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Behavioral decay in aging male *C. elegans* correlates with increased cell excitability

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Abstract

Deteriorative changes in behavioral functions are natural processes that accompany aging. In advanced aged *C. elegans* nematodes, gross decline in general behaviors, such as locomotion and feeding, is correlated with degeneration of muscle structure and contractile function. In this study, we characterized the age-related changes in *C. elegans* male mating behavior to determine possible causes that ultimately lead to age-related muscle frailty. Unlike the kinetics of general behavioral decline, we found that mating behavior deteriorates early in adulthood, with no obvious muscle fiber disorganization or sperm dysfunction. Through direct mating behavior observations, Ca^{2+} imaging and pharmacological tests, we found that the muscular components used for mating become more excitable as the males age. Interestingly, manipulating either the expression of AChR genes or dietary-mediated *ether-a-go-go* family K^+ channel function can reduce the muscle excitability of older males and concurrently improve mating behavior, suggesting a correlation between these biological processes.

Keywords

aging; mating behavior; EAG/ERG K^+ channels; acetylcholine receptors; caloric deprivation

1. Introduction

Adult longevity is under genetic regulation and several molecular pathways present in species ranging from *C. elegans* to mice are implicated in prolonging or shortening the organismal lifespan (Kenyon et al., 1993, Guarente and Kenyon, 2000, Kenyon, 2005). The decline of neuromuscular function that accompanies aging contributes to decreased efficiency in executing behavior outputs (Herndon et al., 2002, Huang et al., 2004). A relatively well-studied model for behavioral decline is *C. elegans* locomotion behavior (Huang et al., 2004, Murakami et al., 2008, Iwasa et al., 2010).

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Conflict of interest

All authors disclose no conflict of interest.

Disclosure statement

No animal and human subjects were used in this study.

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On a solid culture surface, *C. elegans* exhibits sinusoidal body movement by alternating the contraction and relaxation of the dorsal and ventral body wall muscles. The pattern of body wall muscle contraction is produced by interactions between cholinergic excitatory and GABAergic inhibitory motor neurons (McIntire et al., 1993). A young adult animal moves continuously and exhibits a well-coordinated, sinusoidal pattern. As it ages, the body movements become progressively less continuous and coordinated (Herndon et al., 2002, Huang et al., 2004). In addition to ACh and GABA, the neurotransmitters dopamine and serotonin regulate basal slowing of non-starved animals in response to food and enhanced slowing when the animal encounters food after starvation, respectively (Sawin et al., 2000, Chase et al., 2004). During aging, the basal slowing response is increased so that the difference between the two slowing responses is diminished (Murakami et al., 2008). In a liquid environment, *C. elegans* displays an alternative thrashing/swimming locomotory pattern (Pierce-Shimomura et al., 2008). As the animals age, the frequency of body wall muscle contractions during swimming is also decreased (Schreiber et al., 2010).

Motor decline during aging is used in *C. elegans* to identify molecular pathways that extend lifespan when perturbed and subsequently delay the deterioration of locomotion behavior. For instance, reducing insulin-like signaling by limiting the activity of the insulin-like receptor, *daf-2*, can prominently extend lifespan and delay locomotory deterioration (Huang et al., 2004). By performing RNAi screens for aged animals with improved locomotory vigor in liquid, researchers have identified novel molecules that modify the rate of behavioral aging. Down-regulation of EGF signaling, through mutation of the two negative regulators, HPA-1 and HPA-2, or knocking down the level of RAS-related Rag GTPase RAGA-1, can delay the age-related deterioration of swimming ability (Iwasa et al., 2010, Schreiber et al., 2010). These studies identified molecules that attenuate age-related locomotory decline. However, much less is known about the cellular mechanisms that result in this decline in the first place.

Research has shown that locomotory decline in *C. elegans* is associated with sarcopenia in body wall muscles, characterized by deformed nuclei and muscle fiber loss and disorganization (Glenn et al., 2004). This might be true for the decline of behavior at the advanced age. However, the behavior begins to decline at an early phase of aging, when there is no significant loss of muscle fibers (Glenn et al., 2004, Murakami et al., 2008) or significant neuronal morphology change (Herndon et al., 2002, Tank et al., 2011). Manipulation of serotonin signaling suppresses the increased basal slowing response at adult day 4 prior to the timing of sarcopenia onset (Glenn et al., 2004, Murakami et al., 2008). This raises the possibility that the physiological state, which determines the timing and coordination of muscle contraction, might be suboptimal prior to the onset of significant muscle contractile apparatus damage. However, locomotion behavior might be too robust to uncover subtle physiological changes that occur prior to the deterioration of muscle structure.

Unlike general locomotion behavior, *C. elegans* male mating requires precise coordination between the male genital muscles, in conjunction with the locomotion neuromuscular circuit, to be executed successfully. Mating behavior can be divided into the following steps: response, backing, turning, vulva location, spicule insertion and sperm transfer (Liu and Sternberg, 1995). The backing and vulva location behaviors are initiated and executed by tail-located sensory neurons. When the male's fan-shaped tail contacts the cuticle of the hermaphrodite, he uses the locomotory neuromuscular circuit to move backwards, scanning along the hermaphrodite cuticle for the vulva. If the male fails to locate the vulva on one side, he will make a turn at the end of the hermaphrodite and continue backwards locomotion. Once he encounters the vulva, the male will contract sex-specific muscles located in his tail, in order to press against the vulval lips. He then repetitively prods the

vulva with his spicules, in an attempt to breach the tight vulval slit. This is achieved through rapid contractions of the protractor muscles, which are attached to his spicules. Once his spicules partially penetrate, the protractor muscles fully contract allowing complete spicule insertion. Sperm transfer occurs following full spicule penetration (Barr and Garcia, 2006, Liu et al., 2011). Relative to other general *C. elegans* behaviors, the complexity of male mating might render it less robust as the male ages and the physiology of neuromuscular circuits change. The cellular and molecular mechanisms that regulate the spicule insertion step of mating are well described, allowing one to monitor the physiology and functional changes in the circuit as the male proceeds through adulthood (Gruninger et al., 2006, LeBoeuf et al., 2007, Gruninger et al., 2008, LeBoeuf et al., 2011, Liu et al., 2011).

In this study, using *C. elegans* male mating as a model, we determined that mating potency declines significantly at an early phase (the third day of adulthood) of aging, when no obvious sarcopenia or sperm deficiency is detected. We observed that 3-day-old males can sense the hermaphrodite as well as young males, though backing behavior is erratic: the males display much more spicule prodding and complete protraction of their spicules at non-vulval regions of the hermaphrodite. Additionally, when aged males penetrate a hermaphrodite's vulva, a proportion of them withdraw their spicules prior to or during sperm transfer. We used *in-vivo* calcium imaging and pharmacological tests to determine that during adulthood, male sex muscle excitability is increased. We were able to prolong mating potency by reducing cell excitability through genetic mutations of ACh receptors genes or through transient starvation. In conclusion, we hypothesize that the earliest signs of age-related behavioral decline is correlated with an increase of excitability in the sex muscles. The increased excitability leads to uncoordinated contractions of the sex muscles during mating, which in turn reduces successful copulation.

2. Materials and methods

2.1. Strains and medium

Nematodes were grown at 20°C on NGM agar plates seeded with *Escherichia coli* OP50, except for the *pha-1(e2123)* strain, which was maintained at 15°C (Schnabel and Schnabel, 1990). The alleles used in this study were: *lite-1(ce314)* (Edwards et al., 2008) on LGX; *unc-29(e193)* on LGI (Lewis et al., 1980); *pha-1(e2123)* (Schnabel and Schnabel, 1990), *unc-64(e240)* (Brenner, 1974) and *unc-103(n1213)* (Park and Horvitz, 1986) on LGIII; *him-5(e1490)* (Hodgkin et al., 1979), *acr-18(ok1258)* generated by the *C. elegans* Gene Knockout Consortium, *gar-3(gk305)* (Liu et al., 2007), *acr-16(ok789)* generated by the *C. elegans* Gene Knockout Consortium and *egl-2(rg4)* (LeBoeuf et al., 2007) on LG V.

2.2. Mating potency assay

Males were isolated from non-crowded plates, either at the late L4 stage (when cells in the male tail spike have completely migrated anteriorly) or for the cycloheximide assays (see below), after they newly crawled out of their L4 molt. They were kept individually or in groups of 20–30 on 1–2 cm diameter lawns of bacteria. ~30% and ~70% of males that contain the *unc-103(n1213)* deletion (referred to as *unc-103(0)* in the results section) or both the *unc-103(n1213)* and *egl-2(rg4)* deletions (the *egl-2(rg4)* deletion is referred to as *egl-2(0)* in the results section), respectively, display the constitutive protracted spicule phenotype (LeBoeuf et al., 2007, LeBoeuf et al., 2011). Males with constitutive protracted spicules cannot sire progeny, therefore we used animals that did not display the abnormal behavior for the mating potency assays. L4 *pha-1* hermaphrodites were isolated and grown at 20°C one day before the mating potency assays were conducted. A 1-day-old *pha-1* hermaphrodite and an adult male were picked to 5 mm diameter lawns (10 µl of *E. coli*, grown overnight in LB media at 37°C without aeration, was spotted onto an 3.5 mm NGM

agar plate to make a 5 mm diameter lawn) to increase the chance of mating. 2–3 days later, we scored the male as sexually potent if the plate contained cross-progeny. The 0- or 1-day-old males' mating potency under well-fed or starved conditions was set as 100%, and the normalized percentages of potent males for the following days were calculated as follows: normalized percentage of potent males = (non-normalized percent of potent (n)-day-old males / non-normalized percent of potent 1-day-old males) $\times$ 100%.

If males were required to be starved, they were serially transferred to *E. coli*-less NGM agar plates using a mouth pipette and water as a vehicle. To inhibit bacterial growth, the plates contained streptomycin at a final concentration of 30 μ g/ml. An 8 M glycerol ring was applied to the edge of the agar to discourage males from crawling and desiccating on the sides of the plates. After the starvation period, males were transferred using a worm pick to NGM plates containing *E. coli*.

To assay the effects of translational inhibition on male behavior, cycloheximide (Sigma, St. Louis, MO), freshly dissolved in water at a concentration of 20 mg/ml, was added to the surface of NGM plates with or without *E. coli*. The drug was allowed to soak into the plate overnight. The final concentration of cycloheximide in the plates was 250 μ g/ml. This concentration was either lethal to larvae or stalled larval growth. If the CHX treatment was required in an experiment, we waited until the males molted from their L4 cuticle, and then immediately placed them on the CHX-containing plates (LeBoeuf et al., 2011).

2.3. Lifespan assay

L4 males were isolated from non-crowded plates. The males were transferred daily to new OP50-seeded NGM plates. Males were considered dead if no obvious response occurred after gentle prodding with the pick. In the lifespan analysis, males that crawled up the side of the plates were censored on the day they dried.

2.4 Sex muscle fiber observation

To visualize the structure of the sex muscles during aging, we made transgenic *C. elegans* expressing actin tagged at the N-terminus with YFP. The plasmid was constructed as follows: we PCR-amplified the 1.2kb genomic sequence of *act-1*, using primers that contained homologous sequences around the insertion position (next to the YFP gene) of the vector pGW322YFP. The primers that were used to linearize the plasmid pGW322YFP removed the stop codon of the YFP gene. The primers used were: Actin-inf backbone (YFP) F: CATTTCGTAGTAATTCCAAGTACGCGC; Actin-inf backbone (YFP) R: TTTGTATAGTTCATCCATGCCATGTGTAATC; Actin-pGW322YFP inf F: GGCATGGATGAACATATACAAAATGTGTGACGACGAGGTTGCCGC; Actin-pGW322YFP inf R: CAGTTGGAATTCTACGAATGTTAGAAGCACTTGCGGTGAACGATGG. The linearized pGW322YFP vector was then recombined with *act-1* DNA using the Clontech infusion enzyme kit (Clontech, Mountain View, CA) to produce the plasmid pXG30. pXG30 additionally contains the gateway cassette in front of the YFP: actin gene fusion. To express YFP:actin in the sex muscles, we replaced the gateway cassette with the *unc-103E* promoter (Reiner et al., 2006) in pXG30 using LR clonase (Invitrogen, Carlsbad, CA) to construct the plasmid pXG31. 50 ng/ μ l of pXG31 and 150ng/ μ l pUC18 were injected into *him-5(e1490)* males. The transgenic L4 males were picked and visualized using fluorescence microscopy.

2.5. Staining sperm with SYTO-17

The red fluorescence dye SYTO-17 (Invitrogen, Eugene, OR) was diluted in a solution of 100 mM NaCl, 50 mM KH₂PO₄, 3 mM CaCl₂, 3 mM MgSO₄ to a final concentration of 70 μ M. Males were incubated in 1 ml of the dye for three hours in the dark at 20°C. They were

then removed from the dye and paired with hermaphrodites. The next day, sperm in the hermaphrodite's spermatheca were visualized by fluorescence microscopy.

2.6. Assessment of mating behaviors

We observed mating behavior from two to five minutes for each male using a dissecting stereomicroscope. We spaced ten 2-day-old *unc-64(e246)* adult hermaphrodites on a 5 mm diameter bacterial lawn. We then used a mouth pipette and water as a vehicle to place a male in the center of the hermaphrodite group. A hand-held timer or a lab-written Microsoft Excel macro was used to record when a male accomplished certain aspects of mating behavior (Liu et al., 2007). During mating, we recorded the following parameters: when and how many times a male contacted a mate after he was introduced to the bacteria; how long he required to turn successfully at the ends of a mate after he initiates the mating sequence; when and how many times a male contacted the vulva; when and how long the male spent attempting to insert his spicules. A different population of males was used to obtain data for each behavioral metric.

Males cycle between different combinations of mating behavior sub-routines in order to successfully insert their spicules and ejaculate. Some males utilize a very stereotypic sequence of mating sub-steps (such as backing, locating the vulva, and persistent prodding at the vulval slit until penetration), whereas other males adopt rare and unorthodox methods (such as prodding everywhere on the hermaphrodite, or doing brief strong spicule pokes at the vulva for a few seconds and then moving off). Ultimately, the most important outcome of mating behavior is the efficient and rapid transfer of sperm, regardless of the strategy the male uses; sometimes, the stereotypic mode of mating does not result in successful spicule insertion. To differentiate and assign a spectrum of scores for the male, regardless of how he achieves complete penetration and ejaculation, we developed a metric that incorporates how long the male attempts to insert his spicules at the vulva, and whether he was successful at penetration with whatever strategy he employed. So for example, a male that stays in contact with a hermaphrodite, but never encounters the vulva, would get a very low score. A male that meanders around the hermaphrodite, finds the vulva early on, but does not insert in the observation period would be assigned a higher score. However, a male that meanders even more, but at the last few seconds of the observation period, locates the vulva and immediately inserts might get an even better score; although he procrastinated, he got the job done. A male that instantly finds the vulva and penetrates would get a very high score, whereas a male that instantly finds the vulva, but could not insert would get a lower score. However he might get a higher score than a successful procrastinator. The efficiency of spicule insertion, E_{SI} , was calculated from recordings made during the first 120 seconds of contact between the male and the hermaphrodite. If the male successfully inserted his spicules before the 120 seconds were over, then the observation was stopped. $E_{SI} = (\text{time (sec) spent at spicule insertion attempts} / \text{total time (sec) in contact with hermaphrodite, up to 120 sec}) \times (1 / \text{time (sec) in contact with the hermaphrodite, such as backing or turning, but not attempting insertion})$; this parameter penalizes the male for "procrastination" time or confusing other parts of the hermaphrodite as vulval regions $\times (1 + (0 \text{ if no penetration, otherwise a bonus for time of penetration, which is: } (120 \text{ secs} - \text{time (sec) remaining after a successful penetration}) / 120 \text{ sec}))$. A hypothetical E_{SI} of 1.99 would mean that the male located the vulva and inserted his spicules approximately 1 sec after contact with the hermaphrodite; whereas a hypothetical E_{SI} of 0.0 meant that the male spent his first 120 seconds in contact with the hermaphrodite, but not attempting spicule insertion behavior at the vulva.

2.7. Ca²⁺ imaging

The genetically encoded calcium indicator G-CaMP1.3 was used to visualize calcium transients in the male sex muscles. The Gateway reading frame cassette B (Invitrogen) was blunt-end cloned into the XbaI site of plasmid pTG29, a previously described vector containing the GFP-based calcium indicator G-CaMP1.3 (Gruninger et al., 2008). The resulting G-CaMP Gateway destination vector, pTG30 was then recombined using LR clonase (Invitrogen) with the Gateway entry vector pLR22, which contains the *lev-11* promoter (LeBoeuf et al., 2007), to generate the plasmid pTG32. To visualize red fluorescence in the same cells as G-CaMP1.3, the gateway destination vector pGW322DsRed was recombined with pLR22 to generate the plasmid pLR132. pGW322DsRed was generated by cloning an AgeI-SplI fragment containing the mDsRed gene from the plasmid pDC68 (a gift from Dr. Daniel Chase, University of Massachusetts) into the AgeI-SplI site of plasmid pGW322YFP (Garcia and Sternberg, 2003), replacing the YFP gene with the monomeric DsRed gene.

Transgenic animals were generated using standard protocols (Mello et al., 1991). To generate animals that contain G-CaMP expressed in all sex and body wall muscles, an injection mixture containing 10 ng/μl of pTG32, 10 ng/μl of pLR132, 50 ng/μl of pBX1 and 130 ng/μl of pUC18 was injected into *pha-1(e2123);him-5(e1490);lite-1(ce314)* (Granato et al., 1994). A stable transgenic line, which expressed green and red fluorescence at approximately the same intensity, *pha-1(e2123); him-5(e1490); lite-1(ce314); rgEx430* [P*lev-11*: G-CaMP; P*lev-11*: mDsRed], was kept for analysis.

Construction of animals that contain G-CaMP expressed in all male sex muscles was previously described (Gruninger et al., 2008). In short, a mixture containing 12 ng/μl of pLR136 (a plasmid containing both G-CaMP expressed from the *unc-103E* promoter and *pha-1(+)*), 2 ng/μl of pLR132 (a plasmid containing DsRed expressed from the *unc-103E* promoter), 50 ng/μl pBX1 and 136 ng/μl of pUC18 was injected into *pha-1(e2123);him-5(e1490);lite-1(ce314)* to generate the line *pha-1(e2123);him-5(e1490); lite-1(ce314); rgEx197*[P*unc-103E*:G-CaMP; P*unc-103E*:mDsRed].

Transgenic males were separated from hermaphrodites at mid or late L4 stage (a stage where the tail spike hypodermal cells have finished their anterior retraction). At day 1 and day 3, the males were either directly visualized during copulation (without a microscope coverslip), or they were immobilized between a microscope coverslip and 10% (for 1-day-old males) or 8% (for 3-day-old males) Noble agar pads containing Polybead polystyrene 0.1μm microspheres (Polysciences, Inc., WA). Mating and immobilized animals were visualized on an epifluorescence-equipped Olympus BX51 microscope. Using a 40X long working distance objective, immobilized worms were imaged for ~1 minute and mating worms were imaged for ~5 minutes. The G-CaMP and DsRed fluorescence signals at the male tail were recorded simultaneously using a Dual View Simultaneous Image splitter (Photometrics, Tucson, AZ) and a Hamamatsu ImagEM Electron multiplier (EM) CCD camera at the speed of ~30 frames per second.

The Ca²⁺ data were analyzed using the Hamamatsu Simple PCI (version 6.6.0.0) software and Microsoft Excel, as described previously (LeBoeuf et al., 2011, Liu et al., 2011). Four region-of-interests (ROIs), of equal area, were generated in the Simple PCI software. Two of the ROIs were used to measure the background signal in the green and red channel, and two ROIs were used to measure the fluorescence of the male anal depressor muscle and the male spicule protractor muscles in both channels. The mean pixel intensity (MPI) was measured for every ROI in every frame, in each recording. The data were then transferred from Simple PCI to Microsoft Excel. For each frame of the recordings, the values of the background

ROIs were then subtracted from their respective ROIs that quantified the muscle fluorescence.

The red channel was used as a reference to analyze the green channel. The fluorescence measured in the red channel, in theory, should not change during the course of the recording. However, flickering of the mercury arc lamp, movement/contraction artifacts and photobleaching will cause fluorescence changes from frame to frame, which is not due to calcium transients. To correct for these artifacts, in each frame, the background subtracted MPI in the red channel was plotted with respect to time and a one-phase decay curve (to correct for photobleaching) was fitted over the data points using GraphPad Prism (version 4.03). The fitted curve serves as an arbitrary reference to quantify the magnitude of non-interesting fluorescence. For each frame, the background subtracted red channel MPI value was divided by the interpolated value to give a correction value. The inverse of that correction value for each frame was then multiplied by the subtracted green channel MPI of the respective frame. This corrects the values from the green channel, so that the fluorescence changes reflect calcium transients rather than experimental artifacts. The values for each frame of the recording was then calculated as $\Delta F/F_0 = (((\text{corrected MPI (frame } n) - \text{corrected MPI (frame 0(initial frame))}) / \text{corrected MPI (frame 0(initial frame))}) \times 100)$. The values were then plotted with respect to time. Generally photobleaching did not occur in the green channel, but when it was obvious, a one-phase decay curve was fitted over the data points using GraphPad Prism. The inverse proportion of decay, for each frame, was then multiplied to the respective experimental data point to re-adjust the plots.

To compare the data between experiments, we tabulated the number of peaks displaying $\Delta F/F_0$ amplitudes larger than 20%. We also calculated the mean $\Delta F/F_0$ and the standard deviation from the mean using Microsoft Excel and GraphPad Prism; the standard deviation was then plotted and compared using the Mann-Whitney non-parametric statistical test. The standard deviation was used as a simple measure to reflect how much spontaneous activity occurred in the muscles during the recordings.

2.8. Drug-induced spicule protraction

To assay agonist-induced spicule protraction, we dissolved the acetylcholine agonist levamisole (ICN Biomedicals, Aurora, OH) in water to make a stock solution of 1 mM. We then serially diluted the stock solution in water as needed. We added 1 ml of the drug to a three well round-bottom Pyrex titer dish. Five to ten males were then transferred to the drug bath. The males were observed for five minutes at 20°C with a stereomicroscope; they were considered responsive to the drug if their spicules remained protracted for 5 seconds. Drug baths were changed after 30 males were observed. GraphPad Prism 5 software was used to perform statistics on all data.

3. Results

3.1 The mating potency of *C. elegans* males significantly drops by the third day of adulthood

To measure how *C. elegans* male mating behavior changes during adulthood, we performed a male mating potency assay (Materials and methods). In this assay, we paired an individual adult male of different ages with a single 1-day-old adult hermaphrodite containing the *pha-1(e2123)* allele at 20°C. The *e2123* temperature sensitive mutation in *pha-1* allows one to score the presence of adult cross progeny as a rapid indicator of a successful mating event, since any self progeny that are homozygous for the recessive temperature sensitive *e2123* mutation cannot undergo embryonic and early larval development at 20°C. From the mating potency assays, we found that for N2 males, 90%, 68%, 37%, 37% and 36% (n=20 for each) of males were potent at adult day 1, 2, 3, 4 and 5, respectively (Fig 1A), relative to

males that were paired with a hermaphrodite soon after adult molt (denoted as day 0). Statistical analysis indicates that at adult age day 3, the N2 males mating potency declines significantly ($p=0.0031$ for 3-day-old males versus 1-day-old males, and $p=0.0001$ for 3-day-old males versus 0-day-old males Fisher's exact test).

To obtain sufficient numbers of animals for the experiment, N2 males must be maintained through continual large scale crossings with hermaphrodites. For ease of further experiments, we tested if *him-5(e1490)* males have the same kinetics of mating behavioral decline as N2 males. The *e1490* mutation in *him-5* increases the incidence of spontaneous males from 0.1% to 30%, by increasing X-chromosome non-disjunction events during meiosis (Hodgkin et al., 1979, Goldstein, 1986). From assaying the mating potency of *him-5* males, we found that relative to 0-day-old males, 90%, 77%, 54%, 24% and 10% ($n=40$ for each) of the males were potent at day 1, 2, 3, 4 and 5, respectively (Fig 1B). Similar to the behavior of N2 males, there was a significant drop of male mating potency in 3-day-old *him-5* males compared either to 0-day-old males ($p<0.0001$) or 1-day-old males ($p=0.0012$). Because there was little difference between N2 males and *him-5* males in the kinetics of mating potency, we used *him-5* males in the subsequent assays, and refer to them as the wild type for ease of comparison.

The percentage of males that are potent could vary due to the population density of the culture plate, where males originated, or the fluctuations of humidity and temperature of when the assay was conducted. Therefore, we asked at what age (day 2 or day 3 relative to day 1) the mating potency drops with consistent measureable statistical significance. We conducted four mating potency trials on separate days. We found that consistent among the trials, male mating potency at day 3 significantly dropped relative to day 1 or day 2, although the percentage of potent males varied ranging from approximately 30% to 60% (Fig 1C). The quantitative variation indicates that data obtained from experiments should be analyzed and compared within a self-contained experimental setting and not between different settings. Additionally, since the difference of mating potency between 1-day-old and 3-day-old males was robust, these two ages were used for further analysis.

Gems and Riddle reported that males raised together have shorter lifespan than males raised in isolation (Gems and Riddle, 2000). We confirmed this observation (Fig 1D) and realized that rearing males in groups might affect male mating potency. In the assays described above, 20–30 L4 males were reared together on a culture plate and allowed to develop to adults prior to using them in the potency assay; those adult males generally attempted to mate continuously with each other by crawling together and scanning each other, which could damage the males in some unknown aspect. To determine if males raised in a group artificially reduced the male mating potency, we conducted the mating potency assay using males reared in isolation and compared them with the males that were raised in groups of 20. We found that males raised in isolation or in groups have the same mating potency trend at day 1 and day 3 (Fig 1E). At day 5, the solitary-reared males have a slightly, but not significantly higher mating potency. These results indicated that the male mating potency decline at day 3 is not correlated to rearing density.

3.2 The decline in mating potency correlates with defective execution of specific steps in copulation behavior

Multiple factors might contribute to the initial decline of male mating potency at the third day of adulthood, such as loss of contractibility in aging sex muscles, dysfunction of male sperm, or decline in behavioral execution. Previous studies indicated that locomotory decline in advanced aged *C. elegans* hermaphrodite is associated with body wall muscle sarcopenia, characterized by abnormal nuclear structure and muscle fiber disorganization. In hermaphrodites, this phenomenon starts at adult age day 7 (Herndon et al., 2002, Huang et

al., 2004). To determine if males mating potency decline at the third day of adulthood is the consequence of disorganized sex muscles, we examined the overall structure of sex muscles in both 1-day-old, 3-day-old ($n=20$) and 8-day-old Punc-103E:YFP::actin transgenic males ($n=10$), by visualizing the actin-containing thin filaments of the sex muscles (Fig. 2A). In 8-day-old males, we observed muscle deterioration at different levels: some males' sex muscles displayed hole-like structures in the actin filaments (Fig 2A–5); whereas in others, the actin filaments were either disorganized or formed aggregates (Fig 2A–6, 7). However in 3-day-old males, we did not observe muscle sarcomere disorganization similar to 8-day-old males (Fig 2A-1, 2), indicating that the mating potency decline at day 3 is probably due to other reasons.

To determine whether dysfunctional sperm at the third day of adulthood is the primary cause of mating potency decline, we stained sperm DNA in the male's testis with the red fluorescent dye SYTO-17 and tested if hermaphrodites that contained fluorescent sperm from 3-day-old adult males produced cross progeny (Hill and L'Hernault, 2001). From overnight matings between stained 1-day-old males and *pha-1* hermaphrodites, 27/30 hermaphrodites contained stained male sperm in their spermatheca; all 27 hermaphrodites produced cross progeny. From matings between stained 3-day-old males and hermaphrodites, 6/30 hermaphrodites contained stained male sperm in their spermatheca; all 6 hermaphrodites produced cross progeny. In addition, we reasoned that if the decline of a male's ability to sire progeny is due to dysfunctional sperm, mating potency should drop even if hermaphrodites that are easier to mate into are used. The 1-day-old *pha-1* hermaphrodites present a challenge to the males, as they are actively moving and their vulvas have not yet been stretched from repeated egg-laying. To make mating easier for the males, we conducted the mating potency assay using 4-day-old *pha-1* hermaphrodites. These hermaphrodites are less active, have a more stretched out vulval opening from extended egg-laying and are depleted of self-sperm. We found that the percentage of 3-day-old potent males was significantly increased from 37% to 70% ($p=0.02$) when we used older *pha-1* hermaphrodite as mates (Fig 2B). These observations indicate that the sperm of 3-day-old males are still functional. This left the possibility that the reduction in mating potency could be due to a decline in some aspect of behavioral execution.

To confirm this, we observed the mating behavior of 1- and 3-day-old males, which were paired with 2-day-old paralyzed *unc-64(e240)* hermaphrodites, to compare behavioral parameters more uniformly between copulation events. For a male to initiate the mating behavior, he has to rely on the functionality of chemosensory cilia, which sense the chemical and mechanical cues from hermaphrodites (Barr and Sternberg, 1999, Barr and Garcia, 2006). The male starts to scan along the hermaphrodite backwardly when the ray sensory neurons located in his tail contact his mate, likely through chemosensory and mechanical sensation (Koo et al., 2011). When 3-day-old males were introduced to the mating lawn, they took twice as long as 1-day-old males to contact a hermaphrodite (99 ± 52 secs for 1-day-old male, $n=20$; 182 ± 146 secs for 3-day-old males; mean $\pm$ SD, $p=0.02$, unpaired t-test) (Fig 3A). 3-day-old males did not initially explore the new lawn, but after 30 seconds they would initiate exploration and locate a mate within six minutes. Once a male's tail contacted a hermaphrodite, we observed his behavior for up to 5 minutes and recorded how well they executed each step of mating behavior. 3-day-old males immediately initiated mating with the same efficiency as young males; the mean number of contacts before backing behavior was two for both young and old males ($n=20$ for each) (Fig 3B). Thus sufficient numbers of ray sensory neurons located in male's tail are functional to mediate contact response. During scanning, if the male reaches the end of hermaphrodite, he will make a turn to continue scanning along the hermaphrodite cuticle (Loer and Kenyon, 1993). After the 3-day-old males made contact, they took slightly longer than younger males to make a successful turn at the ends of the hermaphrodite (7 ± 3 secs for 1-day-old male,

n=20; 15 ± 15 secs for 3-day-old males; mean $\pm$ SD, $p=0.03$, unpaired t-test) (Fig 3C). Instead of turning, the older males would rub their tails back and forth at the ends of the hermaphrodites or consecutively attempt, abort and reattempt a turn for a few seconds. They would make a successful turn with their tail, but then they would not carry through the turn with continued backward locomotion. Instead, they would move forwards and then backwards again to reattempt the turn. Interestingly, when the aged males were hesitant in their turns, the curvature of their body resembled that of a male trying to insert his spicules into the vulva (Fig 3D). More frequently, the 3-day-old males would stop their locomotion at non-vulva regions, which also accounts for why they took longer to make one turn after contact.

Sensing the vulva is regulated by the hook and post-cloacal sensilla. After a successful turn, 3-day-old males had no trouble locating the vulva, as compared to young males (Fig 3E), indicating that these neurons still function at day 3. However, they were not as efficient as younger animals at maintaining their position over the vulva during spicule insertion attempts or breaching the vulva lips. After the postcloacal sensilla (p.c.s.) neurons and hook neurons sense the vulva, the male stops at the vulva and simultaneously initiates spicules prodding via rhythmic contractions of the protractor muscles (Garcia et al., 2001). Once the spicules partially penetrate the vulva, the SPC neurons will stimulate the protractor muscles to fully contract and consequently, resulting in full spicule insertion (Liu et al., 2011). During the first two minutes of mating, only 15% of 3-day-old males (n=20) were able to insert their spicule completely, whereas 50% of 1-day-old males (n=20) were able to penetrate their mates (Fig 3F). The efficiency of spicule insertion (E_{SI}), a metric that combines how fast they initiate spicule insertion attempts, how tenacious they sustain reattempts and how fast they completely insert their spicules within two minutes (see Materials and methods), was higher for 1-day-old males compared to 3-day-old males (the average E_{SI} was 0.04 for 1-day-old males, n=20 and 0.004 for 3-day-old males, n=20) (Fig 3F). In conclusion, while older males are capable of performing the specific steps of mating behavior, they do so at a lower efficiency than younger males. Therefore, the inefficient behavioral execution exhibited by 3-day-old males during mating with paralyzed hermaphrodites would magnify during copulations with moving hermaphrodites, which would be a likely cause for reduced mating potency.

3.3 Spicule muscle excitability increases during adulthood

During the behavioral observations we noticed that 3-day-old males spent a significant amount of time at areas outside the vulva region and assumed a posture that was reminiscent of spicule insertion attempts. We asked if this increase in apparent spicule insertion attempts outside the vulva region was correlated with increased sex muscle excitability. To address this question, we used G-CaMP, a GFP-derived fluorescent calcium sensor to monitor the Ca^{2+} transients in the sex muscles during mating (Nakai et al., 2001). We observed Ca^{2+} transients in the sex muscles (Fig 4) of 3-day-old males, when they randomly stopped at non-vulval regions. These observations led us to hypothesize that components of the mating circuit might be hyper-excited, resulting in the ectopic spicule insertion behavior. If this was the case, mating relevant components such as the spicule muscles of older males might display more spontaneous Ca^{2+} transients, even when the males are not mating. To test this idea, we imaged the Ca^{2+} transients in the spicule associated muscles from immobilized 1- and 3-day-old males (see materials and methods). Compared to 1-day-old males (Fig 5B), 3-day-old males displayed more Ca^{2+} transients (Fig 5C, D, E).

The increased incidence of Ca^{2+} transients in 3-day-old male sex muscles suggests the non-stimulated excitability level of the mating circuit increases as the male ages. We hypothesized that the increased excitability might result in hypersensitivity of the spicule-associated muscles to agonist stimulation. The cholinergic SPC motor neurons make direct

synapses to the spicule muscles. To respond to secretions from these neurons, the muscles express levamisole (LEV)-sensitive ionotropic acetylcholine receptors (AChRs) made up of subunits encoded by the *unc-38*, *unc-63*, *lev-8*, *lev-1* and *unc-29* genes. To test the hypothesis that increased calcium transients might be related to hypersensitivity to cholinergic stimulation, we used the acetylcholine agonist LEV to stimulate protractor muscle contraction. Low concentrations of LEV can increase the acetylcholine receptors' closed to open probability, causing excitable cells to fire. To ascertain the sensitivity of males to the drug, we bathed 1- and 3-day-old males for five minutes in various concentrations of LEV, and quantified how many males protracted their spicules for greater than 5 seconds. We found that the LEV sensitivity of the spicule muscles for 3-day-old males was approximately seven times greater than compared to younger males (LEV EC₅₀: 884 μ M for 1-day-old males, 100 nM for 3-day-old males) (Fig 6). This result is consistent with the mating behavior observations and increased calcium transients, indicating that the overall excitability of the male mating circuit increases as the male ages.

3.4 Reducing the expression of ACh Receptor genes can increase the mating potency in 3-day-old males

Our pharmacological assay and Ca²⁺ imaging experiments indicated that during aging, the excitability of male mating circuit components such as the male sex muscles is increased. Therefore, we asked if the hypersensitivity of the cholinergic mating circuit components was related to the mating potency decrease in 3-day-old males. To answer this, we reduced the expression level of several acetylcholine receptor (AChR) genes and asked if this manipulation can increase the mating potency in older males.

Male sex muscles express the GAR-3, ACR-18, ACR-16 and UNC-29 AChRs (Liu et al., 2011). We assayed the mating potency of males that contain a single homozygous mutation in these AChR genes. Relative to 1-day-old males, the mating potency of 3-day-old *gar-3(gk305)* and *acr-18(ok1258)* males dropped significantly, and was similar in kinetics to *him-5* males (Fig 7A). In this study, we found that for *acr-16(ok789)* males, there was also a significant drop at day 3 compared to 1-day-old males. *unc-29* is expressed in the body wall muscles and the lack of functional UNC-29 makes the animals severely defective in locomotion, which obviously eliminates the ability of the mutant males to mate. To circumvent this problem, we restored locomotion to the mutant males by driving the expression of an *unc-29* cDNA, using the *acr-8* promoter that expresses in the body wall muscles, but not the male sex muscles (Liu et al., 2011). Similar to *acr-16* males, the body wall restored *unc-29* males mating potency dropped significantly at day 3 relative to 1-day-old males (Fig 7A). Taken together, these results suggested that reducing the function of single AChR genes does not decrease the rate of mating potency decline by day 3.

We then asked if reducing the expression of all four AChR genes could reduce the rate of mating potency decline. Males that are homozygous for mutations in all 4 genes cannot mate; therefore, males that are heterozygous mutant for all of these AChR genes were used to conduct the mating potency assays. We reasoned that one functional genomic copy of these four AChR genes would be sufficient to allow the males to mate, but might also reduce the sex muscle excitability in 3-day-old males and possibly increase mating potency. To test this hypothesis, we assayed the mating potency of the heterozygous mutant males at day 1 and 3. We observed a significant improvement of mating potency at day 3, 55% for heterozygous males compared to 27% for wild-type males (Fig 7B). We then assayed the 1- and 3-day old heterozygous AChR males' response to LEV. We found that the LEV sensitivity of the spicule muscles for 3-day-old heterozygous males was significantly lower than that of similarly aged wild-type males (LEV EC₅₀: 1 μ M for 3-day-old heterozygous males, 100 nM for 3-day-old *him-5* males) (Fig 6). Thus, reducing one functional genomic copy of each AChR gene was sufficient to reduce the excitability of males mating circuit

components on day 3. This observation suggests a correlation between the changes in the excitability of male mating circuit components and the efficiency of copulation behavior during aging.

3.5 Transient starvation in 1-day-old males can reduce sex muscle excitability and increase mating potency in 3-day-old males

Although genetic manipulation can reduce the excitability of sex muscles and increase mating potency in 3-day-old males, we asked if there is another less-invasive strategy that can be applied to wild-type males, in order to achieve the same outcome. Previous work has showed that caloric deprivation can reduce male sex muscle excitability. 3–18 hrs of transient starvation during early adulthood can suppress fictive spontaneous mating behavior and sex muscle spasms caused by deletion of the *unc-103* encoded *ether-a-go-go* related gene (ERG)-like K⁺ channel (Reiner et al., 2006, LeBoeuf et al., 2007, Gruninger et al., 2008). The suppressive effect can last for days after re-feeding, and is partly facilitated by the compensatory increased expression and function of the *ether-a-go-go* (EAG) K⁺ channel encoded by the gene *egl-2* (LeBoeuf et al., 2011).

Since transient starvation can reduce the excitability of components of the mating circuit in young adult males, we asked if it has a similar effect on 3-day-old males. To address this question, we used LEV to stimulate the male spicule protraction circuit in 3-day-old males that were transiently starved for the first 18~20 hr of adulthood. As expected, we found that the sensitivity to LEV of these transiently starved males was significantly lower than that of well-fed 3-day-old males (LEV EC₅₀: 251nM for transiently starved, 3-day-old males; 100nM for 3-day-old males) (Fig 6).

We then asked if transient starvation can also positively affect the mating behavior of 3-day-old males. We found that male mating behavior can be improved by transient starvation during early adulthood. At day 1, 83% (n=69, 100% after normalization) of males after transient starvation can produce the cross progeny versus 93% (n=60, 100% after normalization) of well-fed males. At day 3, a significant of improvement in mating potency was observed in males that experienced transient starvation. 71% (n=70) of starved males can sire progeny, while only 41% (n=60) of well fed males can mate (Fig 8A). In agreement with the mating potency assay, we observed that during mating behavior, the efficiency of spicule insertion (E_{SI}) of 3-day-old males that were transiently starved during early adulthood is significantly higher than their continuously fed cohorts. During 2 minutes of observation, 40% of the transiently starved 3-day-old males inserted their spicules, and the average E_{SI} was 0.02, similar to 18–24 hr males and significantly different from aged well-fed males ($p=0.005$, Mann-Whitney U test) (Fig 3F).

Since caloric restriction has been shown to extend lifespan in *C. elegans* (Klass, 1977, Hosono et al., 1989), it is possible that the increased mating potency of 3-day-old males by transient starvation is a consequence of lifespan extension rather than the modulation of cell excitability. Therefore, we conducted a lifespan assay for both well-fed males and males transiently starved for ~18hrs and then re-fed. We found that the duration of transient starvation that can increase mating potency in 3-day-old males is not sufficient to extend lifespan (Fig 8B). This result is consistent with our hypothesis that increase of the mating potency by transient starvation during early adulthood is correlated with the reduction of cell excitability in 3-day-old males.

3.6 The long term effect of transient starvation on mating potency requires protein synthesis during early adulthood

To address how transient starvation prolongs male mating potency, we first asked if food deprivation slows down the natural, yet undefined progressive changes in the physiology of the mating circuit, so that 3-day-old males can mate better. If so, transiently starving the males anytime from adult molt to day 3 should increase the mating potency of 3-day-old males. Since ~20 hrs starvation of males starting from late L4 or early adulthood was sufficient to improve male mating potency at day 3, we asked if ~20 hrs of starvation at day 2 can also prolong male mating potency. Males were fed for the first 24 hrs of their adulthood, then starved for ~20 hrs, then re-fed for an additional 24 hrs before they were assayed for their mating potency. We found that transiently starving the males at day 2 of adulthood cannot prolong mating potency (Fig 8C), indicating that starvation needs to occur early in a males' development to have lasting effects on mating potency.

We then asked if protein synthesis during starvation is required to increase the mating potency at the third day of adulthood. To address this, we starved the males (immediately after adult molt) in the presence cycloheximide (CHX), a drug that reduces translation by blocking the ribosomal translocation step (Ernest, 1982). After 18–20 hrs on plates with CHX but lacking food, the males were removed from the drug and transferred to plates containing food. We then scored the 3-day-old male mating potency and found that the percentage of potent males was reduced from 73% (n=70) to 56% (n=57) ($p<0.5$) (Fig 8C). This suggests that under normal conditions, protein synthesis occurs in young starved males that later improves mating potency in older males. One consequence of those newly synthesized proteins might be to offset or reprogram the physiological changes that occur in the mating circuit during aging. In contrast to the negative effect of CHX on starved males, continual exposure to CHX during one day of starvation and two days of re-feeding can restore the positive effect of transient starvation; the percentage of 3-day-old males that can sire progeny increased from 56% to 76% (Fig 8C). This indicates that under normal well-fed condition there might be molecules synthesized during adulthood that are detrimental to the physiological state of the mating circuit. We then asked if perturbing protein synthesis in continuous feeding conditions also positively affects the mating potency of 3-day-old males. We found that continuously blocking protein synthesis slightly, although not significantly, affects 3-day-old males (54% compare to 62% of 3-day-old males can sire progeny $p=0.45$), whereas exposure to CHX only at day 1 (54% compared to 50% of 3-day-old males can sire progeny) or only at day 2 (54% compared to 40% of 3-day-old males can sire progeny) have no effect, suggesting that reducing protein synthesis in general does not increase the mating potency. Taken together, these results suggest that early adulthood is a sensitive period when new proteins, synthesized in response to transient starvation, can offset the deteriorative effect of protein synthesis during re-feeding, to prolong the mating potency.

3.7 UNC-103 and EGL-2 potassium channels mediate the effect of transient starvation on mating potency

In previous work, we demonstrated that 3 to 18 hrs of transient starvation can attenuate the excitability of the mating circuit for an extended period of time, through the activity of EAG family K^+ channels. Deletion of the *unc-103*-encoded ERG-like K^+ channel (*unc-103(0)*) leads to an unregulated increase in the excitability of the male mating circuit, causing a percentage of males to display spontaneous spicule-muscle spasms. As a consequence, the attached spicules are protracted constitutively from the cloacal opening (Garcia and Sternberg, 2003, Gruninger et al., 2006). The *unc-103(0)* spontaneous spicule protraction phenotype can be suppressed by starvation, partially through the up-regulation of *unc-103*'s paralog, the *egl-2*-encoded EAG K^+ channel. In wild-type males, robust expression of *egl-2* in the sex muscles occurs approximately two days after adulthood; however, its temporal

expression can be expedited by starvation, and the effects on attenuating cell excitability can last for days (LeBoeuf et al., 2007, LeBoeuf et al., 2011). This led us to ask if the EGL-2 and UNC-103 K⁺ channels could be molecules that respond to short term starvation, attenuate cell excitability, and consequently reduce the rate of male mating decline.

To address the hypothesis, we used *unc-103(0)* and *egl-2(0)* single- and *unc-103(0); egl-2(0)* double-mutant males to test if transient starvation's effect on mating decline is perturbed by mutations in these EAG family K⁺ channel genes. In wild-type males, *egl-2* expression in the male sex muscles is robust at day 2 of adulthood, suggesting that the K⁺ channel might be important during that period. Therefore, we assayed the mating potency of wild type, *unc-103(0)* and *egl-2(0)* single and double mutant males on day 1, 2 and 3. The mating potency kinetics for *unc-103(0)* single mutant males resembled wild type. Mating behavior did not significantly decline until day 3, and transient starvation improved the mating potency (from 18% to 62%, $p < 0.05$ $n=40$) (Fig 9A). Unlike wild type or *unc-103(0)* males, the mating potency of *egl-2(0)* males measurably declined by day 2. However, after starvation, 87% ($n=40$) of males were potent, which was significantly higher than their well fed cohorts (60% $n=40$) (Fig 9B). Therefore, single K⁺ channel mutations do not obviously interfere with processes that occur during and after transient starvation.

Mutations in both K⁺ channels did affect the male's mating potency after starvation. Similar to *egl-2(0)* males, the mating potency of *unc-103(0); egl-2(0)* animals significantly drops at day 2 (from 100% to 44%, $n=39$ and 34 , respectively, $p=0.02$). However, unlike in *egl-2(0)* males, transient starvation did not improve the mating potency of the double mutants (45% for well-fed males vs. 47% for starved males, $n=44$ and 48 , respectively) (Fig 9D). We did observe a slight, but not statistically significant difference (probably due to the limited sample size) in mating potency of *unc-103(0); egl-2(0)* males at day 3 between well-fed and starved conditions. Therefore, we cannot rule out that there are additional molecules that might have a minor involvement in the transient starvation response. We conclude that increase in K⁺ channels function during transient starvation might result in slowing the decline of mating potency during aging.

4. Discussion

Progressive behavioral decline occurs during aging; this decline is relatively easier to investigate at an advanced age since it is more noticeable. Behavioral decline is evidenced in *C. elegans* through slower movement and decreased feeding as they age, similar to other well-studied invertebrate and vertebrate model organisms (Forster et al., 1996, Huang et al., 2004, Simon et al., 2006). The decline in those general behaviors is usually correlated with the loss of cell integrity, such as cytoskeletal disorganization in the worm's body wall and pharyngeal muscle cells (Herndon et al., 2002, Augustin and Partridge, 2009). However, early indicators of behavioral decline can occur prior to the onset of gross morphological and structural changes to the cells. For example, people start losing memory capability in their late 20's, when there are no obvious pathological changes to their brain structure (Salthouse, 2003). Therefore, the study of decline at an advanced age may not allow the identification of mechanisms responsible for early-phase behavioral decline. It would be advantageous to identify and explore a behavioral model that is sensitive to early phase changes in the neuromuscular circuitry during aging.

The complex mating behavior performed by *C. elegans* males offers an ideal model to study the early effects of aging on behavior. Male mating requires accuracy and coordination between the different groups of sex muscles and studying this behavior might uncover subtle physiological changes in the mating circuitry that reduce the efficiency of copulation (Garcia et al., 2001, Liu et al., 2011). In contrast, a general behavior such as locomotion on

solid media has been shown to be robust and require environmental manipulation, such as crawling into a food source or swimming in liquid media, to study the subtle or measurable changes that occur during early aging (Murakami et al., 2008, Hsu et al., 2009). Unlike general behaviors, the *C. elegans* male mating behavior starts to decline significantly at a very early stage of adulthood, when there is no obvious muscle structure disorganization or sperm dysfunction. Hence, we can use mating behavior as a compliment to other successful behavioral models to identify early factors that promote behavioral decline and thus provide potential therapeutic targets to delay the onset of structure dysfunction in neuromuscular cellular components.

To determine the physiological changes that occur in the *C. elegans* male mating circuit during aging, we carefully observed the sub-steps of mating in 1 and 3-day-old males. We found that ectopic spicule insertion behaviors at the non-vulval regions were displayed in the older males. This suggests that the physiological state of the mating circuit (consisting of neurons and muscles) might change during early aging in term of increased excitability. Because of technical challenges, we did not dissect how the individual neurons and muscles in the male mating circuitry might contribute to the phenomenon. The heightened spontaneous sex muscle Ca^{2+} transients in non-mating males and sensitivity to the agonist levamisole (a drug that stimulates receptors expressed on the spicule muscles and not on the spicule neurons) suggest that in 3-day-old males, at least the muscle's excitability increases. However, it is likely that the sensory and motor neurons that stimulate the sex muscle activity might be also hyper-excitable, for as-of-yet determined reasons, similar to the sex muscles. Thus we do not exclude the possibility that sex neuron outputs also change in 3-day-old males and could partially contribute to the muscle responses in the assays.

The excitability of the neuromuscular circuitry is critical to precisely control the execution of behavior. Changes in the excitability of neurons and muscles are associated with behavioral decline during aging among many organisms: reduced cell excitation would lead to the failure of initiating or maintaining behaviors, whereas increased excitability may also result in exaggerated, promiscuous or uncoordinated behaviors. Similar to *C. elegans* males, other invertebrates such as the mollusc *Lymnaea stagnalis* display an increased sensitivity of excitable cell membranes to acetylcholine stimulation with age (Frolkis et al., 1984). In contrast, many reports in vertebrate systems demonstrated that the general excitability in either neurons or muscles is reduced during aging (De Luca et al., 1990, Karakelides and Nair, 2005, Lopes et al., 2007). For instance, the hippocampus-dependent learning ability decreases during aging, which is correlated with a decrease excitability in hippocampal neurons (Wu et al., 2002). However, similar mechanisms might be responsible for the difference in excitability states during aging between invertebrates and vertebrates. In the mammalian brain, calcium imaging studies demonstrated a significant increase of intracellular Ca^{2+} in response to a prolonged synaptic stimulation in hippocampal neurons of aged animals (Thibault et al., 2001). This is consistent with our finding that there are increased Ca^{2+} transients in the male mating circuit. However, the Ca^{2+} current in mammals plays a signaling role, and the increased of Ca^{2+} activates calcium dependent potassium channels, which in turn hyperpolarizes the cell membrane to attenuate excitability (Murchison and Griffith, 1995, 1996, Thompson et al., 1996). Whereas, in the muscles of invertebrates such as *C. elegans*, the influx of Ca^{2+} through voltage-gated calcium channels (VGCCs) propagates depolarizing action potentials, and promotes excitation contraction coupling (Hagiwara and Byerly, 1981, Maryon et al., 1998). Although the idiosyncratic cellular output may differ between aging cells of invertebrates and vertebrates, *C. elegans* male mating behavior can be used as a model to investigate the mechanism of increased Ca^{2+} transients, which appears to be conserved among species during aging.

Manipulation of cell excitability can be used as a strategy to delay the deterioration of behavior. Excitability can be affected by changes to neurotransmitter pathways or the ion channels that maintain appropriate polarization. In the *C. elegans* male mating circuitry, acetylcholine is used to promote sex muscle contractions. Through halving the gene dosage of AChR genes, we found that the excitability of the mating circuitry decreased and concurrently, mating behavior was significantly improved at the third day of adulthood. The promiscuous spicule insertion behavior displayed by 3-day-old males mirrors the previously reported observation that 4-day-old *C. elegans* hermaphrodites display an exaggerated locomotor behavioral response when they enter an area of solid media containing food (Murakami et al., 2008). When young starved hermaphrodites crawl into an area containing their food *E. coli*, they greatly reduce their velocity; this behavior is termed the enhanced slowing response and is regulated by the neurotransmitter serotonin (Sawin et al., 2000). Well-fed 4-day-old *C. elegans* hermaphrodites inappropriately display this behavior, and reducing serotonin signaling, similar to our experiments where we reduce ACh signaling, attenuates the basal slowing response (Murakami et al., 2008). In addition to neurotransmitter signaling, many channels such as sodium, potassium and calcium channels also contribute to establish and maintain the membrane excitability. During aging, one potential reason for the decreased excitability in hippocampal neurons is increased intracellular Ca^{2+} , possibly through modification of Ca^{2+} channels. The increased Ca^{2+} then activated the Calcium dependent potassium channels such as the voltage- and calcium-dependent BK channel, which hyperpolarizes the cell membrane (Murphy et al., 2004). Therefore, decreasing the function of potassium channels is a potential strategy to increase cell excitability and promote learning. Indeed, in aged mammals, increasing neuronal excitability through targeted deletion of a potassium channel subunit, Kv β 1.1, in learning-associated pyramidal neurons in the hippocampal area CA1 enhanced learning and memory functions (Murphy et al., 2004). Taken collectively, the available research suggests that genetic manipulation of neurotransmitter pathways or cell membrane excitability components is a feasible method to delay the deterioration of behavior.

In addition to genetic manipulation, our results demonstrate that transient food deprivation can reduce the excitability of the mating circuit and prolong copulation behavior after re-feeding. During starvation, the excitability of mating circuits is suppressed, probably to focus the animal's attention towards searching for food (Gruninger et al., 2008). Once feeding is resumed, the male's mating ability can be recovered within 1 hr (unpublished results) and subsequently, mating ability is improved at the third day of adulthood. Caloric restriction has a positive effect on organisms ranging from yeast, worms and flies to mammals by extending lifespan. However, unlike in our experiments, prolonged dietary restriction has to be used to extend lifespan (Houthoofd and Vanfleteren, 2006, Houthoofd et al., 2007, Greer and Brunet, 2009). We were able to produce a positive, lasting effect on mating ability using ~20 hrs of food deprivation that does not affect males' lifespan. Thus, the increased mating ability in aged males is not due to lifespan extension but could be a result of long-lasting changes to the mating circuitry produced by transient starvation.

A previous study suggests that the specific onset of food deprivation during *C. elegans* hermaphrodite adulthood is not critical to increase lifespan (Kaeberlein et al., 2006). Food deprivation either starting from the second, fourth or post-reproduction tenth day of adulthood would significantly increase the animal's longevity. In contrast, we found that only newly molted adult males are responsive to short-term starvation, since starving males later in adulthood does not confer any beneficial effects. Taken together, these results suggest that the mechanisms of improving male mating potency by food deprivation differ from that of increased lifespan by caloric restriction.

Caloric restriction may extend lifespan through physiological changes such as increased autophagy, increased resistance to environmental stresses, decreased metabolic oxidative damage and overall decreased translation levels (Masoro, 2005, Hansen et al., 2008). However, protein synthesis is required during starvation to improve mating behavior. Blocking protein synthesis during starvation and during re-feeding can restore the positive effect of starvation. These results suggest that during aging, signaling molecules might accumulate, which increase cell excitability. Young adulthood could be a critical developmental period that can be modified by transient starvation, to produce molecules including potassium channels that will offset the increased excitability. This hypothesis is consistent with our results that UNC-103 and EGL-2 potassium channels mediate the effect of transient starvation.

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Fig. 1.

C. elegans mating potency declines significantly by the third day of adulthood. (A) Relative mating potency of N2 males normalized to 0-day-old males ($n=20$ for each day). The normalized percentage for each day is listed on the top of each bar. Fisher's exact test was used to compare the mating potency between different aged males. Asterisks *, ** and *** indicate the $p<0.05$, 0.01 and 0.0001 respectively. (B) Relative mating potency of normalized *him-5* males ($n=40$ for each day). (C) Four independent trials of mating potency assays in *him-5* males ($n=30$ for each column). (D) Adult lifespan of males maintained in isolation (triangles) or in groups of 20 per plate (squares). ($n=97$ for solitary males; $n=205$ for grouped males); *** $p<0.0001$, Log-rank (Mantel-Cox) Test (E) Normalized mating

potency of *him-5* males reared in groups of 20 or in isolation. The black bar represents males kept in isolation, whereas the light gray bar represents males kept in groups. The number of males assayed for each day is listed on the bottom of each bar.

Fig. 2.

Mating potency decline is not due to sex muscle disorganization or sperm dysfunction. (A) Negative grey scale fluorescent images of the adult male tail muscles. The overall structure of male-specific sex muscles indicated by YFP:ACTIN in 3-day-old male shows no significant difference as compared to that of 1-day-old male. (1) and (3) show the male tail structure with noticeable striated oblique muscles of 1-day-old and 3-day-old males respectively. (2) and (4) show diagonal muscles of 1-day-old and 3-day-old males respectively. Scale bar is 10 μ m. (5), (6) and (7) show disorganized diagonal muscles of 8-day-old males. We observed hole-like structure in the diagonal muscles indicated by *. (ob) oblique muscles; (dgl) diagonal muscles. (B) Mating potency of 3-day-old *him-5* males using 1-day-old and 4-day-old *pha-1* hermaphrodites as mates. Fisher's exact test Asterisks * indicates the $p < 0.05$ (N=40 for each case).

Fig. 3.

Specific steps in mating behavior are affected as the male ages. (A) The time required for the males to initiate mating behavior. Each dot represents the metric of a single male observed. * $p < 0.05$; unpaired t-test. (B) The number of contacts the males made prior to initiating backward locomotion along the hermaphrodites cuticle. (C) The time between the male initiating mating behavior and completing the first turn at the end of the hermaphrodite. * $p < 0.05$; unpaired t-test. (D) The top panel (1) depicts a 1-day-old male moving backwards along the hermaphrodite and turning at the end of his mate. The posture of a 3-day-old male (2) during backward locomotion resembles a male attempting to insert his spicules (3). Scale bar is 100 μm . (E) The number of vulval contacts prior to successful

insertion of spicules. (F) Spicule insertion efficiency (E_{SI}) during 2-minutes of observation. Open symbols indicate that the males successfully inserted their spicules. The number of success insertion are listed on the top of each column. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; Mann-Whitney non-parametric test.

Fig. 4.

An old male displays ectopic spicule insertion behavior at a non-vulva region. The arrows point to the hermaphrodite vulva and the male tail is inside the rectangular box. The green indicates the background fluorescence of G-CaMP. Once Ca^{2+} is released into the muscle cytoplasm, the G-CaMP fluorescence becomes more intense, indicated by the yellow and red false color. On the right are negative grey scale images of the left montage to delineate the outlines of the hermaphrodite and male.

Fig. 5. Immobilized old males display increased Ca^{2+} transients in the sex muscles. (A) A male was immobilized on an agar pad and recorded in the green (left) and red (right) fluorescence channels simultaneously. The mean gray pixel intensity was measured in the rectangular region of interest. (B) Plots of Ca^{2+} transients in 8 representative 1-day-old males. (C) Plots of Ca^{2+} transients in 8 representative 3-day-old males. (D) Standard deviation from the mean $\Delta F/F_0$ for each male. $N=18$ for each group. ** $p < 0.01$; Mann-Whitney non-parametric Test. (E) No. of transient peaks with amplitude larger than 20% ($p < 0.05$) change; Mann-Whitney non-parametric Test.

Fig. 6.

The response of the males' spicule muscles to different concentrations of the ACh agonist LEV. 30 males were assayed for each concentration. The EC₅₀ for each treatment or strain was calculated and listed on the right of each corresponding line.

Fig. 7.

Manipulation of AChR gene dosage can improve the mating potency of 3-day-old males. (A) Relative mating potency in single AChR mutant homozygous males. (B) Relative mating potency in AChRs heterozygous mutants. The number of males assayed for each strain is listed within the bottom of each bar and the normalized percentages are listed within the top of each bar. * $p < 0.05$; ** $p < 0.01$; Fisher's exact test.

Fig. 8. Transient starvation can improve the mating behavior without extending lifespan. (A) Changes in mating potency of transiently starved and well fed aged males. ** $p < 0.01$; Fisher's exact test. (B) Adult lifespan of transiently starved and fed *him-5* males ($n=78$). $p=0.2$ ns; Log-rank (Mantel-Cox) test. (C) The effect of cycloheximide on male mating potency.

Fig. 9.

Potassium channels are required to mediate the effect of transient starvation on mating potency. Numbers at the top of the bars are normalized percentages. Below are the numbers of males assayed. The *unc-103* mutation causes a certain percentage of males to protract their spicules constitutively, the Prc phenotype. Males used in the mating potency assay did not display the abnormal phenotype, and are referred to as non-Prc males. (A) Changes in mating potency of transiently starved and fed aged *unc-103(0)* males. ** $p < 0.001$; Fisher's exact test. (B) Changes in mating potency of transiently starved and well fed aged *egl-2(0)* males. * $p < 0.05$; Fisher's exact test. (C) Changes in mating potency of transiently starved and well fed aged wild-type males. (D) Changes in mating potency of transiently starved and well fed aged *unc-103(0); egl-2(0)* males. * $p < 0.05$; Fisher's exact test.