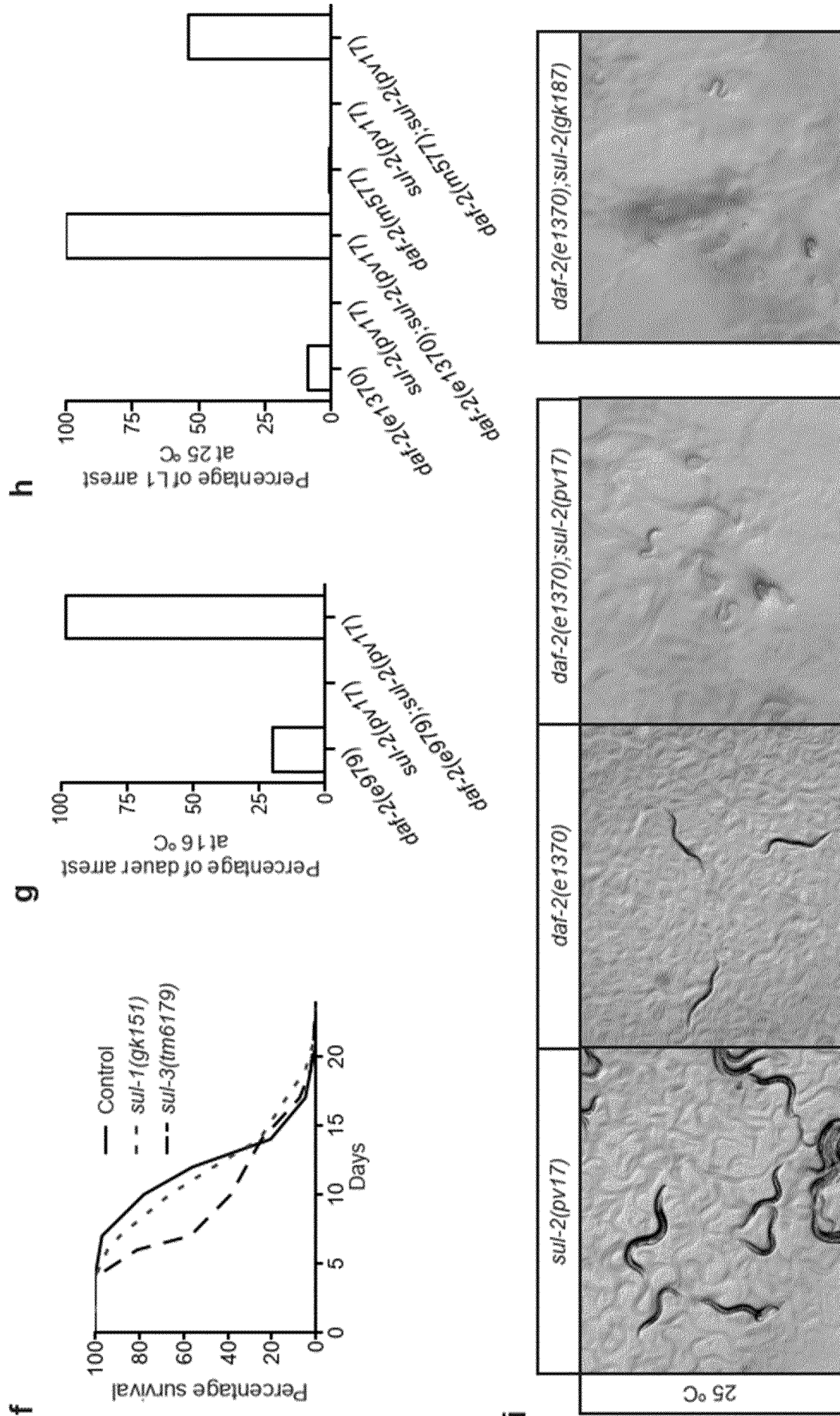


Fig. 5 (cont.)

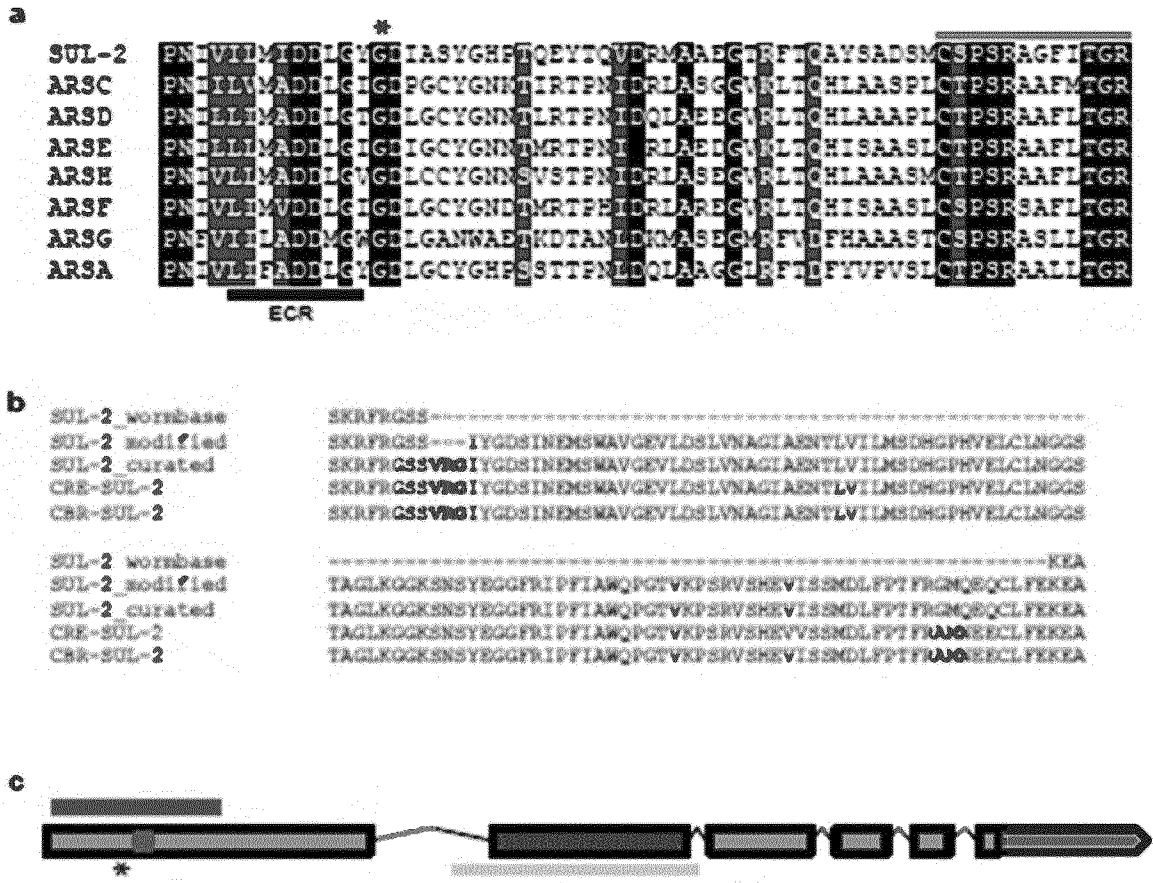


Fig. 6

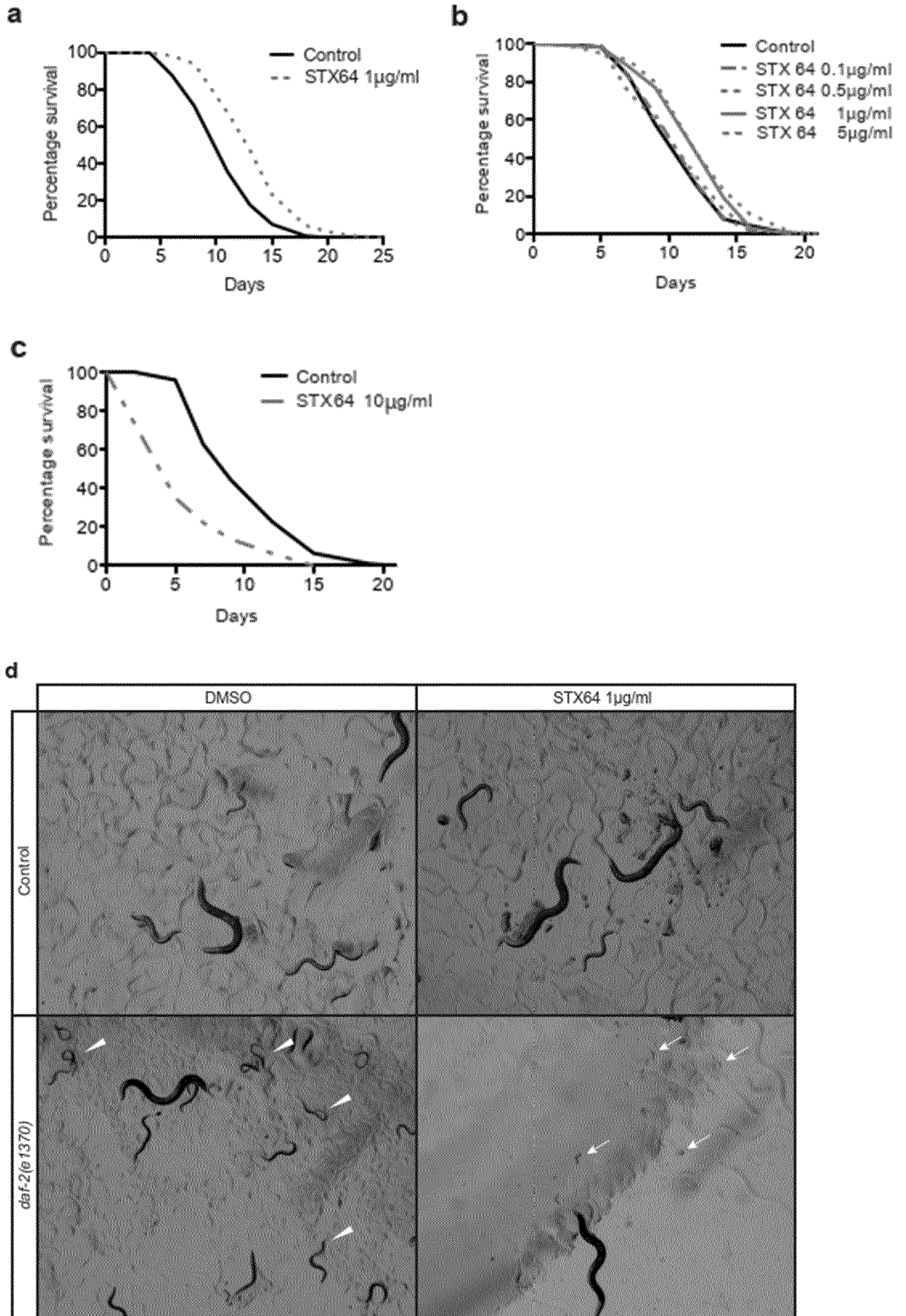


Fig. 7

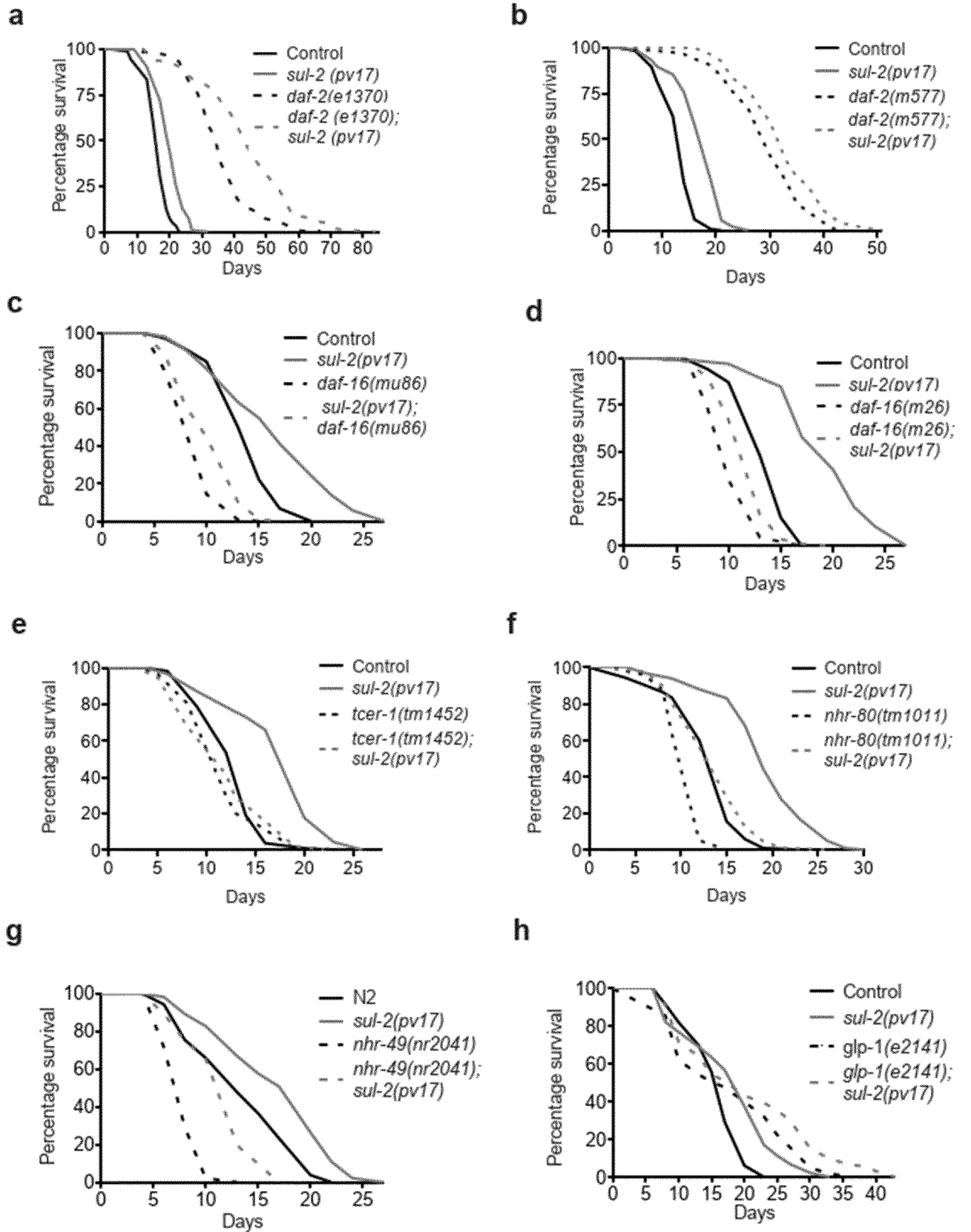


Fig. 8

i

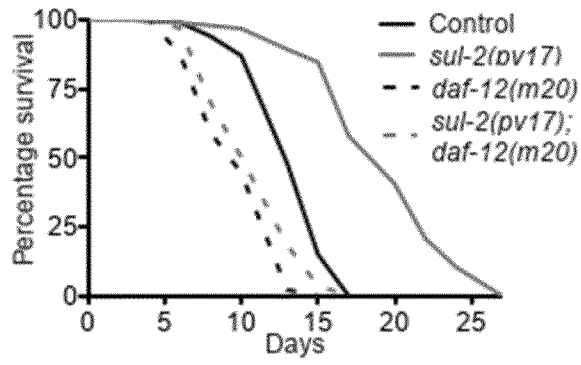


Fig. 8 (cont.)

a

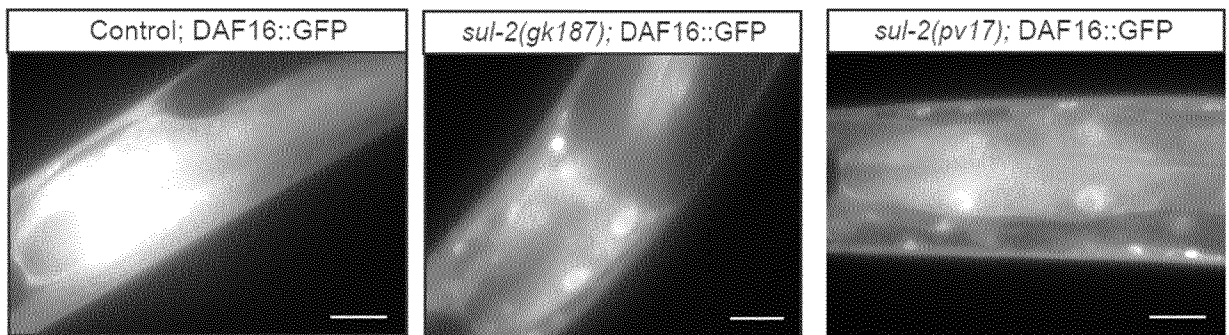


Fig. 9

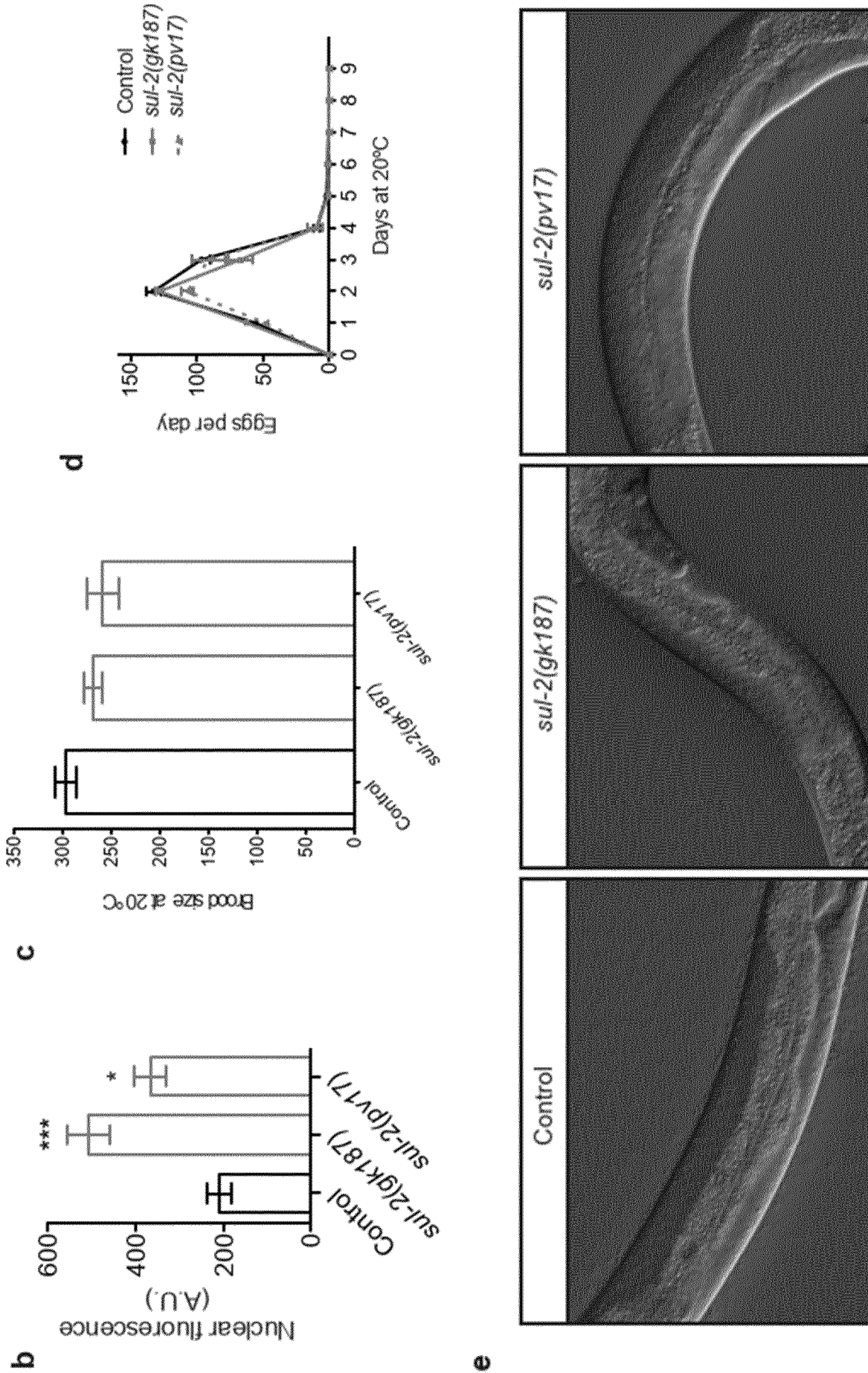


Fig. 9 (cont.)

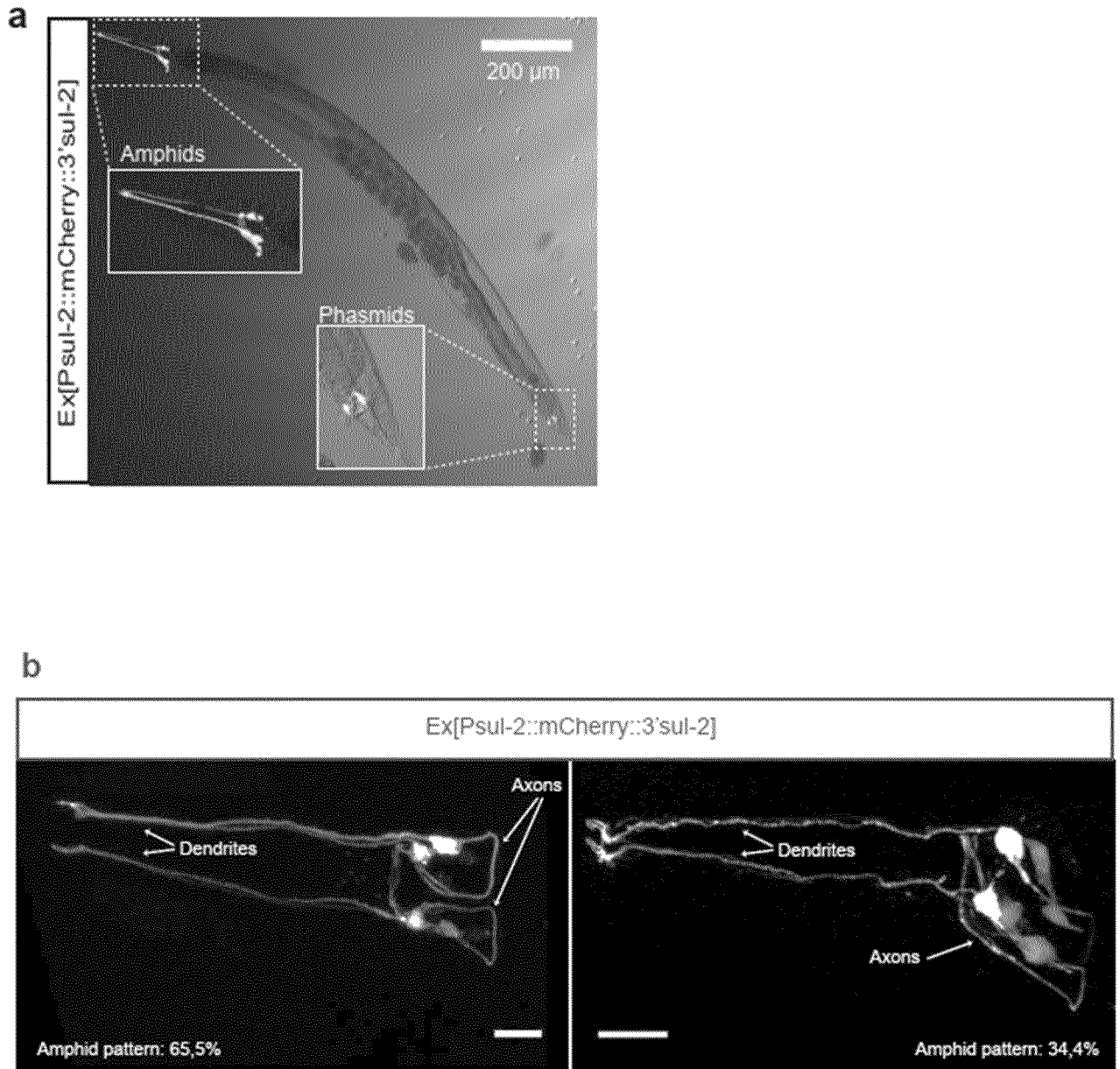


Fig. 10

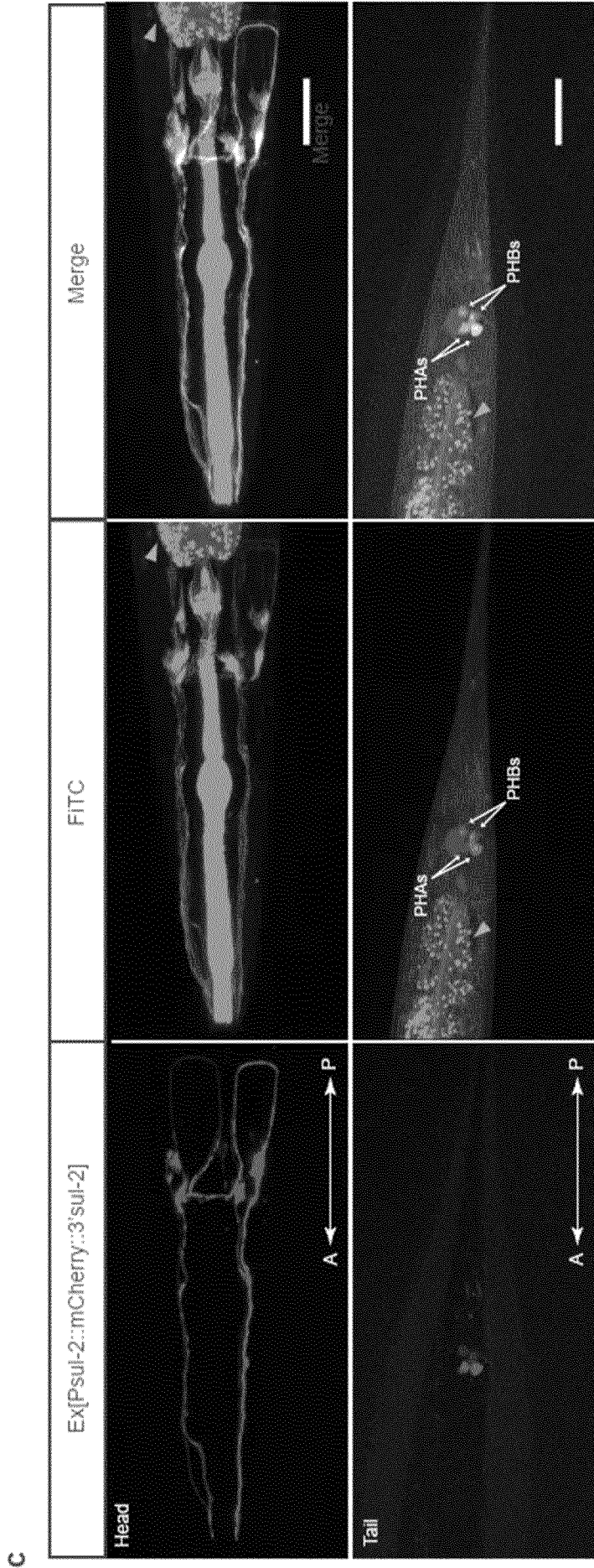


Fig. 10 (cont.)

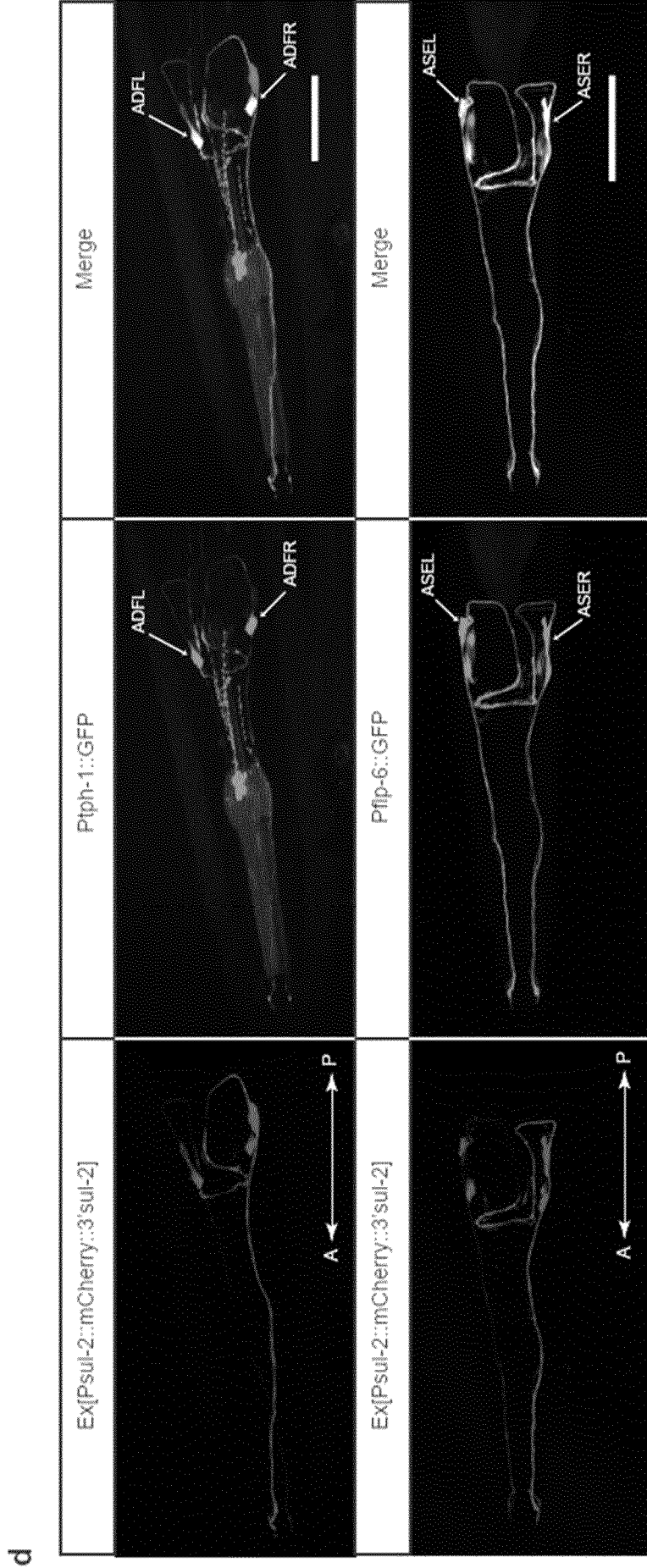


Fig. 10 (cont.)

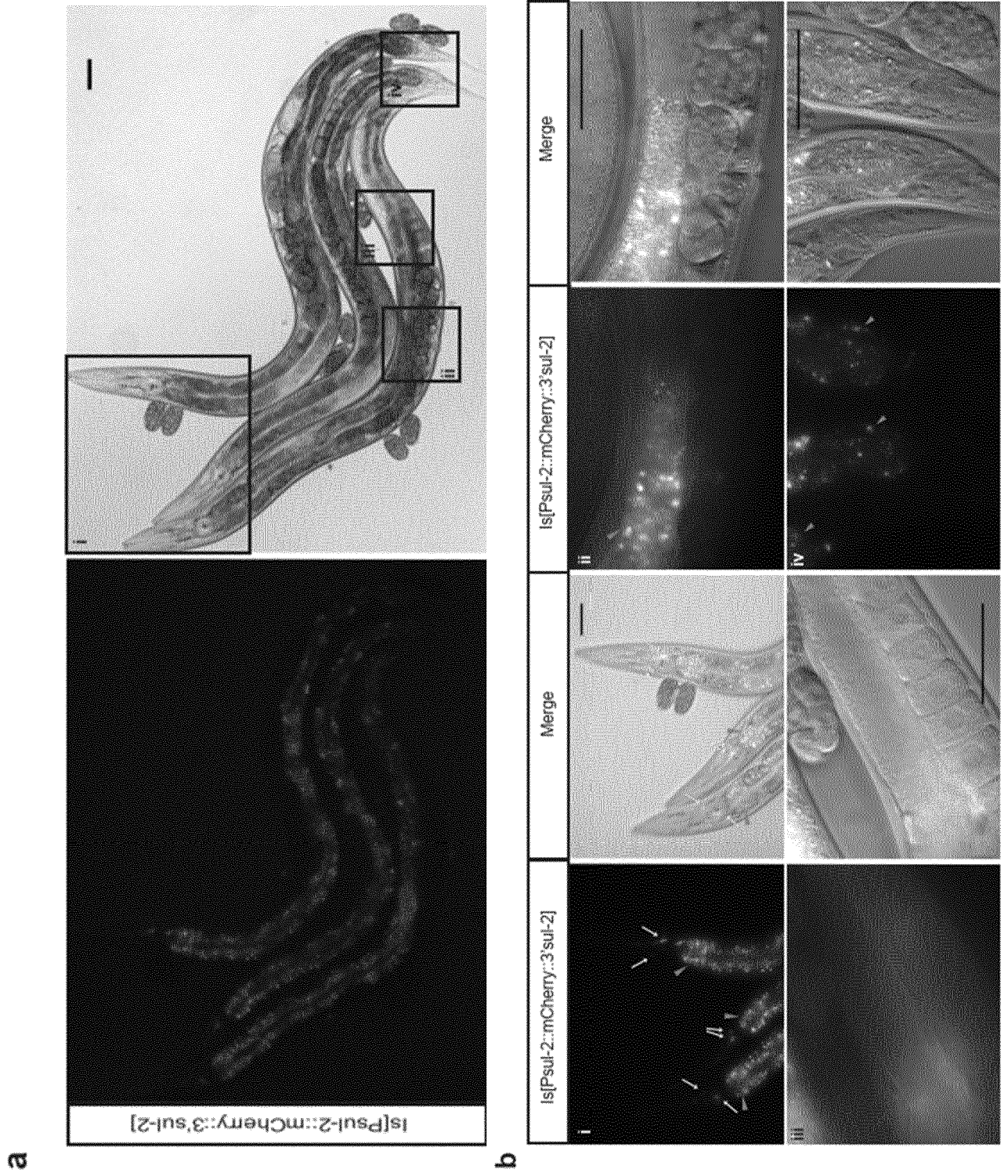


Fig. 11

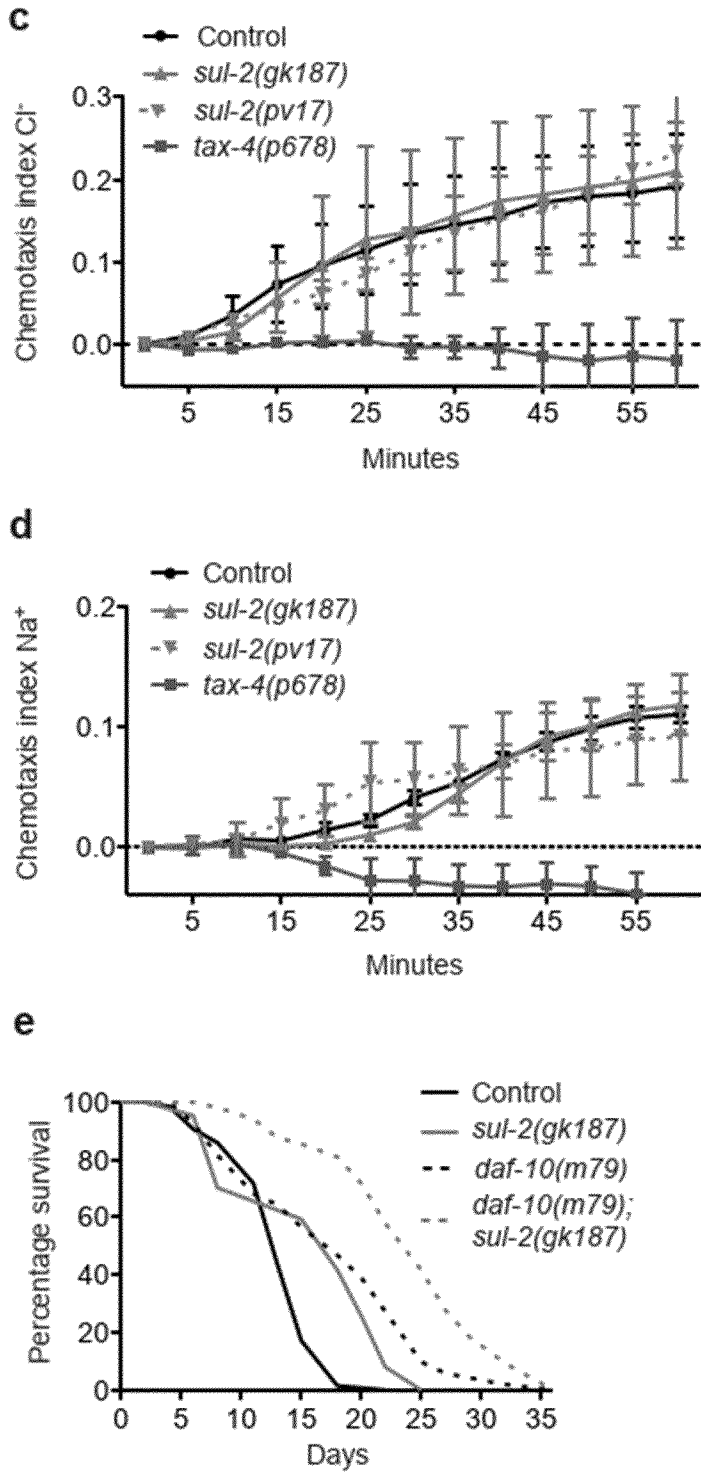


Fig. 11 (cont.)

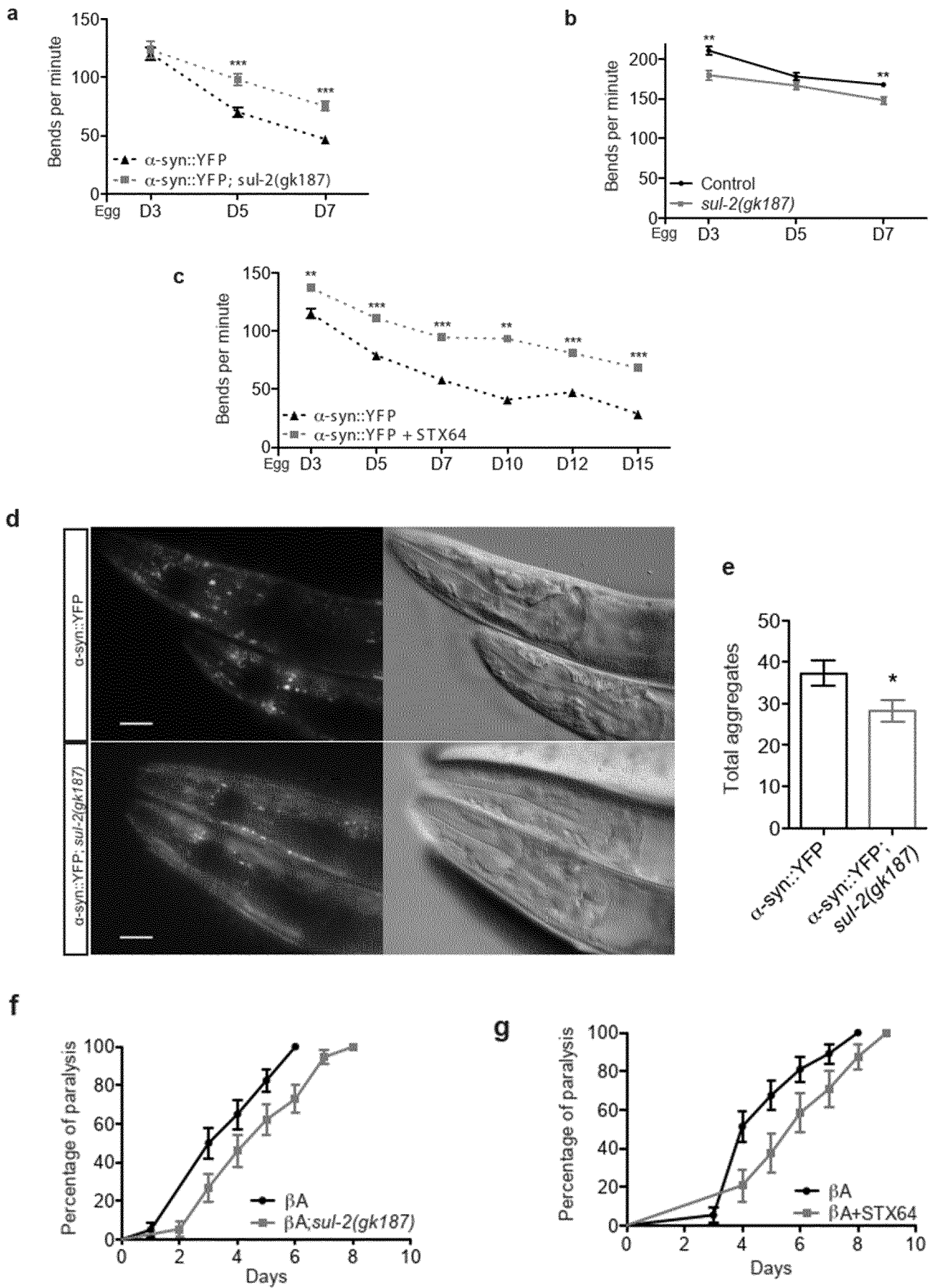


Fig. 12

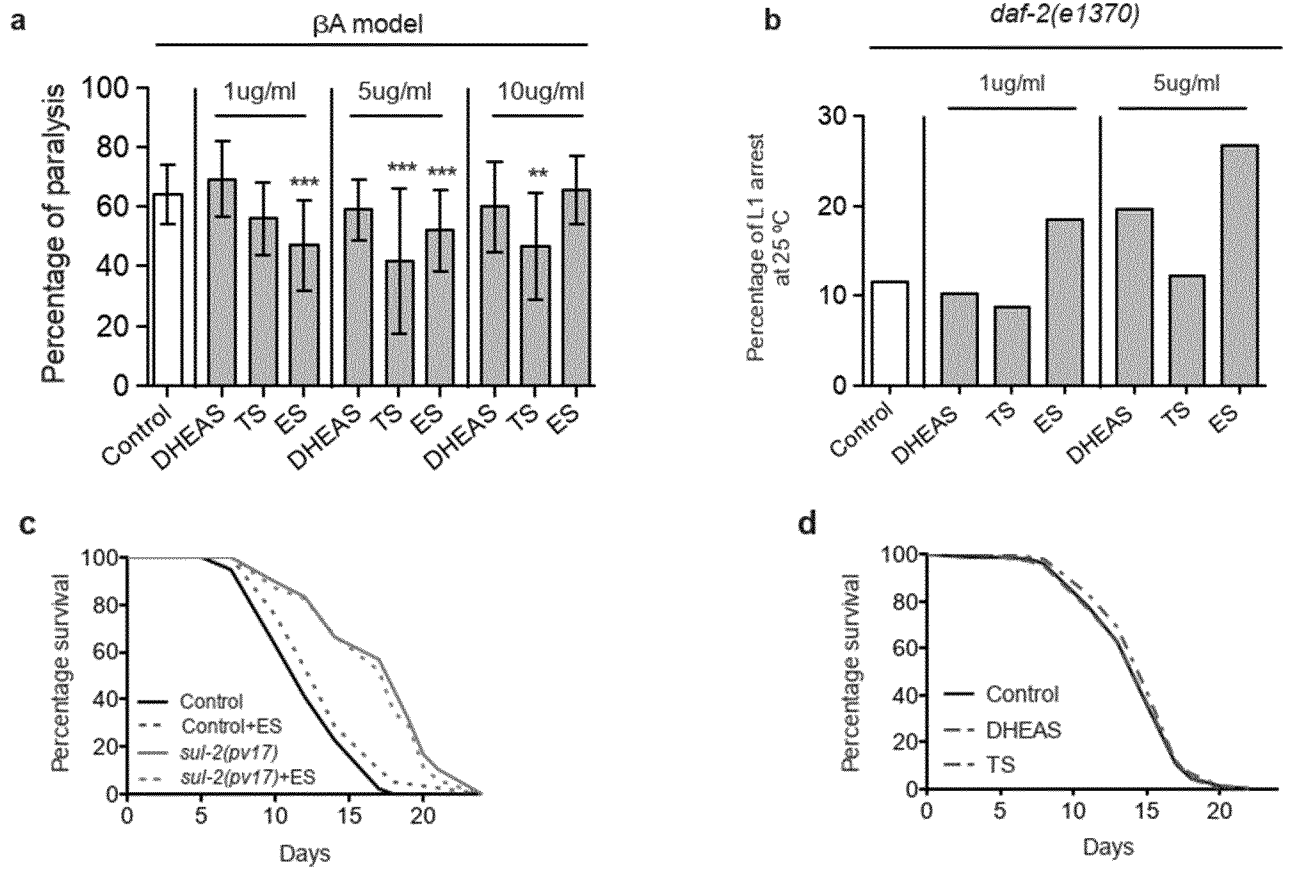


Fig. 13

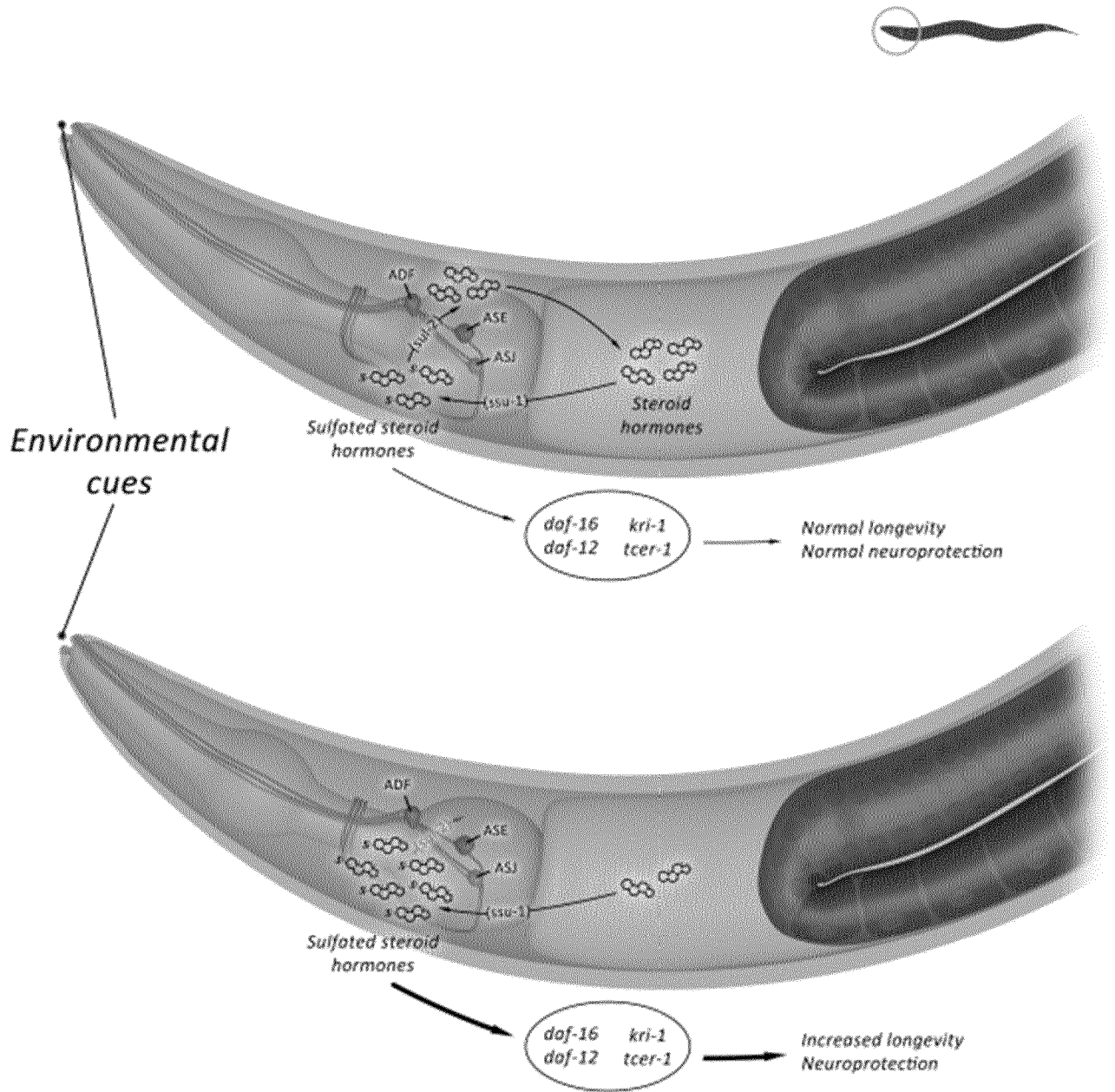
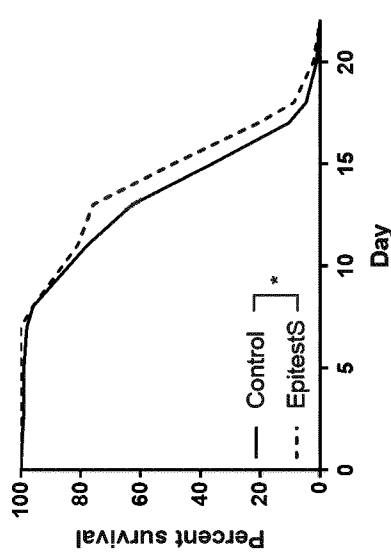
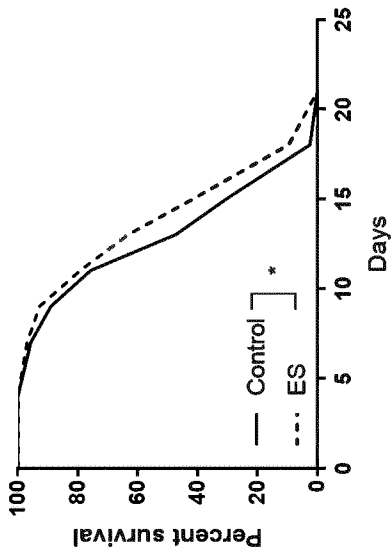
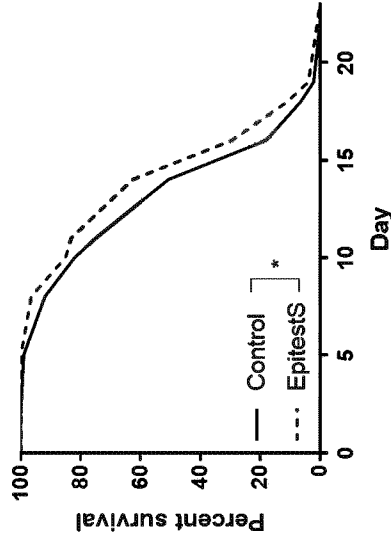
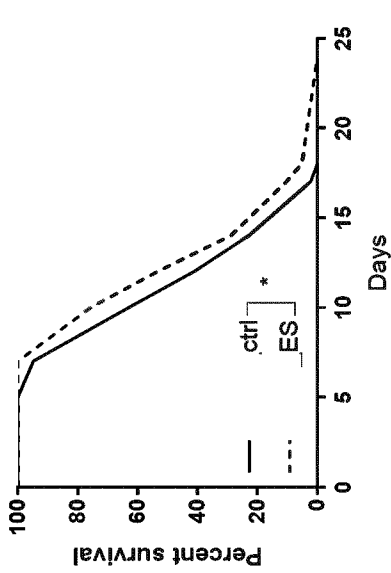
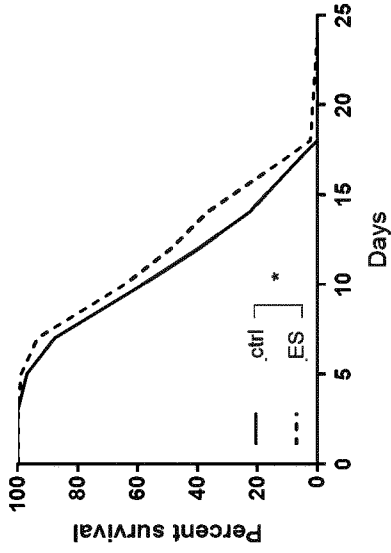


Fig. 14



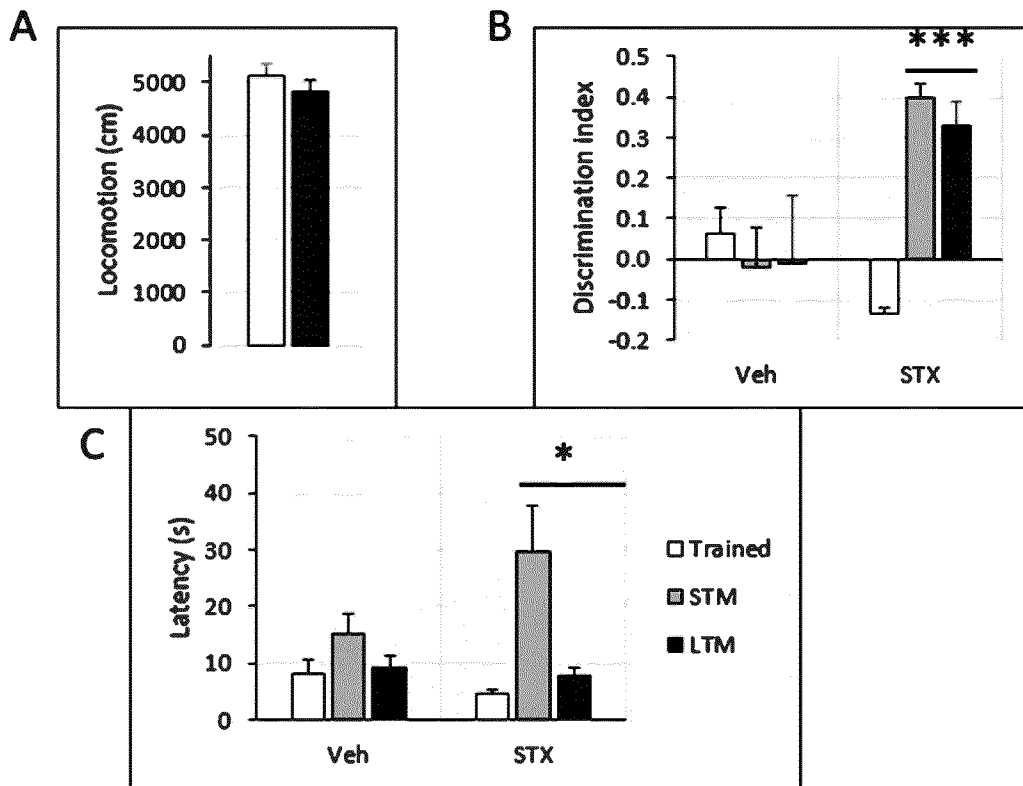


Fig. 16

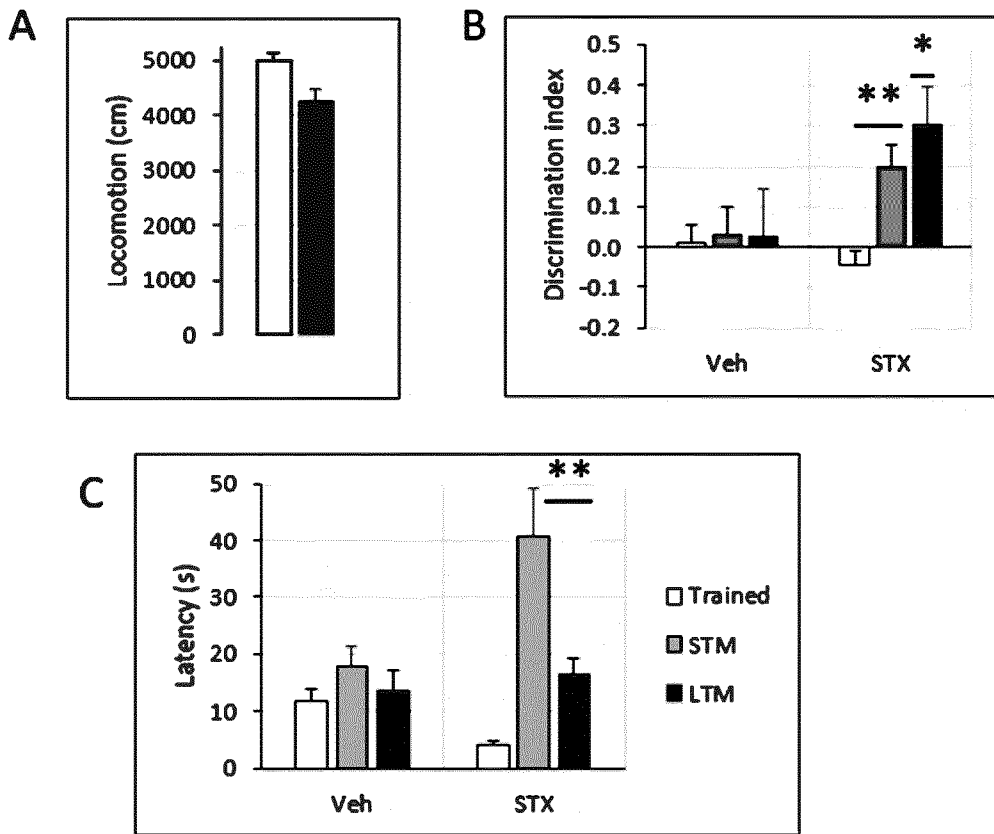


Fig. 17

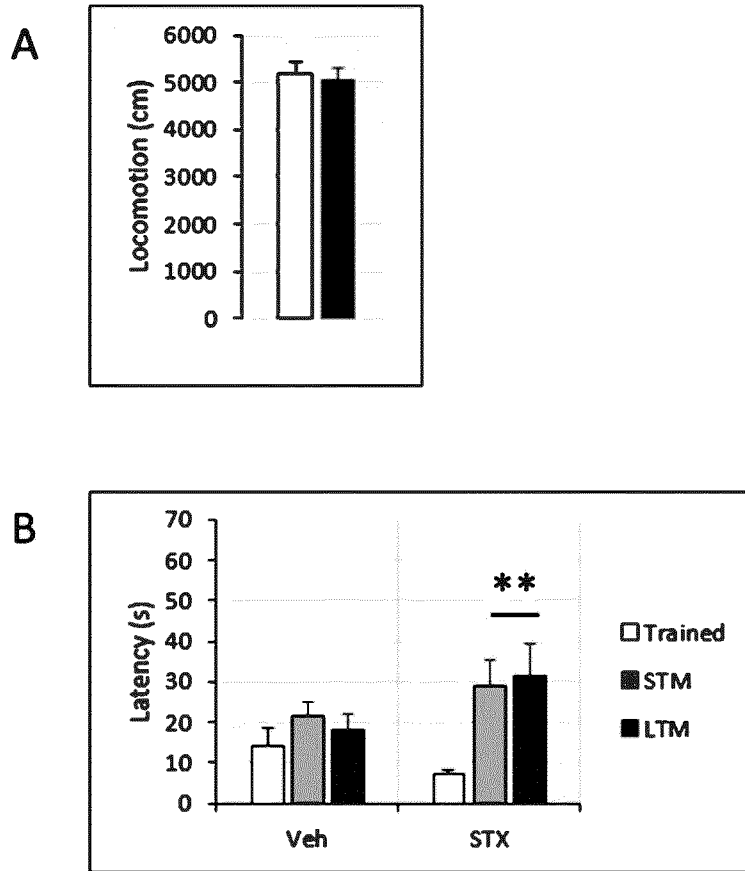


Fig. 18

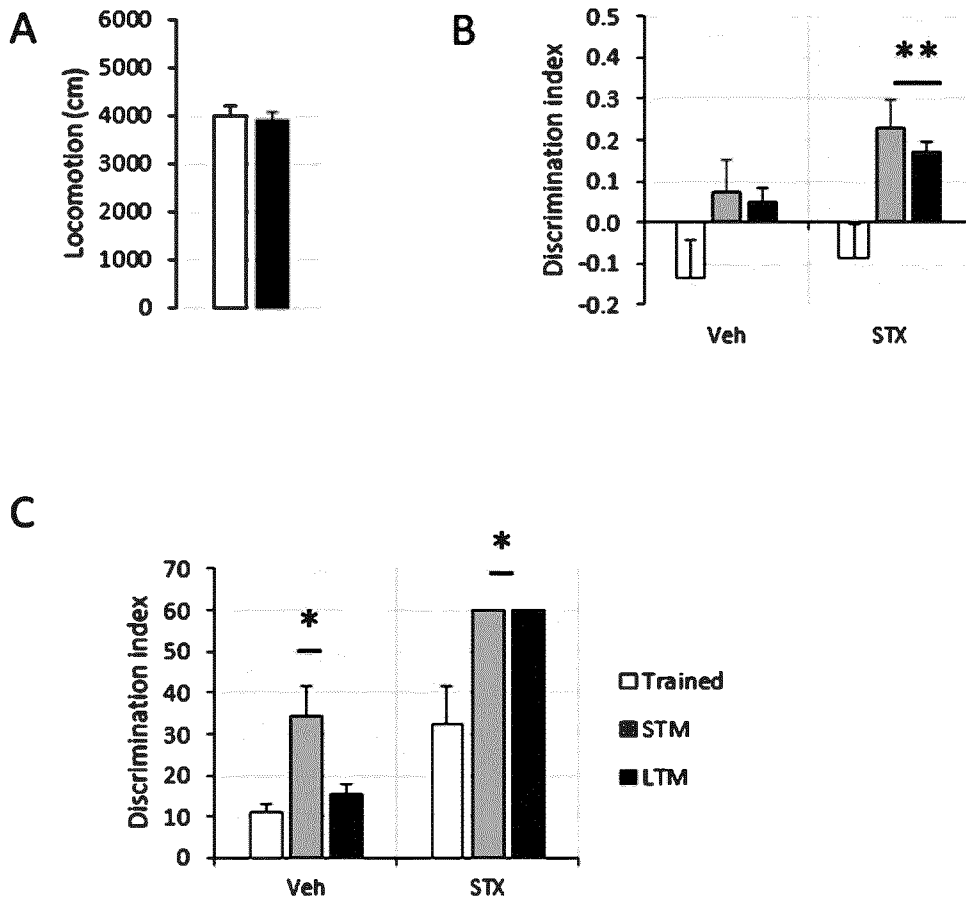


Fig. 19

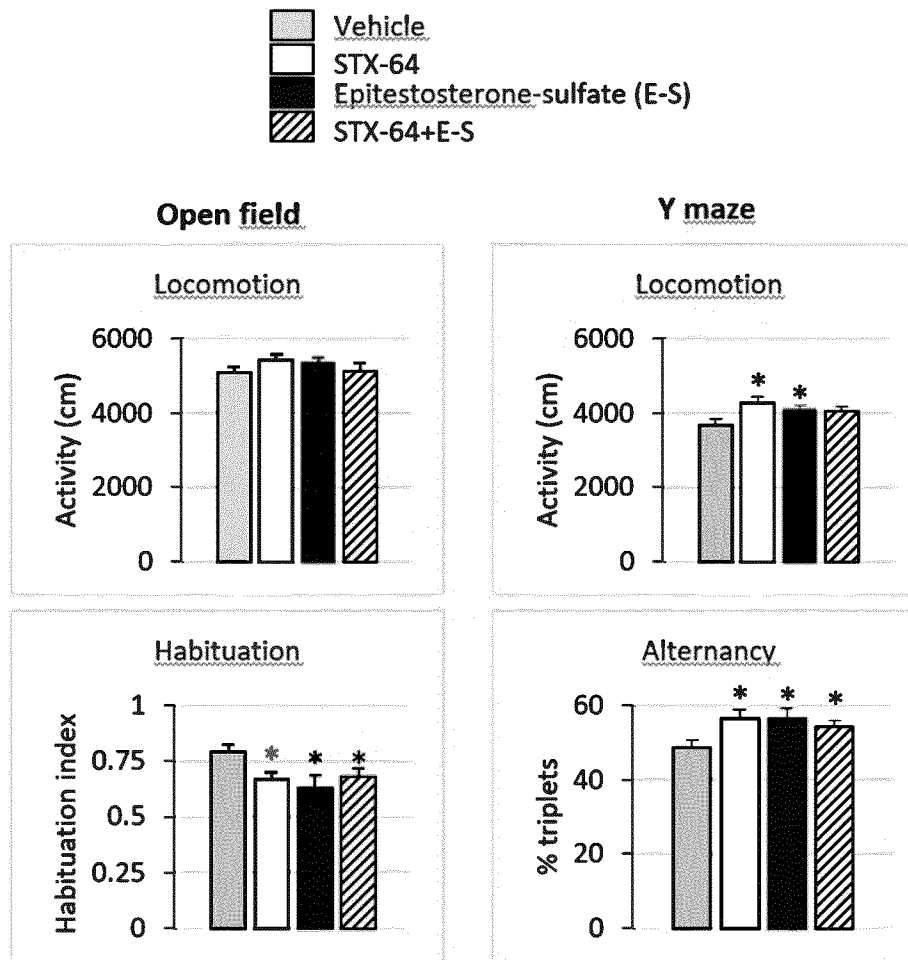


Fig. 20



Learning and memory

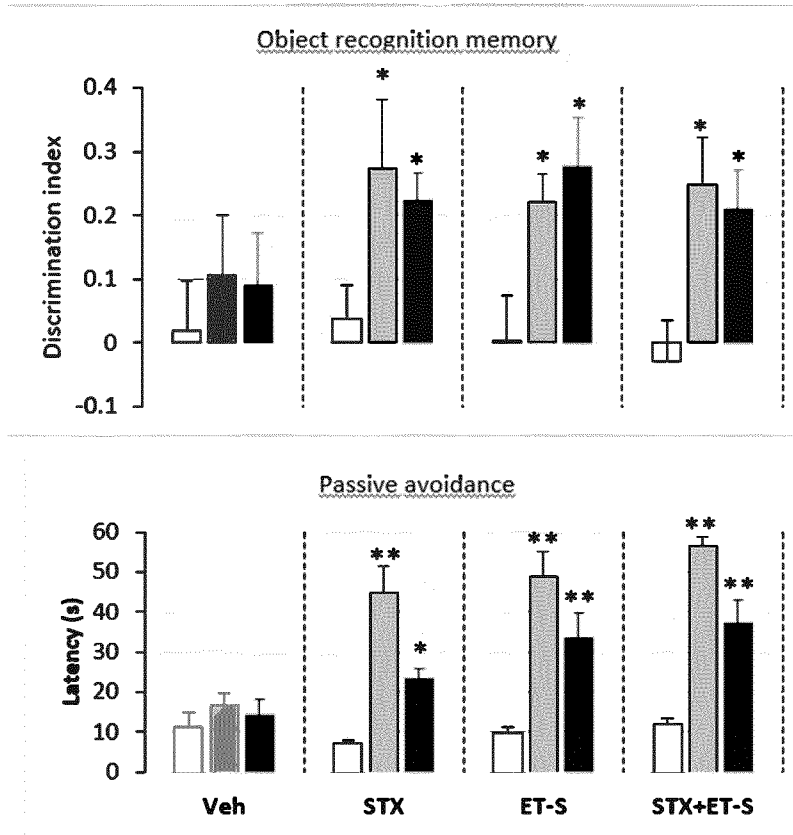


Fig. 21