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Temporally controlled nervous system-to-gut signaling bidirectionally regulates longevity in *C. elegans*

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eLife Assessment

This is an **important** study that addresses the temporal aspects of cell non-autonomous regulation of lifespan. It demonstrates that the same neurons and neurotransmitter have distinct impacts on longevity at different ages. The data **convincingly** supports the authors' claims.

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Abstract

The nervous system modulates aging by secreting signaling molecules to cell-nonautonomously regulate the physiological state of distal tissues such as the gut. However, the underlying mechanisms are not well understood. Here, using *C. elegans* as a model, we identified two distinct neuroendocrine signaling circuits through which motor neurons signal the gut in early life to shorten lifespan but in mid-late life to extend lifespan. Both circuits employ the same neurotransmitter acetylcholine (ACh), while recruiting two different gut ACh receptors ACR-6 and GAR-3 to regulate the transcription factor DAF-16 and HSF-1 in early and mid-late life, respectively. Strikingly, the gut expression of ACR-6 is restricted to early life, whereas that of GAR-3 is confined to mid-late life, providing a potential mechanism for the temporal control of the two circuits. These results identify a novel mechanism that empowers the nervous system to bidirectionally regulate longevity by differentially signaling the gut at different life stages.

Introduction

Aging is a complex physiological process characterized by a progressive functional decline in tissues and organs, leading to the onset of age-related diseases and probability of death[1,2]. The aging process occurs body wide in multiple tissues/organs in a coordinated manner, though different tissues/organs may age at different rates[3-8]. Recent work has uncovered an increasingly important role for the nervous system in orchestrating such a coordinated aging process through tissue-tissue communications[5,9-14]. The nervous system mediates tissue-tissue communications by secreting various signaling molecules such as small neurotransmitters and neuropeptides, which modulate the physiological state of distal tissues. For example, in *C. elegans*, the nervous system can detect and process internal stress signals from mitochondria and external sensory cues, such as temperature and odors, and then communicates with the distal tissue gut to regulate aging in a cell-nonautonomous manner[14-21]. How the nervous system signals distal tissues to regulate aging remains a question under intensive investigation.

The nervous system modulates aging in a complex fashion. Research in the past decade has uncovered both negative and positive impact of the nervous system on aging. The notion that the output of neural cells, including both neurons and glial cells, plays an important role in aging has gained consensus[19,22,23], though conflicting results have been reported as to whether enhanced

neural output is beneficial or detrimental to longevity. For example, several studies have demonstrated that neuronal hyper-excitability shortens lifespan in organisms ranging from *C. elegans* to humans[24-26]. Specifically, the transcription factor REST suppresses neural gene expression in humans and mice, and overexpression of the *C. elegans* REST orthologue *spr-4* suppresses neuronal output and extends lifespan[24]. On the other hand, several studies support the opposing view that enhanced neuronal output extends lifespan. For example, genetic and pharmacological potentiation of motor neuron activity and synaptic transmission at neuromuscular junctions (NMJs) slow down motor aging and extend lifespan in *C. elegans*[27-29]. These seemingly conflicting results reflect the complexity of the role of the nervous system in aging modulation. One possibility is that the nervous system can both negatively and positively regulate aging, depending on the context; however, whether and how the nervous system does so is not well understood.

In this study, we sought to address these questions in *C. elegans*, a widely used genetic model organism for the study of aging. Using the *C. elegans* motor nervous system as a model, we developed a strategy to temporally manipulate the motor neuron output at different life stages. We identified two distinct neuroendocrine signaling circuits through which neuronal output inhibits longevity in early life but promotes it in mid-late life. Both circuits require the neurotransmitter acetylcholine (ACh) as a signaling molecule that signals the gut via two different gut ACh receptors, ACR-6 and GAR-3, to regulate two different transcription factors, DAF-16 and HSF-1, at early and mid-late life stages, leading to shortened and extended lifespan, respectively. Strikingly, the expression of the ACh receptors ACR-6 and GAR-3 in the gut undergoes a temporal switch in early and mid-late life, providing a potential mechanism to temporally control the activity of the two circuits. Our results define the molecular and circuit mechanisms by which the nervous system bidirectionally regulates longevity by differentially signaling the gut at different life stages, highlighting an exquisite role of the nervous system in aging modulation.

Results

Cholinergic motor neurons differentially regulate lifespan at different stages of worm life

To ascertain whether the nervous system promotes or inhibits longevity, we focused on the motor nervous system using *C. elegans* as a model. Previous work has uncovered a role of *C. elegans* cholinergic but not GABAergic motor neurons in the ventral nerve cord in lifespan regulation[28-30]. To further explore the role of cholinergic motor neurons in lifespan control, we ablated the output of these motor neurons by expressing tetanus toxin (TeTx) as a transgene in these neurons using the *acr-2* promoter. TeTx cleaves the SNARE protein synaptobrevin, thereby blocking the release of signaling molecules such as neurotransmitters and neuropeptides from the neurons of interest[31].

Overexpression of TeTx induces characteristic phenotypes of cholinergic deficiency, such as developmental delay and severe locomotion impairment[32], yet does not compromise muscle function (Figure S1A). Surprisingly, such intervention led to a complex effect on the population survival curve by reducing both early mortality and the proportion of long-lived individuals (Figure 1A). Specifically, the 25% lifespan of these worms was prolonged, while their 75% and maximal lifespan were slightly shortened, leading to a mean lifespan slightly increased or unchanged compared to that of wild-type worms. This suggests that inhibiting cholinergic motor neurons may exert temporally distinct effects on survival, leading to decreased individual variation in lifespan. To test this model, we performed the converse experiment by promoting the output of cholinergic motor neurons. This was achieved by expressing in these neurons a transgene encoding syntaxin(T254I), a gain-of-function form of *Drosophila* syntaxin, another SNARE protein required for neuronal exocytosis[33]. Expression of this syntaxin mutant enhances synaptic release without causing vesicle depletion, thereby promoting neuronal output[33], an approach that has previously been successfully applied to manipulate neuronal output in lifespan assays[20]. This transgene simply shortened lifespan without causing a pleotropic effect (Figure

1B), and critically, without inducing motor neuron degeneration (Figure S1B). This suggests that enhancing the output from cholinergic motor neurons in early life may cause an irreversible detrimental effect that overwhelms its beneficial effect in mid-late life.

To further characterize the temporal effect of cholinergic motor neurons on lifespan, we sought to vary the output of cholinergic motor neurons in a temporally controlled manner. To do so, we employed an auxin-induced protein degradation (AID) system to express syntaxin(T254I) fused with a degron tag as a transgene in cholinergic motor neurons[34]. Transgenic worms were then crossed with a line stably expressing TIR1 in these neurons. TIR1 is the plant ubiquitin ligase, an essential component of the AID system[34]. Expression of syntaxin(T254I) can be suppressed by auxin treatment and induced by subsequently removing auxin. Comparable outcomes were obtained with both 0.1 mM and 0.5 mM auxin treatments (Figure S1C-1D). In doing so, we were able to potentiate the output of cholinergic motor neurons in a temporally controlled manner by expressing syntaxin(T254I) at different stages of worm life (Figure 1C). As a control, we first shut down the expression of *syntaxin(T254I)* transgene throughout the entire worm life by treating worms with auxin at all life stages, and found that this had no notable effect on lifespan (Figure 1D). Conversely, enhancing the output of cholinergic neurons throughout the entire worm life (no auxin treatment) shortened lifespan, consistent with the view that the detrimental effect resulting from enhanced motor neuron output in early life overwrites its beneficial effect at later stages (Figure 1E). Indeed, enhancing the output of cholinergic motor neurons during the larval stage only (no auxin from egg to L4) shortened lifespan, confirming the detrimental effect of motor neuron output on lifespan in early life (Figure 1F). Notably, this detrimental effect began to fade when motor neuron output was instead enhanced during the adult stage (auxin from egg to adult stage), and eventually worms became long-lived when motor neuron output was enhanced after Day 5 adulthood (Figure 1G-1L). These data reveal a dichotomous role of cholinergic motor neurons in lifespan regulation, shortening adult lifespan in early life but extending it in mid-late life.

ACh mediates the lifespan-shortening effect of cholinergic motor neurons in early life

How do cholinergic motor neurons differentially control lifespan at different life stages? First, we focused on their lifespan-shortening effect. Cholinergic motor neurons release both small neurotransmitters and neuropeptides[32,35-37]. To determine which type of signaling molecules might be released from motor neurons to shorten lifespan in early life, we first tested *unc-31* mutant worms, which are defective in secreting neuropeptides[38-40]. Loss of *unc-31* exhibited no defect on the ability of cholinergic motor neurons to shorten lifespan, indicating that neuropeptides may not play an important role in this process (Figure 2A and 2B). This led us to test small neurotransmitters. Cholinergic motor neurons release ACh, and indeed, loss of *unc-17* gene, which encodes the sole *C. elegans* vesicular ACh transporter[41], eliminated the lifespan-shortening effect of these motor neurons (Figure 2A and 2C). In *unc-17* mutant worms, no ACh is expected to be released from cholinergic motor neurons. As a control, we also tested mutants deficient in other types of small neurotransmitters, including glutamate (*eat-4*), GABA (*unc-25*), serotonin (*tph-1*), dopamine (*cat-2*), tyramine (*tdc-1*), and octopamine (*tbh-1*), but detected no effect, with the exception of *tph-1*, which showed a modest, partial suppression of the phenotype (Figure S2A-S2F). This observation suggests that the lifespan effects of cholinergic signaling can be modulated by serotonin. These results uncover a key role of the neurotransmitter ACh in mediating the lifespan-shortening effect of cholinergic motor neurons in early life.

The nicotinic AChR ACR-6 acts in the intestine to mediate the lifespan-shortening effect of cholinergic motor neurons

ACh is expected to act through ACh receptors (AChRs). To identify the AChRs that mediate the lifespan-shortening effect of cholinergic motor neurons, we examined all of the 37 *C. elegans* AChR genes, including nicotinic AChRs (nAChRs), muscarinic AChRs (mAChRs), and ACh-gated Cl^- channels[32]. Notably, RNAi of several ACh receptors such as *acr-11* appears to shorten wild-type

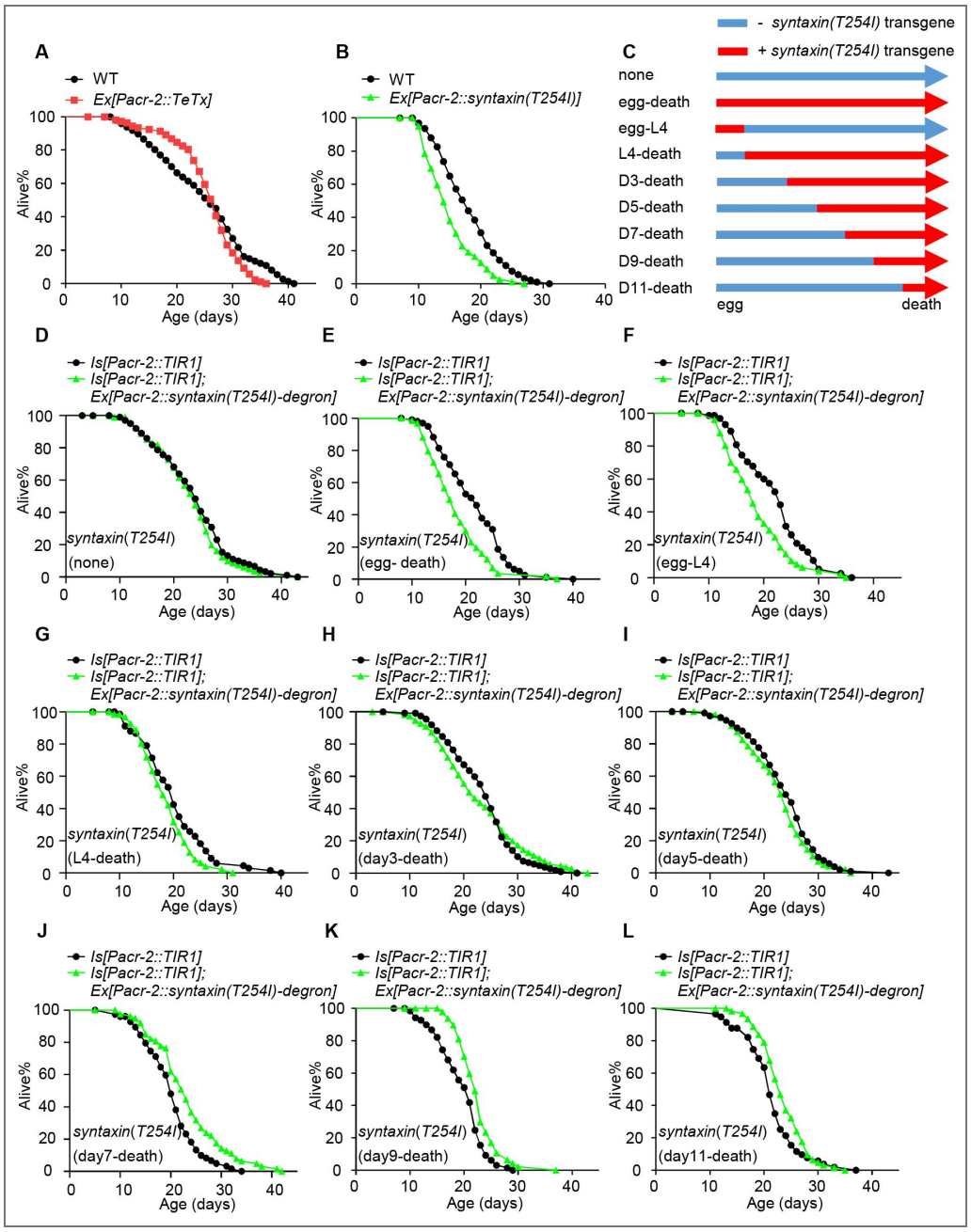

Figure 1. Cholinergic motor neurons differentially regulate lifespan at different stages of worm life.

(A) Ablating the output of cholinergic motor neurons promotes survival at early life stage, whereas inhibits survival at later stages. Tetanus toxin (TeTx) was expressed as a transgene in cholinergic motor neurons using *acr-2* promoter to block exocytosis from these neurons. (B) Promoting the output of cholinergic motor neurons shortens lifespan. The gain-of-function form of *Drosophila* syntaxin(T254I) was expressed as a transgene in cholinergic motor neurons using *acr-2* promotor to potentiate exocytosis from these neurons. (C) Schematic describing the assay. The gain-of-function form of *Drosophila* syntaxin(T254I) fused with a degron tag was expressed as a transgene in cholinergic motor neurons to promote exocytosis from these neurons. Transgenic worms were then crossed with a line stably expressing TIR1 in these neurons. Expression of Syntaxin(T254I) was suppressed by 0.5 mM auxin treatment and induced by subsequently removing auxin. (D) *Syntaxin(T254I)* transgene has slight effect on lifespan when suppressing its expression throughout the entire worm life. (E) *Syntaxin(T254I)* transgene shortens lifespan when inducing its expression throughout the entire worm life. (F) *Syntaxin(T254I)* transgene shortens lifespan when inducing its expression from egg to L4 larval stage. (G-I) *Syntaxin(T254I)* transgene modestly affects lifespan when inducing its expression from L4 larval stage (G), Day 3 (H) or Day 5 (I) adulthood. (J-K) *Syntaxin(T254I)* transgene extends lifespan when inducing its expression from Day 7 (J), Day 9 (K) or Day 11 (L) adulthood. See Table S1 for detailed statistical analysis of lifespan data. Logrank (Kaplan-Meier) was used to calculate p values.

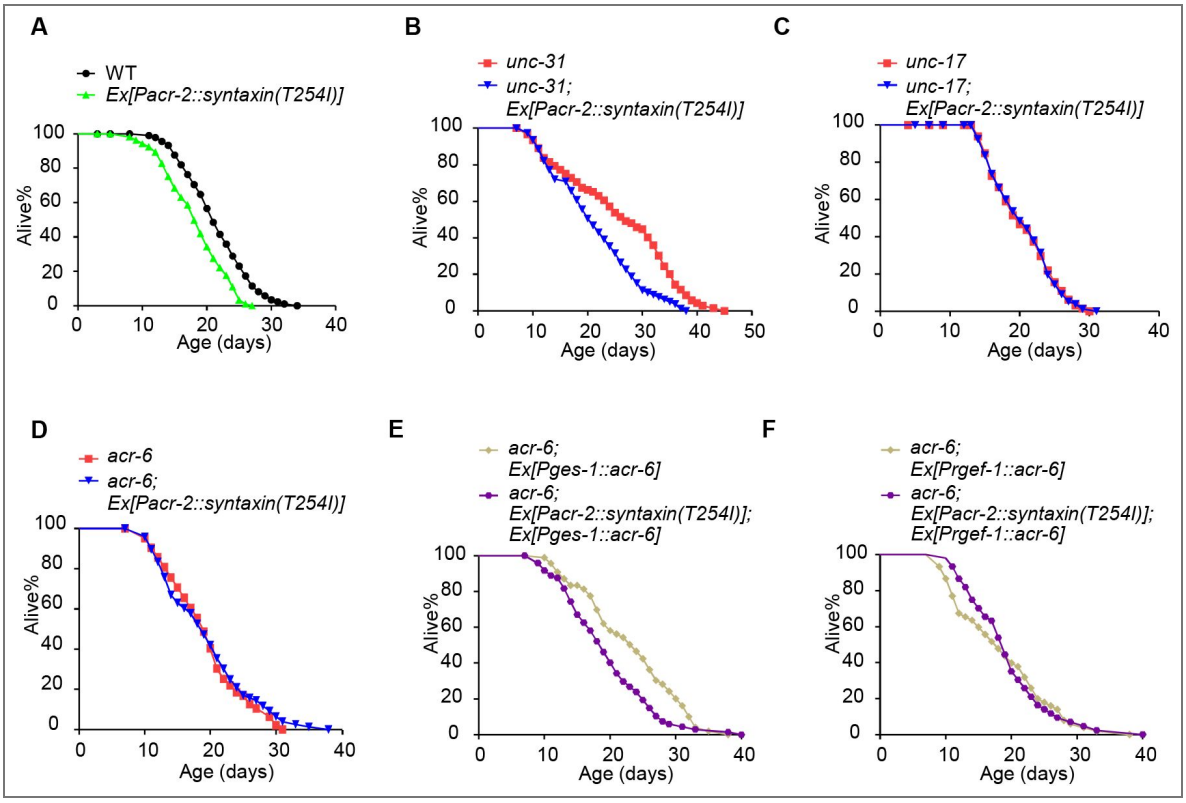

Figure 2. ACh and the nicotinic AChR ACR-6 mediate the lifespan-shortening effect of cholinergic motor neurons in early life.

(A) *Syntaxin(T254I)* transgene shortens lifespan. (B) Loss of *unc-31* exhibits no defect on the ability of *syntaxin(T254I)* transgene to shorten lifespan. (C) Loss of *unc-17* blocks the ability of *syntaxin(T254I)* transgene to shorten lifespan. (D-F) Loss of *acr-6* blocks the ability of *syntaxin(T254I)* transgene to shorten lifespan (D), a phenotype rescued by transgenic expression of wild-type *acr-6* gene in the intestine (E), but not in neurons (F). The *ges-1* and *rgef-1* promoters were used to drive *acr-6* gene expression in the intestine and neurons, respectively. See Table S1 for detailed statistical analysis of lifespan data. Logrank (Kaplan-Meier) was used to calculate p values.

lifespan, whereas RNAi of several other ACh receptors such as *acr-9* extends wild-type lifespan, suggesting lifespan-modulating potential of ACh receptors (Figure S3). By screening strains with each of these 37 AchRs knocked down by RNAi, we identified three candidate genes: *acr-6* (nAChR), *acr-8* (nAChR) and *gar-2* (mAChR) (Figure S3). To validate these candidates, we examined their mutants. Mutation in *acr-6* blocked the ability of cholinergic motor neurons to shorten lifespan, while mutations in the other two candidates did not (Figure 2D and Figure S4A-S4F). This identifies a key role for the nAChR ACR-6 in mediating the lifespan-shortening effect of cholinergic motor neurons. Previous data has shown that cholinergic signaling and ACR-6 may control pharyngeal pumping[42]. As expected, we found that *acr-6* mutation slightly reduced pumping rates (Figure S4G).

We then asked in which tissue ACR-6 regulates lifespan. Transgenic expression of wild-type *acr-6* gene in the intestine (Figure 2E), but not in neurons (Figure 2F), rescued the *acr-6* mutant phenotype, indicating that ACR-6 acts in the intestine. This suggests that ACh released from cholinergic motor neurons may shorten lifespan by acting on its cognate receptor ACR-6 in the intestine, revealing a nervous system-to-gut signaling axis.

Cholinergic motor neurons require the transcription factor DAF-16 in the intestine to regulate lifespan

Aging pathways tend to converge on a subset of transcription factors[12]. We went on to search for a transcription factor that functions in the intestine to mediate the lifespan-shortening effect of cholinergic motor neurons in early life. RNAi of *daf-16*, which encodes a FOXO transcription factor that is a key regulator of lifespan, did not further shorten the lifespan of worms expressing *syntaxin(T254I)* transgene (Figure 3A and 3B); as a control, RNAi of other transcription factor such as *hsf-1*/HSF1, *skn-1*/Nrf2 *and pha-4*/FOXA did (Figure 3C-3E). This suggests that *daf-16* may act in the same pathway as cholinergic motor neurons. DAF-16 regulates lifespan by controlling the expression of its target genes. As expected, the expression level of *sod-3* and *mtl-1*, two commonly characterized DAF-16 target genes, was upregulated in transgenic worms deficient in releasing ACh from cholinergic motor neurons (Figure 3F), and downregulated in transgenic worms with enhanced ACh release from cholinergic motor neurons (Figure S5A), consistent with the notion that DAF-16 acts downstream of cholinergic motor neurons. To obtain further evidence, we assessed the subcellular localization pattern of DAF-16::GFP fusion and found that *acr-6* RNAi notably promoted nuclear translocation of DAF-16, confirming that ACh signaling inhibits DAF-16 activity (Figure S5B).

DAF-16 is known to regulate lifespan primarily in the intestine[4]. To determine whether DAF-16 also functions in the intestine, we performed tissue specific RNAi experiments in *sid-1* mutant background to restrict RNAi to specific tissues. dsRNA against *daf-16* was expressed as a transgene specifically in the intestine. RNAi of *daf-16* in the intestine abolished the ability of cholinergic motor neurons to regulate lifespan (Figure 3G, 3H and Figure S5C-5E). Thus, similar to ACR-6, DAF-16 also appears to function in the intestine, suggesting that the intestine is an important target tissue to which cholinergic motor neurons signals to regulate lifespan. It also suggests that cholinergic motor neurons shorten lifespan by regulating DAF-16 activity in the intestine.

Collectively, the data presented above suggests a model in which cholinergic motor neurons regulate lifespan through a neuroendocrine signaling circuit (Figure 6D, below). In this circuit, cholinergic motor neurons signal the intestine via the neurotransmitter ACh that acts through its cognate receptor ACR-6 to regulate DAF-16 in the intestine, leading to a shortened lifespan (Figure 6D). Because this lifespan-shortening effect results from enhanced motor neuron output in early life and overwrites its beneficial effect at later stages, we propose this signaling circuit mediates the lifespan-shortening effect in early life.

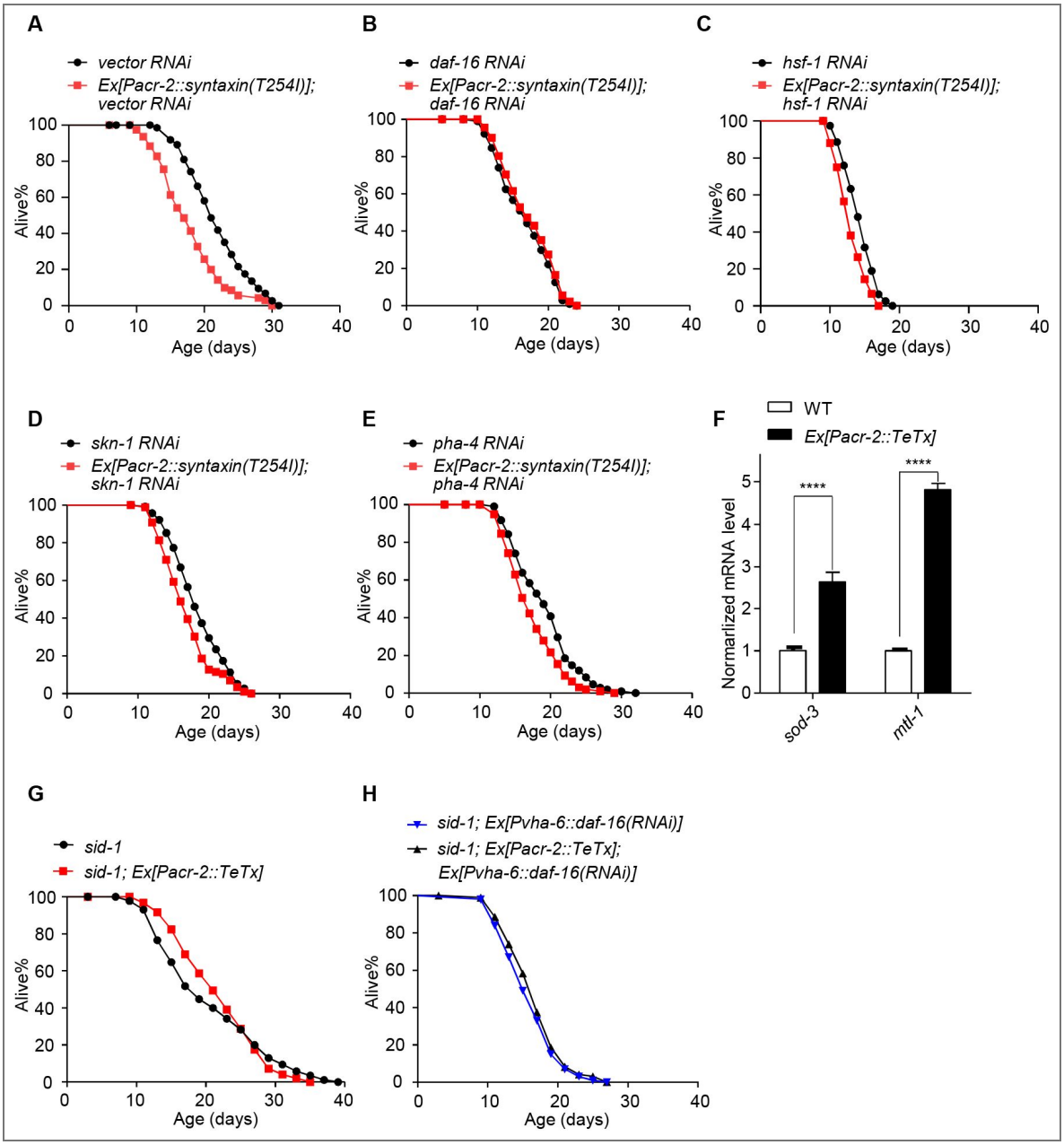

Figure 3. The FOXO transcription factor DAF-16 is required in the intestine for cholinergic motor neurons to regulate lifespan in early life.

(A) *Syntaxin(T254I)* transgene shortens lifespan. (B-E) RNAi of *daf-16* does not further shorten the lifespan of *syntaxin(T254I)* transgenic worms, while RNAi of *hsf-1* (C), *skn-1* (D) and *pha-4* (E) does. (F) qPCR analysis of DAF-16 target genes. qPCR reactions were run in triplicates for each genotype. Day 1 adult worms were detected. Each experiment was repeated three times. Error bars represent s.e.m. ****p< 0.0001 (ANOVA with Bonferroni's test). (G-H) RNAi of *daf-16* in the intestine (H) abolishes the ability of TeTx transgene to regulate lifespan (G). dsRNA against *daf-16* was expressed as a transgene specifically in the intestine using *vha-6* promoter. These experiments were carried out in a *sid-1* mutant background where systemic effect of RNAi is absent. See Table S1 for detailed statistical analysis of lifespan data. Log-rank (Kaplan-Meier) was used to calculate p values.

ACh mediates the lifespan-extending effect of cholinergic motor neurons in mid-late life

Having characterized the mechanisms by which cholinergic motor neurons signal the intestine to shorten lifespan in early life, we then set out to explore how these motor neurons extend lifespan in mid-late life. Given that ACh is the only neurotransmitter known to be released by these motor neurons and its role in mediating motor neuron-to-gut signaling in early life, we asked whether ACh also plays a role in mediating the lifespan-extending effect of these motor neurons in mid-late life. Again, we resorted to the AID system to specifically enhance the output of cholinergic motor neurons in mid-late life by temporally restricting the expression of *syntaxin(T254I)* transgene at this stage. We found that cholinergic motor neurons lost the ability to extend lifespan at mid-late life stage in *unc-17* mutant worms that are deficient in ACh release, revealing an essential role of ACh in mediating the lifespan-extending effect of these motor neurons in mid-late life and suggesting it as a key signaling molecule in this process (Figure 4A and 4B). Thus, the same neurotransmitter ACh appears to mediate both the lifespan-shortening and extending effects of cholinergic motor neurons.

The muscarinic AChR GAR-3 acts in the intestine to mediate the lifespan-extending effect of cholinergic motor neurons in mid-late life

We then asked how the same neurotransmitter ACh secreted from the same type of neurons mediates two opposite effects on lifespan. To address this question, we re-screened all the 37 AChR genes (Figure S6). Two candidate genes emerged from the screen: *gar-2* and *gar-3*, which encode two different mAChRs. RNAi of *gar-2* or *gar-3* completely suppressed the ability of cholinergic motor neurons to extend lifespan in mid-late life (Figure S7A-S7C). *gar-2* and *gar-3* mutant worms exhibited a similar phenotype (Figure S7D, S7E and Figure 4C), suggesting that GAR-2 and GAR-3 may be the receptors for ACh that mediate the lifespan-extending effect of cholinergic motor neurons.

To determine in which tissue GAR-2 and GAR-3 act to regulate lifespan, we specifically knocked down *gar-2* and *gar-3* genes in the intestine and neurons, as both genes are known to be expressed in these two tissues[43-46]. RNAi of *gar-3* in the intestine (Figure 4D and 4E), but not in neurons or the muscle (Figure 4D-4F, and Figure S8A, S8D-S8E), abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stage. Thus, GAR-3 may function in the intestine to regulate lifespan. Surprisingly, RNAi of *gar-2* in the muscle (Figure S8A-S8C), but not in neurons or the intestine (Figure S7F-S7H) had an effect on the ability of cholinergic motor neurons to extend lifespan in mid-late life, indicating that GAR-2 acts in the muscle to regulate lifespan. Given our focus on neuron-gut signaling, we decided to focus on GAR-3 for further characterizations. These results identify GAR-3 as a candidate AChR that acts in the intestine to mediate the effect of the neurotransmitter ACh and cholinergic motor neurons on lifespan extension in mid-late life.

Cholinergic motor neurons require the transcription factor HSF-1 in the intestine to regulate lifespan in mid-late life

In light of our observation that the transcription factor DAF-16 acts in the intestine to regulate lifespan in early life, we wondered if DAF-16 also regulates lifespan in mid-late life. Surprisingly, RNAi of *daf-16* and other transcription factors such as *skn-1* and *pha-4* failed to suppress the lifespan-extending effect of cholinergic motor neurons (Figure S9). By contrast, RNAi of *hsf-1* completely abolished the ability of these motor neurons to extend lifespan in mid-late life (Figure 5A and 5B), suggesting that HSF-1, rather than DAF-16, is required by cholinergic motor neurons to regulate lifespan at mid-late life stage. Additional support came from the observation that the expression of *hsp-16.2* and *pat-10*, two commonly characterized HSF-1 target genes, were upregulated in long-lived worms in which the output of cholinergic motor neurons was enhanced at mid-late life stage (Figure 5C). HSF-1 is known to regulate lifespan primarily in the intestine

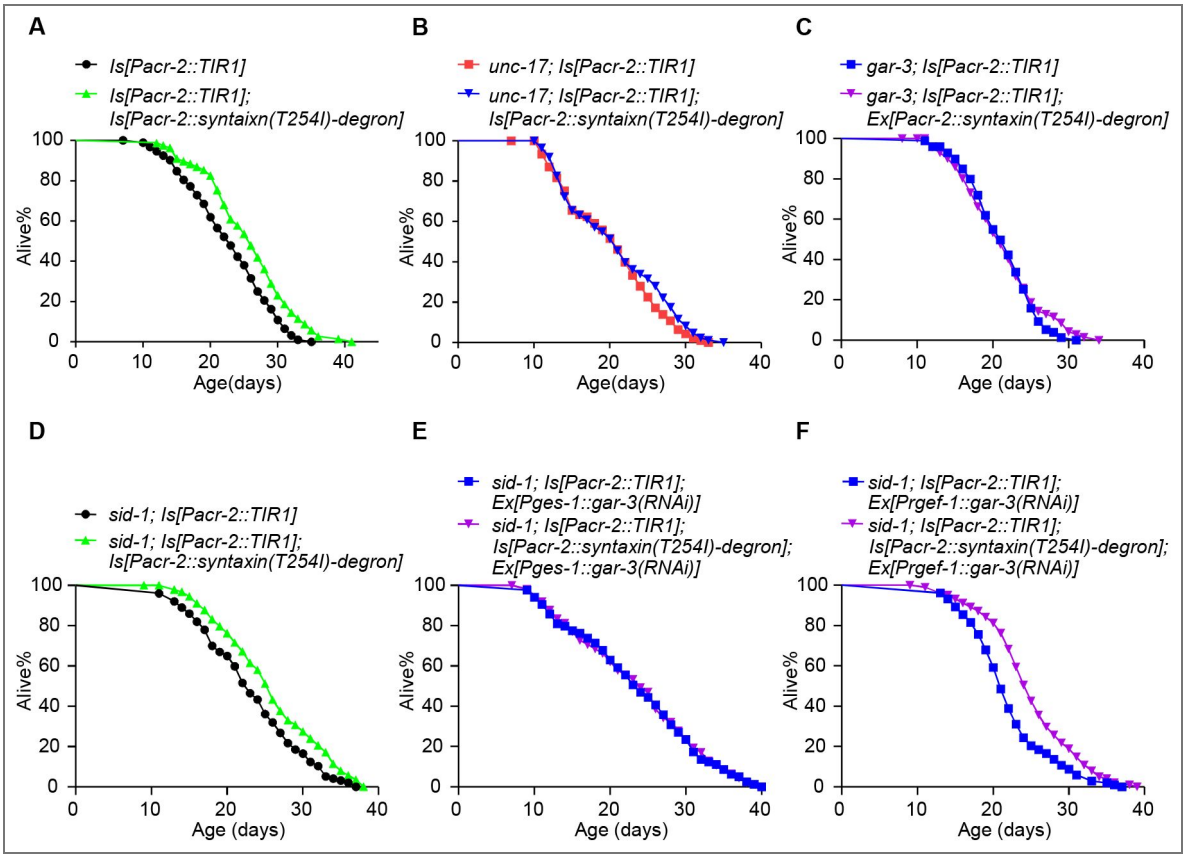

Figure 4. ACh and the intestinal muscarinic AChR GAR-3 mediate the lifespan-extending effect of cholinergic motor neurons in mid-late life.

(A) Enhancing the output of cholinergic motor neurons in mid-late life extends lifespan. The *syntaxin(T254I)* transgene was induced from Day 7 adulthood as described in Figure 1C. (B-C) Loss of *unc-17* (B) or *gar-3* (C) blocks the ability of cholinergic motor neurons to extend lifespan in mid-late life. (D-F) RNAi of *gar-3* in the intestine (E), but not in neurons (F), abolishes the ability of cholinergic motor neurons to extend lifespan in mid-late life (D). dsRNA against *gar-3* was expressed as a transgene specifically in the intestine and neurons using *ges-1* and *rgef-1* promoter, respectively. These experiments were carried out in a *sid-1* mutant background where systemic effect of RNAi is absent. See Table S1 for detailed statistical analysis of lifespan data. Log-rank (Kaplan-Meier) was used to calculate p values.

and neurons[47,48]. To identify in which tissue HSF-1 functions, we inactivated *hsf-1* specifically in the intestine or neurons by RNAi (Figure 5D-5F). RNAi of *hsf-1* in the intestine but not in neurons abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stage. Thus, HSF-1 appears to function in the intestine to regulate lifespan, suggesting that cholinergic motor neurons extend lifespan in mid-late life through HSF-1 in the intestine.

In summary, this set of data suggests a model in which cholinergic motor neurons signal the distal tissue intestine in mid-late life to extend lifespan through a distinct neuroendocrine circuit (Figure 6D). Similar to the lifespan-shortening circuit described above, this circuit is also formed by cholinergic motor neurons and the intestine, and employs the same neurotransmitter ACh; however, it recruits a different AChR GAR-3 to regulate a different type of transcription factor HSF-1 in the intestine, leading to the opposite outcome in lifespan (Figure 6D).

Temporal control of ACR-6 and GAR-3 expression in the intestine at different life stages

The identification of two distinct neuroendocrine circuits provides a potential explanation to the opposing effects of cholinergic motor neurons on lifespan at different life stages. However, it prompts an important question as to how these two circuits are temporally controlled at different stages of worm life. One possibility is that the expression of some components in the two circuits are temporally regulated at different life stages. To test this idea, we sought to examine the expression of the two AChRs ACR-6 and GAR-3 in the intestine. Using CRISPR/Cas9 genome editing, we inserted a mCherry tag at the C-terminal end of the *acr-6* and *gar-3* gene to label the endogenous ACR-6 and GAR-3 protein. At the larval stage, ACR-6::mCherry fluorescence was detected in the intestine, while GAR-3::mCherry was not (Figure 6A). Remarkably, beginning at the adult stage, ACR-6::mCherry expression started to decrease and became undetectable at Day 2 adulthood (Figure 6B). On the other hand, GAR-3::mCherry began to show expression at the adult stage and became prominent at Day 2-4 adulthood (Figure 6A and 6B). We were unable to examine GAR-3::mCherry expression at Day 5 adulthood and beyond, due to the interference by increasingly bright gut auto-fluorescence even in the *glo-4(ok623)* background with reduced gut auto-fluorescence[49] (Figure S10). Nevertheless, these results unveil a clear temporal switch in the intestinal expression of ACR-6 and GAR-3 at different life stages (Figure 6C). Though additional mechanisms may also contribute, such a dichotomy in the intestinal expression of ACR-6 and GAR-3 at larval and adult stages provides a potential mechanism that underlies the temporal control of the two neuroendocrine circuits, leading two opposing outcomes in lifespan.

Discussion

Research in the past decade has revealed an increasingly important role of the nervous system in orchestrating a body-wide aging process by signaling distal tissues, particularly the gut[3,4,11,12,14-19,30]. How the nervous system signals the gut to regulate aging, however, is not well understood, and work in this area has yielded some seemingly inconsistent data. This points to a model in which nervous system-gut signaling can both positively and negatively regulate aging, highlighting a complex role of the nervous system-gut signaling axis in aging[17,20,30]. Nervous system-gut communications may differentially regulate aging through spatial mechanisms. For example, different neurons may play distinct roles in aging by positively and/or negatively regulating longevity via secreting distinct neurotransmitters and neuropeptides[17,18,20,50-52]. By sharp contrast, whether and how the nervous system temporally regulates aging is poorly understood. Here, using the *C. elegans* motor nervous system as a model, we identified a temporal mechanism by which the nervous system both positively and negatively regulates lifespan by temporally controlling nervous system-to-gut signaling at different life stages. Specifically, we identified two distinct neuroendocrine signaling circuits by which cholinergic motor neurons act to shorten lifespan in early life, whereas extending lifespan in mid-late life. The two circuits employ the same neurotransmitter ACh, but recruit two different AChRs ACR-6 and GAR-3 that act in the intestine to regulate two different transcription factors DAF-16 and HSF-1 to shorten and extend lifespan at early and mid-late life stage, respectively. To the best of our knowledge, this represents the first

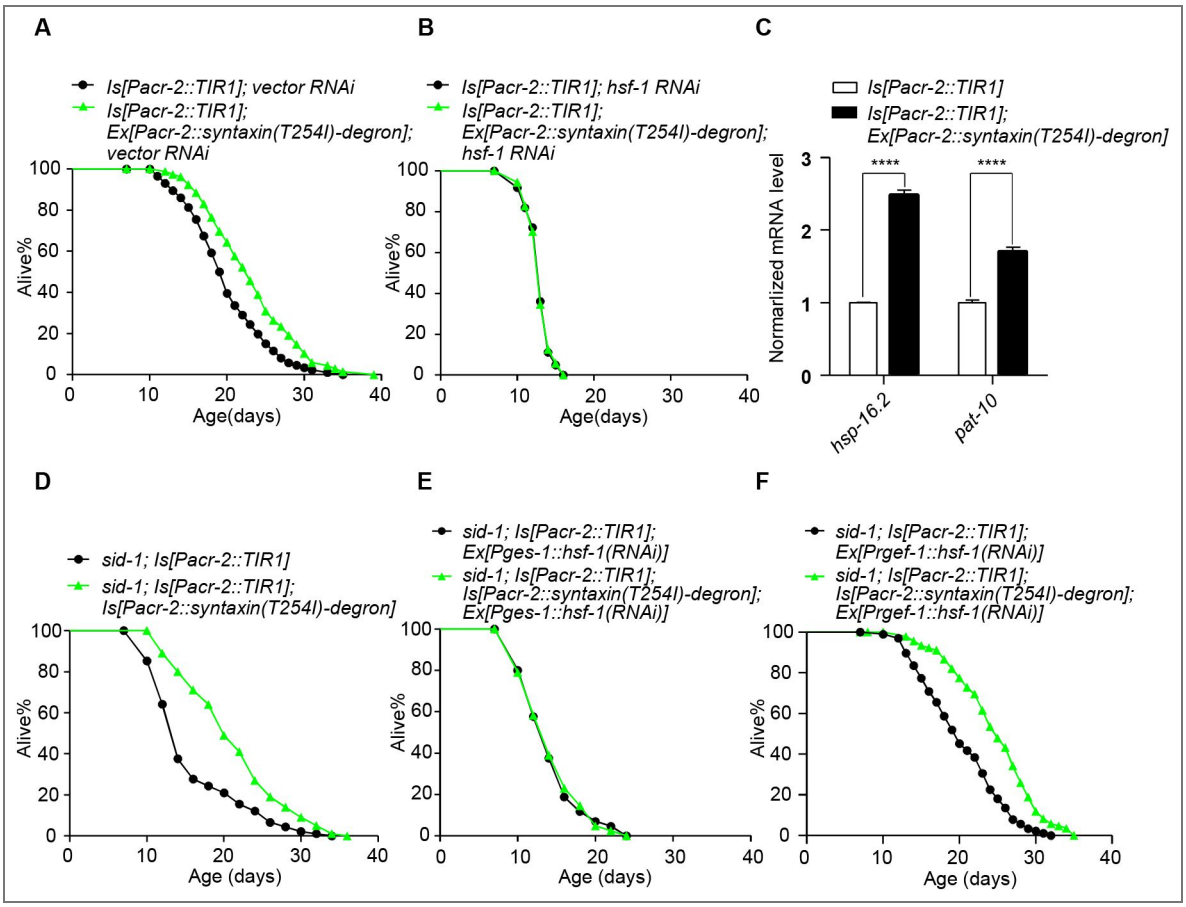

Figure 5. The transcription factor HSF-1 is required in the intestine for cholinergic motor neurons to regulate lifespan in mid-late life.

(A-B) Enhancing the output of cholinergic motor neurons in mid-late life extends lifespan (A). This effect was fully suppressed by RNAi of *hsf-1* (B). The *syntaxin(T254I)* transgene was induced from Day 7 adulthood as described in Figure 1C. (C) qPCR analysis of HSF-1 target genes. qPCR reactions were run in triplicates for each genotype. Day 10 adult worms were detected. Each experiment was repeated three times. Error bars represent s.e.m. ****p< 0.0001 (ANOVA with Bonferroni's test). (D-F) RNAi of *hsf-1* in the intestine (E), but not in neurons (F), abolishes the ability of cholinergic motor neurons to extend lifespan in mid-late life (D). dsRNA against *hsf-1* was expressed as a transgene specifically in the intestine and neurons using *ges-1* and *rgef-1* promoter, respectively. These experiments were carried out in a *sid-1* mutant background where systemic effect of RNAi is absent. See Table S1 for detailed statistical analysis of lifespan data. Logrank (Kaplan-Meier) was used to calculate p values.

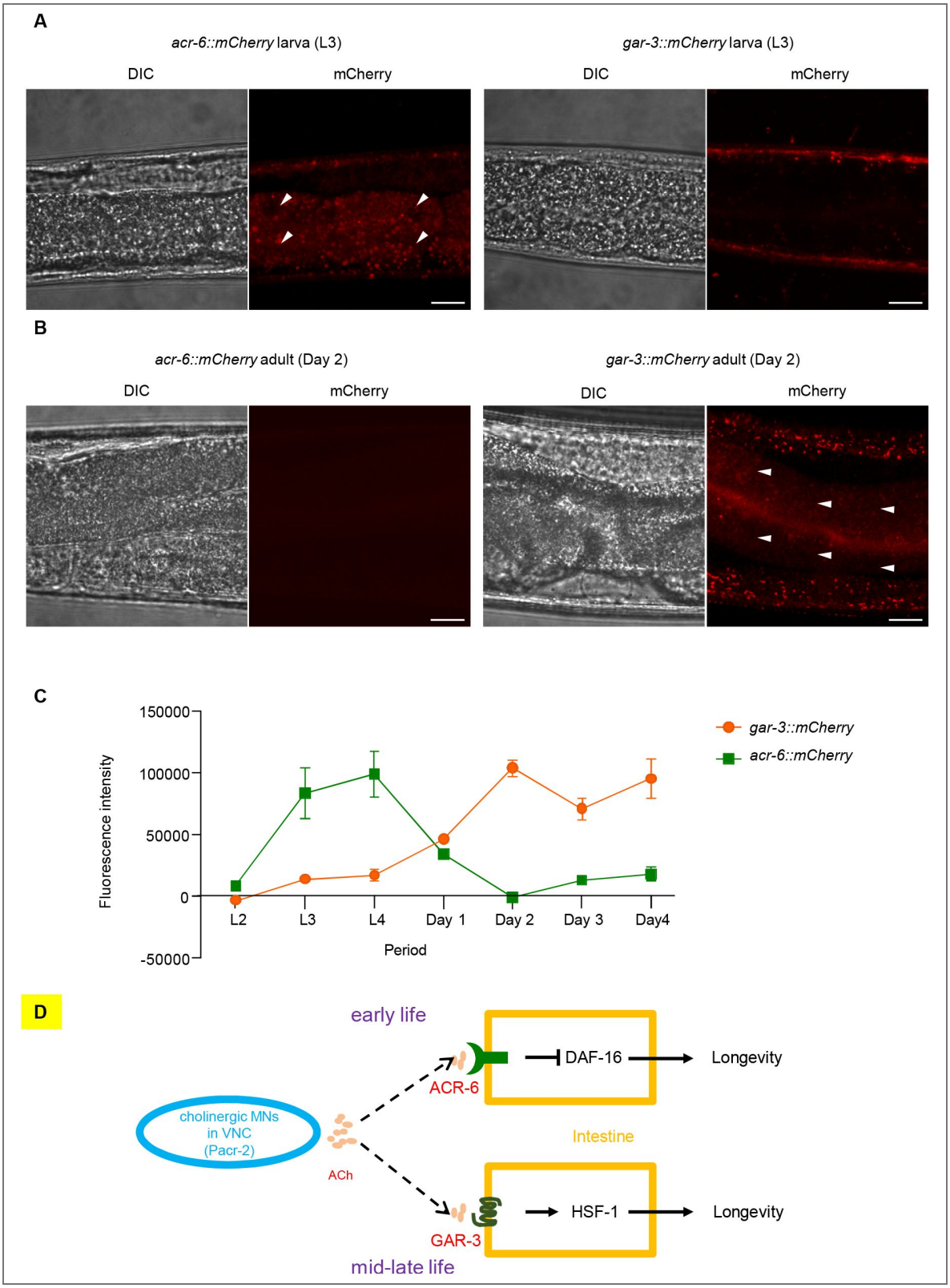

Figure 6. Temporal control of ACR-6 and GAR-3 expression in the intestine at different life stages.

(A-B) The expression pattern of endogenous ACR-6 and GAR-3 proteins at L3 Larva stage (A) and Day 2 adult stage (B). Arrowheads point to the area of the intestine. Scale bars, 20 μm. (C) Quantification curves summarizing the data in (A) and (B). The entire intestinal area was selected for measurement. Error bars represent s.e.m. n ≥ 10. (D) A schematic model illustrating the two cholinergic motor neurons-to-gut signaling circuits that act in early life to shorten lifespan and in mid-late life to extend lifespan.

such example illustrating how the nervous system may differentially regulate lifespan by temporally controlling nervous system-to-gut signaling. Nevertheless, it is still unclear whether other neuronal populations share similar temporal regulatory mechanisms. A previous study reported that inhibiting cholinergic neurons activity (using *unc-17* promoter) extends lifespan regardless of timing[24], which is different to the temporal lifespan regulation we observed in cholinergic motor neurons (using *acr-2* promoter). This discrepancy is likely due to differences in subsets of neurons, as the *unc-17* promoter labels a broad repertoire of cholinergic neurons, while the *acr-2* promoter mainly marks cholinergic motor neurons[53]. Thus, the distinct lifespan-modulating effects of cholinergic motor neurons may be overshadowed by opposing contributions from other cholinergic subtypes when a mixed population is manipulated. Alternatively, both activation and inhibition of cholinergic activity may perturb neurotransmission balance, leading to similar effects on lifespan[54]. It will be interesting to test these hypotheses in future studies.

One interesting observation is that the expression level of ACR-6 and GAR-3 in the intestine features a temporal switch at the larval and adult stages. The appearance followed by disappearance of ACR-6 expression in the intestine at the larval and adult stages nicely correlate with the temporal window under which cholinergic motor neurons shorten lifespan. This provides a potential mechanism by which cholinergic motor neurons shorten lifespan in early life. Remarkably, the temporal expression pattern of GAR-3 in the intestine follows a reciprocal pattern: it is not expressed at the larval stage and only appears at the adult stage, providing a potential mechanism for the lifespan-extending effect exerted by cholinergic motor neurons in mid-late life. It should be pointed out that the intestinal expression of GAR-3 emerges at Day 1 adulthood, earlier than the time point at which the beneficial role of cholinergic motor neurons began to take effect. Several factors might have contributed to this delay. For example, the lifespan-shortening signaling in the intestine might not have been completely shut off during early adulthood, particularly considering that the role of the transcription factor DAF-16 might be long-lasting. This might antagonize the effect of the lifespan-extending signaling initiated by GAR-3 in early adulthood. Additionally, though GAR-3 expression can be detected in early adulthood, it might take some time for it to fully engage other signaling components, which might cause a delay for the lifespan-extending signaling to take effect. Future studies are needed to elucidate the mechanism that directs the temporal switch of ACR-6 and GAR-3 expression in the intestine.

Though the temporally-controlled dichotomous expression pattern of ACR-6 and GAR-3 in the intestine provides a potential mechanism underlying the opposing effects of cholinergic motor neurons on longevity, we do not exclude the possibility that additional mechanisms may apply. For example, the expression pattern of other components in the two neuroendocrine circuits may undergo a similar temporal shift; similarly, their function may also be subjected to temporal control. An alternative mechanism based on differential levels of cholinergic signaling could also contribute to the observed lifespan effects. Our work provides an entry point to understand how temporal mechanisms may contribute to the complex roles of the nervous system as well as nervous system-gut communications in aging.

Materials and methods

Strains and genetics

Wild-type: N2. *Ex263a[Pacr-2::TeTx::sl2::yfp]. Is4a[Pacr-2::TIR1::sl2::yfp]. Is4a; Ex329a[Pacr-2::syntaxin(T254I)-degron::sl2::mCherry]. Ex294a[Pacr-2::syntaxin(T254I)::sl2::mCherry]. unc-31(e169). unc-31; Ex294a. cat-2(e1112). cat-2; Ex294a. tbh-1(n3247). tbh-1; Ex294a. tph-1(mg280). tph-1; Ex294a. eat-4(ky5). eat-4; Ex294a. unc-25(e156). unc-25; Ex294a. tdc-1(n3419). tdc-1; Ex294a. unc-17(e245). unc-17; Ex294a. acr-6(ok3117). acr-6; Ex294a. acr-8(ok1240). acr-8; Ex294a. gar-2(ok520). gar-2; Ex294a. acr-6; Ex321a[Pges-1::acr-6::sl2::yfp]. acr-6; Ex294a; Ex321a. acr-6; Ex324a[Prgef-1::acr-6::sl2::yfp]. acr-6; Ex294a; Ex324a. sid-1(qt9). sid-1; Ex263a. sid-1; Ex380a[Pvha-6::daf-16 (RNAi)+Pvha-6::sl2::mCherry]. sid-1; Ex263a; Ex380a. sid-1; Ex371a[Prgef-1::daf-16 (RNAi)+Prgef-1::sl2::mCherry]. sid-1; Ex263a; Ex371a. Is4a; Is5a[Pacr-2::syntaxin(T254I)-degron::sl2::mCherry]. unc-17; Is4a. unc-17; Is4a; Is5a. gar-3(gk337); Is4a. gar-3; Is4a; Ex329a. sid-1; Is4a. sid-1; Is4a; Is5a.*

sid-1; Is4a; Ex359a[Pges-1::gar-3(RNAi)+Pges-1::sl2::cfp]. sid-1; Is4a; Is5a; Ex364a[Pges-1::gar-3(RNAi)+Pges-1::sl2::cfp]. sid-1; Is4a; Ex395a[Prgef-1::gar-3(RNAi)+Prgef-1::sl2::cfp]. sid-1; Is4a; Is5a; Ex398a[Prgef-1::gar-3(RNAi)+Prgef-1::sl2::cfp]. gar-2; Is4a. gar-2; Is4a; Ex329a. sid-1; Is4a; Ex357a[Pges-1::gar-2(RNAi)+Pges-1::sl2::cfp]. sid-1; Is4a; Is5a; Ex363a[Pges-1::gar-2(RNAi)+Pges-1::sl2::cfp]. sid-1; Is4a; Ex390a[Prgef-1::gar-2(RNAi)+Prgef-1::sl2::cfp]. sid-1; Is4a; Is5a; Ex392a[Prgef-1::gar-2(RNAi)+Prgef-1::sl2::cfp]. sid-1; Is4a; Ex373a[Pges-1:hsf-1(RNAi)+Pges-1::sl2:cfp]. sid-1; Is4a; Is5a; Ex374a[Pges-1:hsf-1(RNAi)+Pges-1::sl2:cfp]. sid-1; Is4a; Ex384a[Prgef-1::hsf-1(RNAi)+Prgef-1::sl2::cfp]. sid-1; Is4a; Is5a; Ex385a[Prgef-1::hsf-1(RNAi)+Prgef-1::sl2::cfp]. acr-6(xu129a[acr-6::mCherry]). gar-3(xu130a[gar-3::mCherry]). ieSi57[Peft-3::TIR1]. ieSi57[Peft-3::TIR1]; Ex329a. Is4a; Ex847a[Pvha-6::daf-16 (RNAi)+Pvha-6::sl2::mCherry] line 1. Is4a; Is5a; Ex847a[Pvha-6::daf-16 (RNAi)+Pvha-6::sl2::mCherry] line 1. Is4a; Ex848a[Pvha-6::daf-16 (RNAi)+Pvha-6::sl2::mCherry] line 2. Is4a; Is5a; Ex848a[Pvha-6::daf-16 (RNAi)+Pvha-6::sl2::mCherry] line 2. Is4a; Ex859a[Pmyo-3::gar-2(RNAi)+Pmyo-3::sl2::mCherry] line 1. Is4a; Is5a; Ex859a[Pmyo-3::gar-2(RNAi)+Pmyo-3::sl2::mCherry] line 1. Is4a; Ex860a[Pmyo-3::gar-2(RNAi)+Pmyo-3::sl2::mCherry] line 2. Is4a; Is5a; Ex860a[Pmyo-3::gar-2(RNAi)+Pmyo-3::sl2::mCherry] line 2. Is4a; Ex857a[Pmyo-3::gar-3(RNAi)+Pmyo-3::sl2::mCherry] line 1. Is4a; Is5a; Ex857a[Pmyo-3::gar-3(RNAi)+Pmyo-3::sl2::mCherry] line 1. Is4a; Ex858a[Pmyo-3::gar-3(RNAi)+Pmyo-3::sl2::mCherry] line 2. Is4a; Is5a; Ex858a[Pmyo-3::gar-3(RNAi)+Pmyo-3::sl2::mCherry] line 2. zIs356[daf-16::gfp + roller].

Mutant strains were outcrossed at least four times before use. The *acr-6, acr-8, acr-15, acr-16, acr-19, acr-21, acr-24, gar-1, acc-1, acc-2, acc-4, deg-3, lev-8, pbo-5, eat-2* and *unc-63* RNA interference (RNAi) clones were generated in the laboratory, while other RNAi clones were from the Ahringer library and were confirmed by sequencing.

Microinjections were performed using standard protocols. Each plasmid DNA listed above in the transgenic line was injected at a concentration of 50 ng/μL. Each marker for RNAi was co-injected at a concentration of 25 ng/μL.

Worm synchronization

Strains were grown at 20°C for at least three generations before lifespan determination. Twenty Day 2 adult worms were transferred to fresh 60 mm nematode growth medium (NGM) plates. The adult worms were removed from the plates after laying eggs for 4 hours. The plates were placed at 20°C for two days. L4 larva were used to start the normal lifespan or RNAi assays.

Lifespan assay

Lifespan assays were performed on 60 mm NGM plates at 20°C as previously described[15]. ~120 worms of each genotype were used for lifespan assays and transferred every other day to fresh NGM plates. Survival rate was scored every 1 – 2 days. Worms were censored if they crawled off the plate, bagged, or exhibited protruding vulva. In all cases, the first day of adulthood was scored as day 1. Lifespan data was analyzed with GraphPad Prism 8 (GraphPad Software, Inc.) and IBM SPSS Statistics 21 (IBM, Inc.). Log-rank (Kaplan-Meier) was used to calculate *P* values. All lifespan assays were repeated at least twice. 2 μg/mL 5-Fluoro-2'-deoxyuridine (FUDR) was included in assays involving TeTx transgene worms, *unc-31* and *unc-17* mutant worms, which show a defect in egg laying.

RNA interference (RNAi) assay

RNAi was performed using the RNAi-compatible OP50 bacterial strain OP50(xu363) and HT115 as previously described[55]. RNAi plates included carbenicillin (25 μg/mL) and isopropyl-β-D-thiogalactoside (0.5 mM). OP50(xu363) or HT115 bacteria with vector or RNAi plasmid were seeded on RNAi plates 2 days before experiment.

Auxin treatment

Auxin treatment was performed by transferring worms to bacteria-seeded NGM plates containing 0.5 mM natural auxin indole-3-acetic acid (G&K Scientific) as previously described[34]. Auxin was dissolved in cholesterol solution (25 g/L) and KP buffer (2.5 g/L) to avoid additional ethanol.

Cholesterol solution and KP buffer with auxin were added to NGM agar that has cooled down to about 60°C before pouring the plates. Expression of syntaxin(T254I) can be suppressed by auxin treatment and restored in 24 hours following auxin removal.

Microscopy

Different stage worms were anesthetized with 5 mM sodium azide (Sigma) on 2% agarose pads. Confocal images were acquired by FLUOVIEW FV3000 series of confocal laser scanning microscopes (Olympus) equipped with a 100X objective as Z-stacks and were processed using ImageJ (NIH). Maximum intensity Z-projection images were shown to illustrate the endogenous ACR-6 and GAR-3 protein expression pattern. To quantify the ACR-6 and GAR-3 protein expression level in the intestine, maximum intensity Z-projection were used and the entire intestine area were selected by ImageJ.

CRISPR genome editing

ACR-6::mCherry and GAR-3::mCherry are both knockin alleles made by injecting RNP mixtures as described previously[56]. In brief, repair templates were amplified using unmodified primers with 70 bp homology arms against the target genes from linker-mCherry plasmid. The RNP mixture was assembled at 37°C for 15 min and contained Cas9 protein, tracrRNA, and crRNA (all from IDT). crRNA corresponding DNA target sequences are 5'-ATTCACGGATTGATAGCAAT-3' (*acr-6*), and 5'-ATTCACGGATTGATAGCAAT-3' (*gar-3*). The double-strand donor templates were melted right before adding them to the assembled RNPs [57]. Microinjection quality was scored using a co-injection marker, PRF4::*rol-6 (su1006)* plasmid. In general, 50 P0 were injected, singled, and maintained at 25°C for 3 days. Two plates with the highest number of F1 rollers (usually more than 20 rollers for a good injection) were selected, and 48 F1 rollers from each plate were picked and singled for further genotyping[56]. The positive insertions were confirmed by Sanger sequencing. Using this CRISPR-RNP technology, the coding sequence of mCherry and a 27 bp linker sequence were inserted immediately after the last amino acid codon of the *acr-6* gene on chromosome I and of *gar-3* gene on chromosome V, respectively.

Levamisole paralysis assay

Worms were synchronized at the L4 larval stage one day prior to the assay. The following day, 20-30 adult worms were transferred to a plate with 1 mM levamisole hydrochloride and prodded every 10 minutes for 2 hours to assess movement. Worms that showed no response to this harsh touch were classified as paralyzed.

Nuclear translocation of DAF-16::GFP

L3 larva stage worms were anesthetized with 5 mM sodium azide (Sigma) on 2% agarose pads. Confocal images were acquired by FLUOVIEW FV3000 series of confocal laser scanning microscopes (Olympus) equipped with a 20X objective as Z-stacks. Image processing and quantification of DAF-16::GFP nuclear localization were carried out using ImageJ (NIH). For each worm, the nuclear localization level was calculated as the percentage of intestinal cells showing nuclear GFP signal.

Data availability

The datasets generated and analyzed during this study are either included within the manuscript or are available from the authors upon request.

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Additional information

Author contributions

L.X., C.H. and L.C. conducted the experiments and analyzed the data. L.X., C.H., L.C., X.Z.S.X., and J.L. interpreted the data. L.C., X.Z.S.Z., and J.L. wrote the article with assistance from other authors.

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Additional files

Supplemental Figure 1-10.

Supplemental Table 1.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript addresses the temporal patterns in how cholinergic signaling to the gut affects the lifespan of the worm C. elegans, which should make the manuscript of wide interest to those who study aging, as well as those who study the brain-gut axis in health and disease. The authors show that early acetylcholine (ACh) signaling to the intestine via the ACR-6 receptor shortens worm lifespan, which depends on the DAF-16/FOXO transcription factor. However, later ACh signaling to the intestine via the GAR-3 receptor extends lifespan, which in turn depends on the heat shock factor HSF-1. The authors also show a potential mechanism through which these two temporal patterns of ACh signaling might be coordinated to influence longevity in the worm, and possibly in other animals.

Strengths:

The authors observed that the functional ablation of acr-2-expressing cholinergic neurons in C. elegans (Pacr-2::TeTx) produced a lifespan curve that intersects the lifespan curve of a wild-type population. The first quartile of Pacr-2::TeTx worms shows a longer lifespan than the first quartile of wild-type worms, whereas the last quartile of Pacr-2::TeTx worms exhibits a shorter lifespan than wild type. These observations raised the hypothesis that cholinergic neurons have two opposing effects on longevity: an early longevity-inhibiting effect and a later longevity-promoting effect. Much of the data support the authors' conclusions.

The authors have also addressed the points raised in the previous review.

https://doi.org/10.7554/eLife.97829.2.sa3

Reviewer #3 (Public review):

I very much enjoyed reading Lingxiu Xu et al.'s paper "Temporally controlled nervous system-to-gut signaling bidirectionally regulates longevity in C. elegans," where they investigate the mechanisms by which motor neurons regulate lifespan in C. elegans worms. In this paper, they first discover that interfering with synaptic release in cholinergic motor neurons affects lifespan. Using mutants and gene knockdowns they show that these effects are due to the neurotransmitter acetylcholine. They show that the effects these motor neurons on lifespan are opposite, depending on timed genetic interventions promoting synaptic release. If these interventions occur during development, lifespan is shortened, but if they occur starting on day 7 of adulthood, then lifespan is lengthened. They then show that the transcription factor daf-16 is required for the former effect, while the transcription factor hsf-1 is required for the latter one. In addition, these early and late effects, they find, required the acetylcholine receptors acr-6 and gar-3, respectively, and intestinal expression of these genes rescues the respective phenotypes. Interestingly, tagging the endogenous acr-6 and gar-3 genes with mCherry, they find that the ACR-6 and GAR-3 proteins are expressed in the intestine, ACR-6 during development and GAR-3 during adulthood. Based on these findings they propose a model where acetylcholine from motor neurons regulates lifespan by modulating different receptors expressed at different times. These receptors, in turn, affect lifespan in opposing ways via different transcription factors.

Comments on revisions:

I am grateful to the authors for their effort to address my comments and suggestions, and for the thoughtful discussion of their efforts to strengthen the claims supporting their model.

https://doi.org/10.7554/eLife.97829.2.sa2

Reviewer #4 (Public review):

This is a very interesting study, where the authors discovered two neuroendocrine signaling circuits with opposite effects on organismal longevity elicited by motor neurons at different ages.

Interestingly, both systems employ the same neurotransmitter (that is, acetylcholine) and signal the intestine. However, one has effects on early life to shorten lifespan whereas the other system is activated in mid-life to extend lifespan. At the mechanistic level, this bidirectional regulation is possible through the recruitment of two different ACh receptors in the gut: ACR-6 and GAR-3. The authors found that ACR-6 expression in the intestine is restricted to early life, whereas GAR-3 expression in the gut is confined to mid-late life. Interestingly, ACR-6 modulates the transcription factor DAF-16, but GAR-3 regulates HSF-1.

The study combines different approaches, including inducible systems (AID) which are critical for the conclusions of the paper. The conclusions are well supported by the experiments and results. The data provide a potential mechanism for the temporal control of lifespan and shed light on the complex role of the nervous system in organismal aging. These results can have important implications to understand how organismal aging is regulated in a temporal manner by cell non-autonomous mechanisms.

The paper has significantly improved after addressing all the Reviewers' comments and I did not observe significant weaknesses in the study.

https://doi.org/10.7554/eLife.97829.2.sa1

Author Response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

> *While the authors have proved their hypothesis by temporally increasing the activity of cholinergic neurons at different life stages through the auxin-inducible degron system, their work raises two major concerns. First, they might want to discuss the conflicting data from Zullo et al (Nature 2019, vol 574, pp 359-364). For example, the authors show that increasing the activity of acr-2-expressing neurons after the 7th day of adulthood increases lifespan. However, Zullo et al (2019) show that the reciprocal experiment, inhibiting cholinergic neuron activity on the 1st day or the 8th day of adulthood, also increases lifespan. Is this because the two studies are using different promoters, that of the acr-2 ACh receptor (this work) versus that of the unc-17 vesicular ACh transporter (Zullo et al., 2019)? The two genes are expressed in different subsets of cells that do not completely overlap. CeNGEN shows that acr-2 is expressed in motor and non-motor neurons, but some of these neurons are also different from those that express unc-17. Is it possible that different cholinergic neurons also have opposite lifespan effects during adulthood? Or is it because both lack of signaling and hypersignaling can lead to a long-life phenotype? Leinwand et al (eLife 2015, vol 4, e10181) previously suggested that disturbing the balance in neurotransmission alone can extend lifespan. A simple discussion of these possibilities in the Discussion section is likely sufficient. Or can the auxin treatment and removal be confounding factors? Loose and Ghazi (Biol Open 2021, vol 10, bio058703) show that auxin IAA alone can affect lifespan and that this effect can depend on the time the animal is exposed to the auxin.*

We thank the reviewer for the thoughtful comments and valuable suggestions. In response, we have expanded the Discussion section to address the points raised, as detailed below.

We fully agree with the reviewer that the different results between our study (activating *acr-2*-expressing neurons) and Zullo et al. (inhibiting *unc-17*- expressing neurons) are most likely due to the distinct cholinergic neurons targeted. Our new preliminary data further support this neuron-specific model, as inhibition of acetylcholine synthesis at mid-late life stages produces opposing lifespan effects in different cholinergic neurons. At the same time, we cannot rule out the alternative possibility raised by the reviewer (eLife, 2015) that both activation and inhibition of neuronal activity may extend lifespan by similarly disrupting the balance of neurotransmission. This hypothesis requires further experimental validation in the context of cholinergic motor neurons. Regarding the potential technical concern related to auxin exposure (Biol Open, 2021), our control experiments using 0.5 mM auxin did not show non-specific lifespan effects.

Accordingly, in the revised manuscript, we have discussed the first two possibilities in the Discussion by stating (page 17-18): "Nevertheless, it is still unclear whether other neuronal populations share similar temporal regulatory mechanisms. A previous study reported that inhibiting cholinergic neurons activity (using *unc-17* promoter) extends lifespan regardless of timing[2], which is different from the temporal lifespan regulation we observed in cholinergic motor neurons (using *acr-2* promoter). This discrepancy is likely due to differences in subsets of neurons, as the *unc-17* promoter labels a broad repertoire of cholinergic neurons, while the *acr-2* promoter mainly marks cholinergic motor neurons[53]. Thus, the distinct lifespan-modulating effects of cholinergic motor neurons may be overshadowed by opposing contributions from other cholinergic subtypes when a mixed population is manipulated. Alternatively, both activation and inhibition of cholinergic activity

may perturb neurotransmission balance, leading to similar effects on lifespan[54]. It will be interesting to test these hypotheses in future studies."

> *Second, the daf-16-dependence of the early longevity-inhibiting effect of ACh signaling needs clarification and further experimentation. The authors present a model in Figure 6D, where DAF-16 inhibits longevity. This contradicts published literature. Libina et al (Cell 2003, vol 115, pp 489-502) have shown that intestinal DAF-16 increases lifespan. From the authors' data, it is possible that ACh signaling inhibits DAF-16, not promotes it as they have drawn in Figure 6D.*

We thank the reviewer for this important point. We agree that intestinal DAF-16 promotes longevity. Our original model Figure 6D aimed to show that the larval pathway shortens lifespan by inhibiting DAF-16, not that DAF-16 itself shortens lifespan. The arrowhead style used in the original Fiugure 6D might have given an impression that DAF-16 shortens lifespan. Our apologies. We have now fixed this error in Figure 6D. In addition, as suggested, we have performed additional *daf-16* experiments (see below).

> *In Figure 3F, the authors used Pacr-2::TeTx, which inhibits cholinergic neuron activity, to show an increase in the expression of DAF-16 targets. Why did the authors not use the worms that express the transgene Pacr-2::syntaxin(T254I), which increases cholinergic neuron activity? What happens to the expression of DAF-16 targets in these animals? Do their expression go down? What happens if intestinal daf-16 is knocked down in animals with increased cholinergic neuron activity, instead of reduced cholinergic neuron activity?"*

Thanks for these insightful questions. In Figure 3F-H, we used TeTx instead of syntaxin(T254I) to investigate the function of DAF-16 in the early stage pathway based on the two main reasons. First, *Pacr-2::TeTx* transgene extends lifespan in early life by inhibiting cholinergic activity, which provides a genetic background complementary to that of syntaxin(T254I) for characterizing the role of DAF-16. Second, TeTx pathway is expected to activate DAF-16 and upregulate its target genes. This approach is more sensitive than measuring gene downregulation in *Pacr-2::syntaxin(T254I)* transgenic worms.

We fully agree with the reviewer that performing the corresponding experiments in the syntaxin(T254I) background would strengthen the overall evidence. As suggested, we have now examined the expression of DAF-16 target genes in Pacr-2::syntaxin(T254I) transgenic worms, and performed intestine-specific RNAi of *daf-16* in the same background. We found that these worms exhibit downregulation of DAF-16 target genes. Furthermore, intestinal daf-16 knockdown did not further shorten the already reduced lifespan of these transgenic worms. Together, these results from both the TeTx and syntaxin(T254I) lines confirms that cholinergic motor neurons require DAF-16 in the intestine to regulate lifespan. These new data has now been described in Figure S5A-5D (page 11-12): "As expected, the expression level of sod-3 and mtl-1, two commonly characterized DAF-16 target genes, was upregulated in transgenic worms deficient in releasing ACh from cholinergic motor neurons (Figure 3F), and downregulated in transgenic worms with enhanced ACh release from cholinergic motor neurons (Figure S5A), consistent with the notion that DAF-16 acts downstream of cholinergic motor neurons.", and "RNAi of daf-16 in the intestine abolished the ability of cholinergic motor neurons to regulate lifespan at early life stage (Figure 3G, 3H and Figure S5C-S5E)."

> ***Recommendations for The Authors:***
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> ***Reviewer #1 (Recommendations for The Authors):***
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> *(1) "The Methods section needs to be clarified/expanded."*
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> *(a) "For example, are the authors using indole-3-acetic acid or a synthetic auxin? How long does it take for syntaxin to be made after the removal of the auxin?"*

We have now included auxin information and recovery time in the Method for auxin treatment by stating (page 24): "natural auxin indole-3-acetic acid (G&K Scientific)", and "Expression of syntaxin(T254I) can be suppressed by auxin treatment and restored in 24 hours following auxin removal."

> *(b) "How much FUDR was used in some of the lifespan assays?"*

2 µg/mL FUDR was used in some of the lifespan assays. We have now included the concentration in the Method for lifespan assay by stating (page 23 line 526): "2 µg/mL 5-Fluoro-2'-deoxyuridine (FUDR) was included in assays involving TeTx transgene worms, unc-31 and *unc-17* mutant worms, which show a defect in egg laying."

> *(c) "In line 494 of the Methods section, worms were anesthetized with 50 mM sodium azide. That concentration seems a bit high."*

It is an error indeed. We used 5 mM NaN3. This has now been fixed in the text and in line 548.

(d) "What are the concentrations of the transgenes used in the extrachromosomal arrays?"

We have now included the concentrations in the Method for strains and genetics by stating (line 507-509 on page 22): "Microinjections were performed using standard protocols. Each plasmid DNA listed above in the transgenic line was injected at a concentration of 50 ng/µL. Each marker for RNAi was co-injected at a concentration of 25 ng/µL."

> *(2) "Gene expression can vary in different parts of the worm intestine. Do the measurements in Figure 6C represent the entire intestine or only certain parts of the intestine?"*

We have now included the intestine area used for quantification in the Method for microscopy by stating (page 24): "and the entire intestine area was selected by ImageJ", and in the legends of Figure 6C by stating (page 36): "The entire intestinal area was selected for measurement."

> *(3) "In Figure S1C, does tph-1 have a slight effect? Might serotonin partly counteract the effects of ACh?"*

We thank the reviewer for raising this interesting point regarding the potential role of serotonin. We have re-examined our data in Figure S2C (the original Figure S1C) and agree that loss of *tph-1* partly counteracted the lifespan-shortening effect of Pacr-2::syntaxin*(T254I)* transgene in early life stage, thought the whole-life suppression effect is slight. To assess whether the *acr-2* promoter-driven manipulation might directly affect serotonergic neurons, we checked the CeNGen. We found that the transcript expression of *acr-2* can be detected in serotonergic neurons (ADF, HSN, and NSM), but the levels are extremely low. In this regard, it is unlikely that the Pacr-2::syntaxin*(T254I)* transgene exerts its primary effect by substantially altering serotonin release. While a potential indirect interaction between cholinergic and serotonergic signaling in lifespan regulation remains, it falls beyond the primary focus of the current study. We would like to follow up this in future studies. We have now pointed this out in the text by stating (page 9):"As a control, we also tested mutants deficient in other types of small neurotransmitters, including glutamate *(eat-4)*, *GABA (unc-25)*, *serotonin (tph-1)*,

dopamine (cat-2), *tyramine (tdc-1)*, and *octopamine (tbh-1)*, but detected no effect, with the exception of *tph-1*, which showed a modest, partial suppression of the phenotype (Figure S2A-S2F). This observation suggests that the lifespan effects of cholinergic signaling can be modulated by serotonin."

> *(4) "Where else is GAR-2 expressed? Might there be redundancies between neuronal and intestinal GAR-2?"*

We appreciate this insightful question. Based on available single-cell gene expression atlases of C. elegans at both embryonic and adult stages[1,2], *gar-2* expression has been detected not only in neurons and the intestine, but also in additional tissues such as the muscle. Regarding the observed lack of effects upon neuronal or intestinal *gar-2* RNAi on the ability of cholinergic motor neurons to extend lifespan in mid-late life, and also suggested by another reviewer, we performed muscle-specific RNAi experiments. Together with our previously presented data, the results show that intestinal (but not neuronal or muscle) RNAi of *gar-3* abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stages, while muscle-specific (but not neuronal or intestinal) RNAi of *gar-2* suppresses this effect. This finding indicates that GAR-3 and GAR-2 mediate cholinergic signaling in distinct peripheral tissues, with GAR-3 primarily in the intestine and GAR-2 primarily in muscle, to produce their effects on longevity. Given our focus on neuron-gut signaling, the role of GAR-2 in the muscle will be further investigated in future studies. The new data have now been described in Figure S8 by stating (page 13-14): "RNAi of *gar-2* in the intestine (Figure 4D and 4E), but not in neurons or the muscle (Figure 4D-4F, and Figure S8A, S8D-S8E), abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stage. Thus, GAR-3 may function in the intestine to regulate lifespan. Surprisingly, RNAi of *gar-2* in the muscle (Figure S8A-S8C), but not in neurons or the intestine (Figure S7F-S7H) had an effect on the ability of cholinergic motor neurons to extend lifespan in mid-late life, indicating that GAR-2 acts in the muscle to regulate lifespan."

(1) Packer, J. S. et al. A lineage-resolved molecular atlas of C. elegans embryogenesis at single-cell resolution. Science 365, doi:10.1126/science.aax1971 (2019).

(2) Roux, A. E. et al. Individual cell types in C. elegans age differently and activate distinct cell-protective responses. Cell Rep 42, 112902, doi:10.1016/j.celrep.2023.112902 (2023).

(3) Chun, L. et al. Metabotropic GABA signalling modulates longevity in C. elegans. Nat Commun 6, 8828, doi:10.1038/ncomms9828 (2015).

(4) Izquierdo, P. G. et al. Cholinergic signaling at the body wall neuromuscular junction distally inhibits feeding behavior in Caenorhabditis elegans. J Biol Chem 298, 101466, doi:10.1016/j.jbc.2021.101466 (2022).

> *(5) "In line 344, please correct "fwork" to "work"."*

This has now been fixed.

> *(6) "In line 360, please correct "acts" to "act"."*

This has now been fixed.

> *(7) "Please check citations within the main text. Some of the citations do not fit the cited material. For example, in line 112, reference 28 is not about GABAergic neurons."*

We thank the reviewer for pointing out these important details. We have now carefully checked and corrected the citations throughout the manuscript as suggested.

> ***Reviewer #2 (Recommendations for The Authors):***

> *(1) "How are the authors assessing the efficacy of the TeTx manipulations in their strains? Likely TeTx has a concentration-dependent effect. Are there any phenotypes associated with the loss of cholinergic signaling? Also, does TeTx expression in cholinergic neurons alter the neuronal activity of other associated neurons, or alter muscle integrity?"*

Thanks for the question. Our observations show that overexpression of TeTx results in defects including small size, slow growth, egg-laying deficiencies, and severe locomotion impairment, which are all associated with the loss of cholinergic signaling. While we did not directly examine the activity of interconnected neurons in our strains, we tested the muscle integrity by recording muscle reaction to 1 mM levamisole and found that overexpression of TeTx does not affect muscle integrity. To circumvent these pleiotropic complications, we instead employed *Syntaxin(T254I)* transgenic worms, which exhibits only slight locomotion defects, to further characterize the temporal effect of cholinergic motor neurons on lifespan. This data has now been described in Figure S1A by stating (page 6): "Overexpression of TeTx induces characteristic phenotypes of cholinergic deficiency, such as developmental delay and severe locomotion impairment[32], yet does not compromise muscle function (Figure S1A)."

> *(2) "The authors are expressing TeTx throughout the lifespan of the animal, including during development. How does this contribute to the organismal phenotype?"*

As described above, chronic TeTx expression from egg stage results in developmental delay, which is similar to the development phenotype of *unc-17* mutant worms defective in acetylcholine transmission. However, *unc-17* mutation has no effect on lifespan[3], which is different from TeTx overexpression, indicating that the developmental delay caused by TeTx overexpression may not affect the lifespan phenotype.

(3) Chun, L. et al. Metabotropic GABA signalling modulates longevity in C. elegans. Nat Commun 6, 8828, doi:10.1038/ncomms9828 (2015).

> *(3) "A previous study has shown that increasing cholinergic activity by altering ACR-2 expression can cause neurodegeneration (DOI: https://doi.org/10.1523/JNEUROSCI.1515-10.2010). Does overexpressing syntaxin, or AID-mediated degradation of syntaxin cause motor neuron degeneration, which could also contribute to the lifespan phenotype?"*

We thank the reviewer for raising this important point regarding potential motor neuron degeneration. In response, we performed confocal microscopy to assess the motor neurons. We found that worms expressing the transgene *Pacr-2::syntaxin::mCherry* do not exhibit a defect in the number or morphology of labeled neuronal cell bodies compared to control worms expressing *Pacr-2::mCherry*. This observation indicates that chronic, increased cholinergic activity through syntaxin overexpression, under our experimental conditions, does not induce motor neuron degeneration. This data has now been described in Figure S1B by stating (page 7): "This transgene simply shortened lifespan without causing a pleotropic effect (Figure 1B), and critically, without inducing motor neuron degeneration (Figure S1B)."

> *(4) "Figures 1I-1L: The authors do not show how long it takes for the expression of syntaxin to be restored following the removal of auxin from plates. This would be important to assess the age-dependent effects of neuronal signaling."*

We thank the reviewer for pointing this out. In general, complete restoration of syntaxin expression occurred within 24 hours after auxin withdrawal. We have now pointed this out in the text by stating (the last sentence on page 24):"Expression of *syntaxin(T254I)* can be suppressed by auxin treatment and restored in 24 hours following auxin removal."

> *(5) "In Figures S1A-E: Although the mutant backgrounds decrease the lifespan of animals expressing the Pacr2::syntaxin(T254I) transgene, the lifespan of these transgenic animals*

> *appears to be extended compared to what was shown in Figure 1B. Is this the case? (can these experiments be repeated alongside wild-type N2s to assess if their lifespan is indeed extended compared to the N2?). Also, if so, could it be that the lifespan effects are modified to different extents by other small neurotransmitters?"*

We thank the reviewer for pointing this out. All the experiments presented in current Figure S2 (original Figure S1) were performed with wild-type N2 controls, which are now included in the updated Figure S2. This data shows that, in the *Pacr-2::syntaxin(T254I)* transgenic background, loss of *unc-25 (GABA)* or *tph-1* (serotonin) leads to a further extension of lifespan, while loss of other genes had no effect. Importantly, while *unc-25* mutation also extends lifespan in wild-type worms, *tph-1* mutation does not. This observation indicates that the lifespan effects of cholinergic signaling can be modulated by serotonin. We have now pointed this out in the text by stating (page 9):"As a control, we also tested mutants deficient in other types of small neurotransmitters, including glutamate *(eat-4),*, *GABA (unc-25)*, serotonin *(tph-1)*, dopamine *,(cat-2)*, tyramine *(tdc-1)*, and octopamine *(tbh-1)*, but detected no effect, with the exception of *tph-1*, which showed a modest, partial suppression of the phenotype (Figure S2A-S2F). This observation suggests that the lifespan effects of cholinergic signaling can be modulated by serotonin."

> *(6) "RNAi of several of the receptors appear to modulate wild-type lifespan. Although I understand that this is not the main focus of the manuscript, the fact that this occurs should be mentioned in the results and discussed later on."*

We thank the reviewer for pointing this out. As suggested by the reviewer, we have now pointed this out in the text by stating (page 9):"Notably, RNAi of several ACh receptors such as *acr-11* appears to shorten wild-type lifespan, whereas RNAi of several other ACh receptors such as *acr-9* extends wild-type lifespan, suggesting lifespan-modulating potential of ACh receptors (Figure S3)."

> *(7) "Cholinergic signaling and ACR-6 have been previously shown to regulate pharyngeal pumping/feeding behavior. (https://doi.org/10.1016/j.jbc.2021.10146 "). Could the requirements for ACR-6/cholinergic signaling in longevity be related to caloric restriction/nutritional intake which in turn could be expected to alter DAF-16 and HSF-1 activity? These previous studies should be referenced and discussed."*

Thanks for the suggestion. As suggested by the reviewer, we have examined the pumping rate of *acr-6* mutant worms. Our results showed that *acr-6* mutation slightly reduced the pumping rate. As the decrease is relatively minor, we do not expect a major DR effect, though we cannot completely rule out such a possibility. Furthermore, as *acr-6* acts in the pharynx to regulate pumping but in the intestine to regulate the role of cholinergic signaling in lifespan, we do not expect this would have a major contribution to our pathway. This new data has now been described in Figure S4I. As suggested by the reviewer, we have now pointed this out in the text by stating (page 10): Previous data has shown that cholinergic signaling and ACR-6 may control pharyngeal pumping[42]. As expected, we found that *acr-6* mutation slightly reduced pumping rates (Figure S4G)."

> *(8) "The expectation for the studies in Figure 3/DAF-16, is that animals expressing Ex[Pacr-2::syntaxin(T254I)], should have downregulated DAF-16 in the intestine. This needs to be shown through some method (increased daf-16 activation upon loss of cholinergic signaling does not necessarily imply that the converse is also true)."*

We thank the reviewer for the insightful suggestion. The reviewer has suggested us performing additional measurements to confirm that DAF-16 is the downstream transcription factor in the intestine. Specifically, the reviewer suggested testing if *syntaxin(T254I)* transgene signaling could inhibit DAF-16 activity. We have now followed the reviewer's suggestion by performing two different assays. First, as also suggested by the first reviewer, we detected the

expression of DAF-16 target genes in *Pacr-2::syntaxin(T254I)* transgenic worms, which exhibited downregulation of these genes, consistent with the notion that increasing cholinergic motor neuron activity inhibits DAF-16. This data has now been described in Figure S5A. Second, we performed an assay to detect DAF-16 subcellular localization pattern in the intestine. We found that *acr-6* RNAi notably promotes nuclear translocation of DAF-16, suggesting that ACR-16 inhibits DAF-16, which is consistent with our model. This new data has now been described in Figure S5E. As suggested by the reviewers, we have now pointed this out in the text by stating (page 11): "As expected, the expression level of *sod-3* and *mtl-1*, two commonly characterized DAF-16 target genes, was upregulated in transgenic worms deficient in releasing ACh from cholinergic motor neurons (Figure 3F), and downregulated in transgenic worms with enhanced ACh release from cholinergic motor neurons (Figure S5A), consistent with the notion that DAF-16 acts downstream of cholinergic motor neurons. To obtain further evidence, we assessed the subcellular localization pattern of DAF-16::GFP fusion and found that *acr-6* RNAi notably promoted nuclear translocation of DAF-16, confirming that ACh signaling inhibits DAF-16 activity (Figure S5B)."

> *(9) "Similarly, it would be good to have additional lines of evidence that signaling through GAR-3 impinges on HSF1, and that the lifespan effects are not due to non-specific effects of hsf-1 knockdown, which could lead to several un-related deficiencies and compromise lifespan (Figure 5b)."*

We thank the reviewer for the valuable suggestions. The reviewer correctly noted that the observed lifespan effect from hsf-1 RNAi could involve non-specific deficiencies. In response, we performed an assay to detect HSF-1 subcellular localization in the intestine upon *gar-3* overexpression by using the strain EQ87 *(iqIs28[pAH71(hsf-1p::hsf-1::gfp) + pRF4(rol-6)])*. We found that the induced nuclear translocation of HSF-1 was weak. This result suggests that GAR-3 may modulate HSF-1 activity through a mechanism distinct from, or more subtle than, robust nuclear accumulation, or that its effect is highly dependent on the expression level and timing.

> *(10) "Figure 6: An N2 control should be provided to assess the specificity of the mCherry signal from the intestine (given autofluorescence in the animals' gut)."*

Thanks for the suggestion. As suggested by the reviewer, we have now included the control in Figure S10.

> ***Reviewer #3 (Recommendations for The Authors):***
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> *(1) "While the model is consistent with the data, there are alternatives that were not addressed. Additionally, there are some deficiencies in the interpretation of results that should be addressed, in my opinion. Possibly most importantly given the claims, the authors should address an alternative model: that it is the level of acetylcholine signaling that matters. Is it possible that the level auxin-inducible degradation of syntaxin(T254I) in acr-2 expressing cells is age dependent, such that one level increases lifespan and the other shortens it, and that the timing doesn't matter at all? A chronic dose response to auxin concentration would address if the level of syntaxin is a non-monotonic determinant of lifespan."*

We sincerely thank the reviewer for raising this important alternative model. The reviewer suggested that the apparent temporal effect we observed might instead be explained by an age-dependent change in the efficiency of AID system in degrading syntaxin(T254I) in *acr-2* expressing cells. That is, different levels of acetylcholine signaling, rather than timing, produce opposite lifespan outcomes. We agree that this is a formal possibility that our current data cannot fully rule out. On the other hand, other data in the manuscript suggests otherwise. For example, the expression of ACR-6 and GAR-3 in the intestine exhibited a temporal switch in early and mid-late life, providing support for a time-dependent

mechanism. In addition, the differential requirement of the downstream transcription factors DAF-16 and HSF-1 in the early and mid-late life, respectively, provides further evidence supporting a temporal mechanism. Thus, while we agree that the possibility raised by the reviewer cannot be formally ruled out, the temporal mechanism we proposed may play an important role.

The reviewer suggested performing a chronic dose-response experiment with varying auxin concentrations. Actually when we first employed the AID system to temporally manipulate motor neuron output at different life stages, we tested potential effects of auxin concentration. Using the soma-expressed TIR1 system, we found that, restoring *syntaxin(T254I)* activity from day 10 of adulthood extends lifespan, regardless of whether the prior suppression was maintained with 0.1 mM or 0.5 mM auxin. This suggests that the pro-longevity effect is likely not triggered by differences in the efficacy of prior suppression within this concentration range. We acknowledge that the tested dose range may not cover potential threshold concentrations. Furthermore, we cannot exclude the possibility of a non-linear relationship between auxin concentration and degradation efficiency. We agree that a comprehensive chronic dose-response analysis remains a valuable future direction, and we plan to employ more precise tools in the future to investigate the interplay between signal level and temporal context in lifespan regulation. The auxin concentration data have now been described in Figure S1C-1D by stating (page 7): “Comparable outcomes were obtained with both 0.1 mM and 0.5 mM auxin treatments (Figure S1C-1D).” As suggested by the reviewer, we have discussed the alternative model in the Discussion by stating (page 19): “An alternative mechanism based on differential levels of cholinergic signaling could also contribute to the observed lifespan effects.”

> *(2) “Several times, including in several section headings, it is claimed that daf-16 (eg line 205-206) and acr-6 (eg line 185-186) function "early in life". This was not tested, so the claim is not warranted. For instance, these genes could act later in life to respond to signals made or sent early in life, or they could act both early and late, or only early (as they claim).”*

We thank the reviewer for this precise and important clarification. The reviewer is correct that our genetic interventions do not by themselves define the temporal window.

Our experimental rationale was based on the observation that the lifespan-shortening effect of *Pacr-2::syntaxin(T254I)* expression is similar whether it is induced throughout life or specifically during larval stages (early life), indicating the detrimental effect results from enhanced motor neuron output in early life. Therefore, we used the lifelong expression paradigm as a tool to genetically dissect the downstream pathway triggered by early-life neuronal activation. We acknowledge the reviewer's point that this design does not formally prove that *daf-16* or *acr-6* acts only in early life; they could be required continuously or again later. However, we would like to note that our expression data show that the gut expression of ACR-6 is restricted to early life, which is consistent with a primary early-life function in this context.

To reflect this more accurate interpretation, we have revised all relevant statements, including section headings. We now consistently state that daf-16 is required for the lifespan-shortening effect of cholinergic motor neuron, rather than claiming it functions "in early life". We have also toned down the discussion regarding their temporal function by stating (page 12): “Because this lifespan-shortening effect results from enhanced motor neuron output in early life and overwrites its beneficial effect at later stages, we propose this signaling circuit mediates the lifespan-shortening effect in early life.”

> *(3) “In line 118, they note that such intervention led to a complex effect on the lifespan curve "by initially promoting worm's survival followed by inhibiting it at later stages." I think that while findings from later experiments support a time-dependent lifespan effect*

> *stemming from syntaxin function in the cholinergic motor neurons, this experiment's TeTx expression in those neurons is not time-dependent. Lifespan is an endpoint measure, so there is no sense in which a non-timed perturbation has an early or late effect on an individual. Rather, the effect on survival they observed is at the population level, their intervention increases the average lifespan while decreasing the worm-to-worm variation in lifespan."*

We thank the reviewer for the critical and precise comment regarding our interpretation of the survival curves of TeTx transgenic worms. As suggested by the reviewers, we have revised the text by stating (page 6): "Surprisingly, such intervention led to a complex effect on the population survival curve by reducing both early mortality and the proportion of long-lived individuals (Figure 1A). Specifically, the 25% lifespan of these worms was prolonged, while their 75% and maximal lifespan were slightly shortened, leading to a mean lifespan slightly increased or unchanged compared to that of wild-type worms. This suggests that inhibiting cholinergic motor neurons may exert temporally distinct effects on survival, leading to decreased individual variation in lifespan."

> *(4) "The layout of the plots separating the responses of wild type and mutants to different panels makes it often difficult to interpret the results. For instance, do acr-6, gar-3, and other receptor mutants or knockdowns affect lifespan on their own? If they do, it matters to the interpretation whether they live longer or shorter than the wild type: which of the mutants phenocopy the lack of a lifespan-extending signal that activates them? Which phenocopy lacks a lifespan-shortening signal that activates them? Could they phenocopy the effect of an inhibitory signal? And critically, are the effects of these mutants on lifespan consistent with their model?"*
>
> *"The paper would be stronger if they determined when ACR-6 and GAR-3 functions are necessary and sufficient. Is it possible that the receptor doesn't matter, just that there be one of the two expressed in the intestine, and that other mechanisms determine the lifespan response to modulation of syntaxin(T254I)? What does time-dependent knockdown of these receptors do to daf-16 and hsf-1 localization and to the transcription of the targets of these transcription factors?"*

We thank the reviewer for these insightful comments. We have addressed the points as follows:

As suggested, we have reorganized the lifespan data in Figure S4 to directly compare wild type and mutant/RNAi conditions within the same panels. This new presentation clarifies the autonomous effects of these genes. The data shows that loss of *acr-6* or *gar-2* (via RNAi or mutation) has minimal effect on lifespan. Notably, *acr-8* RNAi shortens lifespan, whereas the *acr-8* mutation does not, supporting our hypothesis of tissue-specific or compensatory roles for this receptor, as detailed in our following response to point (5). The reviewer's key question regarding when these receptors are necessary and sufficient is central to our model. We agree with the reviewer that complementary loss-of-function experiments with temporal precision, such as time-specific knockdown of the two receptors, would provide even stronger evidence. To this end, we attempted to generate endogenous degron-tagged alleles of *acr-6* and *gar-3* to apply the AID system for precise, stage-specific degradation. Unfortunately, despite multiple design attempts and screening efforts, we were unable to obtain homozeygous strains with the desired genomic edits using the same gRNA we used to knock in mCherry or other gRNAs. This is rather frustrating. Consequently, we are currently unable to perform the ideal temporally controlled loss-of-function experiments suggested by the reviewer.

> *(5) "Why does RNAi but not mutation of acr-8 and gar-2 suppress the lifespan shortening effect of Pacr-2::syntaxin(T254I)?"*

Thanks for this important question regarding the differential effects of feeding RNAi versus mutation of *acr-8* and *gar-2*. The discrepancy likely arises from the potential off-target effects of RNAi. RNAi is not strictly specific as it may target other related genes, generating a non-specific effect, whereas precise mutations in *acr-8* and *gar-2* alone may not produce the same effect.

> *(6) "sid-1(-); Ex[Pacr-2::tetx lives longer than sid-1(-); in daf-16(+) worms in Figure 3G; so it is very hard to interpret the lack of effect of Pacr-2::tetx in daf-16(-) worms, since this transgene behaves differently in sid-1 mutants than in wild type worms. This would be clear if the two plots were combined (appropriately, since it is the same experiment). It looks like daf-16 RNAi has a shortening effect in the sid-1 mutant, but not in in sid-1 mutants expressing Pacr-2::text."*

Thanks for this helpful suggestion. As suggested by the reviewer, we have now merged Figure 3G and 3H into one figure to present as Figure S5F. This combined presentation clarifies the comparison and shows that intestinal *daf-16* RNAi shortens lifespan in both sid-1 mutants and *sid-1* mutants expressing *Pacr-2::TeTx*.

> ***Reviewer #4 (Recommendations for The Authors):***
>
> *(1) "Lines 50-52: I would replace "leading to increased incidents in age-related diseases and probability of death" with "leading to the onset of age-related diseases and increased probability of death". Instead of "such an aging process" I would use "the aging process"."*

This has now been fixed.

> *(2) "Figure 2E-F: By rescuing the expression of ACR-6 in neurons or intestinal cells alone, the authors show that the release of ACh from cholinergic neurons has effects on the intestine to shorten lifespan. Is ACR-6 expressed in other tissues (e.g. muscle?) It might be interesting to assess whether ACh also regulates lifespan through activating the ACR-6 receptor in other tissues or specifically targets the intestine. This question is partially answered with the tissue-specific RNAi experiments for DAF-16, but it is possible that ACR-6 also modulates other pathways beyond the tested transcription factors."*
>
> *Analyzing the role of other tissues could also be applied to understand how GAR-3 influences lifespan. Along these lines, it would be interesting to expand the tissue-specific knockdown experiments for GAR-3 to other tissues. More importantly, these experiments can address whether activation of ACR-6 and GAR-3 can also have different effects on lifespan by regulating distinct tissues in addition to the intestine, and not only due to temporal expression patterns. For instance, whereas DAF-16 regulates lifespan primarily through its effects in the intestine, HSF1 could have effects on additional tissues. Although it would interesting to perform these experiments, I understand that the authors main focus is the nervous system-gut axis.*

We thank the reviewer for the insightful suggestions regarding the potential tissue-specific functions of ACR-6 and GAR-3. As noted in our response to point #6, endogenous expression imaging indicates that ACR-6 and GAR-3 are primarily expressed in neurons and the intestine with weak expression of GAR-3 in the muscle, so we tested the muscle. We found that muscle-specific RNAi of *gar-2* abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stages, whereas muscle-specific RNAi of *gar-3* does not. This result further supports that GAR-3 primarily exerts this effect in the intestine.

> *(3) "Can the authors specify in the corresponding figure legend at what age they tested sod-3 and mtl-1 expression in Pacr-2::TeTx worms (Figure 3F)? This is important to*

> *support the conclusions of the paper. Along these lines, can the authors also specify at what age they quantified the expression of HSF-1 targets (Figure 5F)."*

Thanks for the suggestion. As recommended, we have now provided the worm age in Figure 3F (day 1 adult) and Figure 5F legends (day 10 adult).

> *(4) "To further strengthen the authors' conclusions, it might be interesting to examine the intracellular localization of DAF-16 in the intestine of Pacr-2::TeTx and syntaxin(T254I) worms compared to controls."*

We thank the reviewer for this valuable suggestion, which was also raised by another reviewer. In response, we examined the subcellular localization of DAF-16 in the intestine. Direct imaging in the *Pacr-2::TeTx or Pacr-2::syntaxin(T254I)* backgrounds was technically challenging because their fluorescent protein tags (YFP or mCherry) would interfere with the detection of DAF-16::GFP. Therefore, we adopted an alternative approach by modulating the activity of acr-6, the intestinal acetylcholine receptor that transmits cholinergic signals from motor neurons to DAF-16. We found that *acr-6* RNAi promotes the nuclear translocation of DAF-16. These new data are presented in Figure S5E by stating (page 11): "To obtain further evidence, we assessed the subcellular localization pattern of DAF-16::GFP fusion and found that *acr-6* RNAi notably promotes nuclear translocation of DAF-16, confirming that ACh signaling modulate DAF-16 activity (Figure S5B)."

> *(5) "The results with gar-2 RNAi are fascinating. I am very curious (and I assume potential readers too) about what tissues mediate the mid-late life effects of GAR-2 in longevity. Perhaps the authors could add experiments in a couple of other tissues known to regulate organismal lifespan (e.g. muscle). However, I totally understand why the authors focused on GAR-3, especially because both GAR-3 and ACR-6 have effects on the intestine and this is sufficient for the main conclusions of the paper."*

We sincerely thank the reviewer for the insightful suggestion and for highlighting the potential role of GAR-2. In response, we performed muscle-specific RNAi experiments. Together with our previously presented data, the results show that intestinal (but not neuronal or muscle) RNAi of *gar-3* abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stages, while muscle-specific (but not neuronal or intestinal) RNAi of *gar-2* suppresses this effect. This finding indicates that GAR-3 and GAR-2 mediate cholinergic signaling in distinct peripheral tissues, with GAR-3 primarily in the intestine and GAR-2 primarily in the muscle, to produce their effects on longevity. Given our focus on neuron-gut signaling, the role of GAR-2 will be investigated in future studies. The new data have now been described in Figure S8 by stating (page 13-14): "RNAi of *gar-3* in the intestine (Figure 4D and 4E), but not in neurons or the muscle (Figure 4D-4F, and Figure S8A, S8D-S8E), abolished the ability of cholinergic motor neurons to extend lifespan at mid-late life stage. Thus, GAR-3 may function in the intestine to regulate lifespan. Surprisingly, RNAi of *gar-2* in the muscle (Figure S8A-S8C), but not in neurons or the intestine (Figure S7F-S7H) had effect on the ability of cholinergic motor neurons to extend lifespan in mid-late life, indicating that GAR-2 acts in the muscle to regulate lifespan."

> *(6) "Figure 6: It seems that the genes are also expressed in the muscle. Can the authors include images of other tissues in supplementary figures?"*

Thanks for the suggestion. As suggested by the reviewer, we have now included images of whole worms expressing mCherry, which was knocked in the endogenous locus off *gar-3* or *acr-6* by CRISPR in Figure S10. However, we did not detect strong expression of *gar-3* or *acr-6* in the muscle under the conditions examined, which may be limited by the low endogenous protein expression level of the two genes in the muscle, though the CeNGEN website shows they are expressed in the muscle. Determining the precise spatiotemporal expression profiles

of these receptors will likely require more sensitive methods. We plan to address this important question in future studies by using such refined approaches.

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