

Neuronal Serotonin Release Triggers the Heat Shock Response in *C. elegans* in the Absence of Temperature Increase

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Summary

Background: Cellular mechanisms aimed at repairing protein damage and maintaining homeostasis, widely understood to be triggered by the damage itself, have recently been shown to be under cell nonautonomous control in the metazoan *C. elegans*. The heat shock response (HSR) is one such conserved mechanism, activated by cells upon exposure to proteotoxic conditions such as heat. Previously, we had shown that this conserved cytoprotective response is regulated by the thermosensory neuronal circuitry of *C. elegans*. Here, we investigate the mechanisms and physiological relevance of neuronal control.

Results: By combining optogenetic methods with live visualization of the dynamics of the heat shock transcription factor (HSF1), we show that excitation of the AFD thermosensory neurons is sufficient to activate HSF1 in another cell, even in the absence of temperature increase. Excitation of the AFD thermosensory neurons enhances serotonin release. Serotonin release elicited by direct optogenetic stimulation of serotonergic neurons activates HSF1 and upregulates molecular chaperones through the metabotropic serotonin receptor SER-1. Consequently, excitation of serotonergic neurons alone can suppress protein misfolding in *C. elegans* peripheral tissue.

Conclusions: These studies imply that thermosensory activity coupled to serotonergic signaling is sufficient to activate the protective HSR prior to frank proteotoxic damage. The ability of neurosensory release of serotonin to control cellular stress responses and activate HSF1 has powerful implications for the treatment of protein conformation diseases.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is implicated in the stress response of all animals, and its enhanced release

modulates physiological and metabolic adaptation to adverse conditions [1, 2]. Anticipation of danger can trigger serotonin release [3]. In addition, experimentally manipulating serotonin signaling in mammalian models can increase stress hormone production and elicit aversive behaviors, even in the absence of the actual stressor [1, 2]. Thus, although it is unclear whether enhanced release of serotonin is specific to particular stressors or whether it is a nonspecific response associated with increased arousal, serotonergic signaling allows organisms to rapidly adjust their physiology, metabolism, and behavior in expectation of imminent danger.

Exposure to unfavorable environments can also cause macromolecular damage [4, 5]. However, cellular mechanisms aimed at repairing damage and maintaining homeostasis are widely understood to be triggered by the damage itself, either through damage to the cell that activates the response [4, 6–8] or, as has been suggested by recent experiments, through the activation of a stress response in other “sending” cells of an organism that are subject to proteotoxic conditions [9]. Indeed, there is little evidence to date for the preemptive activation of cellular stress responses prior to macromolecular damage. One key homeostatic mechanism by which cells protect themselves against protein damage is through the heat shock response (HSR) and activation of the highly conserved transcription factor, heat shock factor 1 (HSF1) [6, 7, 10]. HSF1 upregulates heat shock protein (HSP) genes that act as molecular chaperones to maintain protein conformation under stress, refold misfolded proteins, and target irreversibly damaged proteins for degradation [6, 7, 10]. Thus, HSF1 activation suppresses protein misfolding and toxicity in numerous animal models of protein conformational diseases, such as Alzheimer’s, Parkinson’s, and Huntington’s diseases [6, 10, 11].

We have previously shown that in *C. elegans*, the HSR is regulated cell nonautonomously by the animals’ AFD thermosensory neuronal circuitry [12, 13]. In addition, other groups have also shown nonautonomous regulation of HSF1 and other cellular stress responses by the nervous system [9, 12–16]. Thermosensory neurons in *C. elegans* are exquisitely sensitive and detect changes as small as 0.05°C above ambient temperature [17]; hence, they can arguably be excited by temperature increments well below those that cause cellular damage. Therefore, a central question that arises from the observation that thermosensory neurons control the HSR of peripheral tissue is whether the nervous system plays an instructive role in activating the HSR upon sensing temperature increase, prior to macromolecular damage, or whether it plays a more general, permissive role in allowing the stress response to be triggered by macromolecular damage. An instructive role involving signaling pathways from neuronal cells to peripheral tissue could activate protective mechanisms against macromolecular damage in anticipation of its actual occurrence. This would not only be adaptive for organisms but, if identified, could suggest powerful strategies to counteract diseases of protein conformation.

Here, we tested whether excitation of the AFD thermosensory neuronal circuitry was sufficient to activate the HSR in distant tissues, even in the absence of heat, through

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serotonergic signaling. To do this, we used optogenetics to excite specific neurons combined with live imaging of HSF1 dynamics in noninnervated gonad nuclei of live, intact animals. We found that excitation of thermosensory and serotonergic neurons alone can activate the HSR in other cells through regulated serotonin release and protect these cells from protein aggregation.

Results

Optogenetic Excitation of *C. elegans* AFD Thermosensory Neurons Activates HSF1

To assess whether thermosensory activity was sufficient to induce the HSR in another cell, we used optogenetics [18] to locally stimulate the two AFD thermosensory neurons in *C. elegans* while maintaining animals at 20°C (Figure 1A). Optogenetic activation was conducted in a previously characterized transgenic strain that expresses the blue-light activated cation channel channelrhodopsin-2 (ChR2) only in the AFD neurons (AFD::ChR2). Live imaging of HSF1 dynamics following optogenetic activation was conducted in germ cell nuclei, which are not innervated. To visualize HSF1, we used transgenic *C. elegans* ubiquitously expressing a single copy of HSF1::GFP under its own promoter (see [Supplemental Experimental Procedures](#) available online). In this strain, HSF1 is present diffusely throughout nuclei at ambient temperatures of 20°C–22°C and upon heat shock relocalizes rapidly to stress-induced nuclear puncta (Figures 1B, 1C, S1A, and S1B). These HSF1 nuclear stress granules are a well-accepted readout for HSF1 activation in multiple model systems [19–21], and many have been shown to correspond to transcriptionally active sites in *C. elegans* [22]. Light activation of ChR2 requires the small molecule all-*trans* retinal (ATR), which in *C. elegans* is supplied through food [18]. The need to supplement exogenous ATR for the activation of ChR2 allowed us to control for the nonspecific effects of handling, light exposure, phototoxicity, immobilization, etc. that might activate HSF1, as we could mock activate animals expressing ChR2 and HSF1::GFP but grown in the absence of ATR by exposure to the exact same light regimen and use them as controls.

We first verified that neither ATR treatment nor manipulations required for the optogenetic experiments activated the HSR. We did this by examining the localization of HSF1 and measuring *hsp70* mRNA levels in mock-activated, control animals (Figures S1C–S1E). Next, to discount the possibility that the manipulation of the animals as required for optogenetic activation caused protein misfolding for unknown, albeit improbable, reasons, we assessed the effects of mock activation on two metastable proteins expressed in *C. elegans* tissue: an expanded polyglutamine (polyQ) expressed in body wall muscle cells [23] and an endogenous temperature-sensitive dynamin allele known to be expressed in neurons, intestine, and pharyngeal muscle [24]. The polyglutamine Q35::YFP is a construct containing 35 glutamine residues fused to YFP that aggregates in an age-dependent manner in body wall muscle cells. Q35::YFP can serve as a folding reporter to assess endogenous misfolding, as its aggregation increases upon misfolding of endogenous *C. elegans* proteins [25] and upon heat shock (Figure S1F). The temperature-sensitive dynamin mutants, *dyn-1(ky51)*, show decreased motility upon misfolding of dynamin that can occur upon a temperature shift or other misfolding events [24]. Therefore, the numbers of aggregates in Q35::YFP animals and the motility of *dyn-1(ky51)* animals would provide us with a measure of misfolding, were

it to occur due to our experimental protocol. Following mock activation, both Q35::YFP-expressing animals and *dyn-1(ky51)* animals continued to show similar levels of misfolding as control animals not subjected to mock activation, indicating that the experimental procedure itself did not trigger overt misfolding (Figures S1G and S1H).

Having established that our methods were not proteotoxic, we proceeded to test whether optogenetic stimulation of the AFD neurons could activate HSF1. In all animals, prior to the light-induced excitation of the AFD neurons, HSF1 was present diffusely throughout nuclei (Figures 1D and 1F). This diffuse localization persisted in the control animals grown in the absence of ATR, even after illumination of the AFD neurons with 490 nm light (Figure 1E). Only 12% ($n = 25$) of the animals displayed any evidence of HSF1::GFP nuclear puncta (Figure 1H), with as few as $6.1\% \pm 3.4\%$ nuclei ($n = 1,503$) showing HSF1 puncta (Figure 1I), perhaps corresponding to basal HSF1 activity. In contrast, light stimulation of the AFD thermosensory neurons resulted in the formation of HSF1::GFP puncta in 78.4% of the experimental animals ($n = 37$) grown on ATR (Figures 1F–1I). This occurred as early as 10 min postactivation, and by 20 min, HSF1 was activated in 25%–79.3% of nuclei per gonad (average = $48.7\% \pm 4.7\%$ gonadal nuclei; $n = 1,746$; Figure 1I). These observations show that excitation of the AFD neurons alone is sufficient to initiate a signaling mechanism to activate HSF1 in distal tissues, even in the absence of temperature increase.

AFD Thermosensory Neuronal Activity Enhances Serotonin Release from the ADF and NSM Serotonergic Neurons

In *C. elegans*, as in mammals, neurosensory regulation of serotonin levels controls many aversive behavioral and physiological responses to noxious stimuli [26–28]. Given the role of serotonin in regulating adaptive physiological changes in anticipation of danger [1–3], we examined whether thermosensory neuronal control of the HSR could be mediated by serotonin. Although the AFD thermosensory neurons are themselves not serotonergic, we had previously seen that during heat stress, the neuronal control of the HSR was dependent on chemosensory input that modulated the metabolic state of the animal [12]. If serotonin release triggered by thermosensory neuronal activity was a signaling mechanism for the nonautonomous activation of the HSR, we predicted that (1) activation of the AFD thermosensory neurons, either optogenetically or upon exposure to increasing temperatures, should enhance serotonin release, (2) inhibiting AFD thermosensory neuronal activity should diminish this release of serotonin, and (3) inhibiting serotonin signaling should diminish the HSR. We tested each of these predictions.

Optogenetic excitation of the AFD thermosensory neurons caused an increase in pharyngeal pumping within the first minute of light exposure (Figure 1J). Enhanced pumping is a well-characterized consequence of serotonin release by serotonergic neurons [29–31]. This occurred only in AFD::ChR2 animals grown on ATR and not in animals grown in the absence of ATR (Figure 1J), and it was consistent and significant, despite the lower pharyngeal pumping rates typically seen in animals that are immobilized, as for our experiments [31]. The increase in pharyngeal pumping stimulated by excitation of the AFD neurons could be inhibited with 100 μ M gramine [30], a competitive serotonin antagonist (Figure 1J). Thus, optogenetic excitation of AFD thermosensory neurons was sufficient to cause serotonin release.

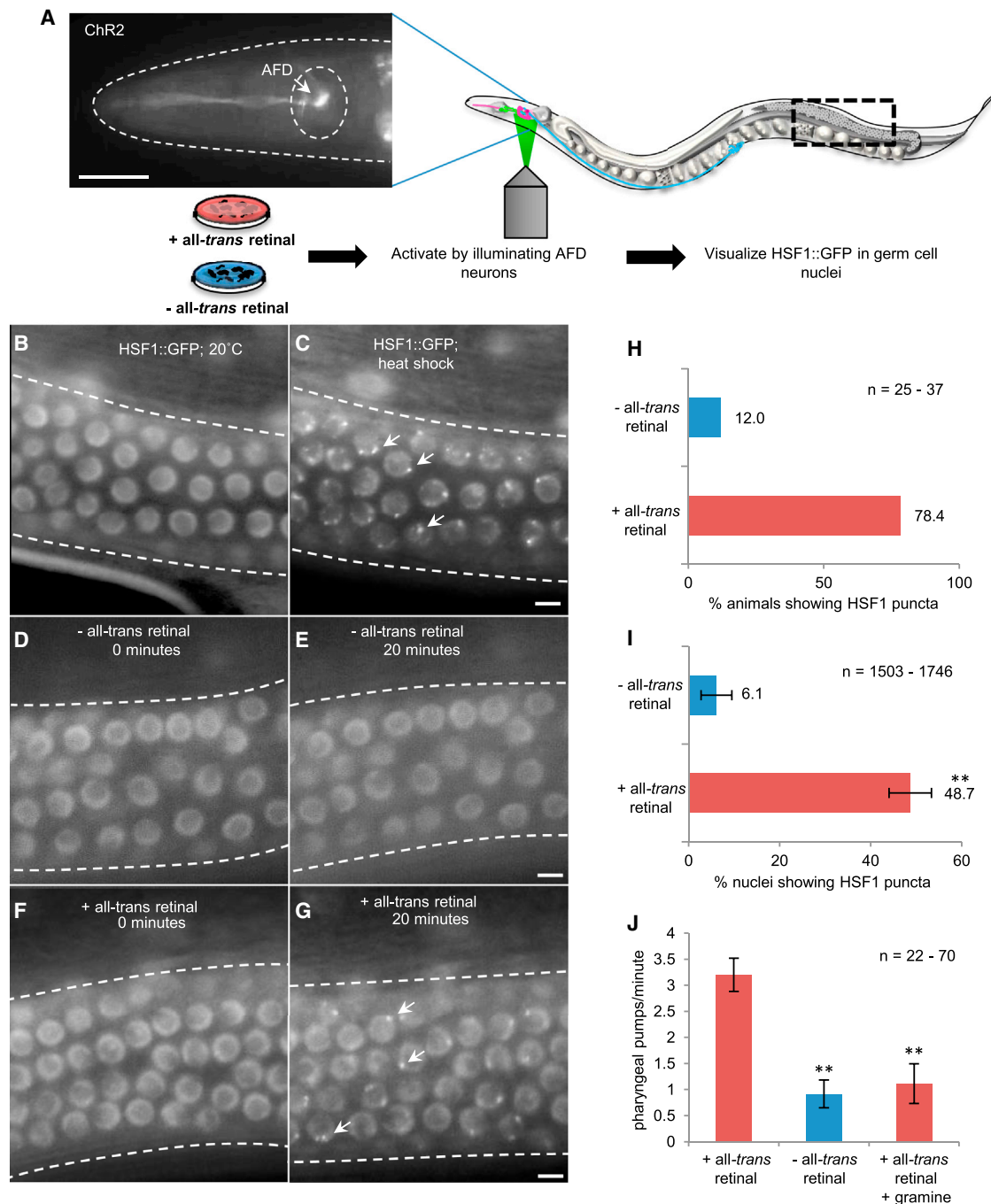


Figure 1. Optogenetic Stimulation of AFD Thermosensory Neurons in *C. elegans* Activates HSF1 and Elicits Serotonin Release in the Absence of Stress
 (A) Experimental setup: localized illumination of AFD neurons in individual *C. elegans* expressing Chr2 in AFD neurons (the scale bar represents 50 μm). Wavelength = 490 nm. Experimental animals were grown in the presence of all-trans retinal (+ATR) and control animals in the absence of all-trans retinal (-ATR). HSF1::GFP was monitored in germ cell nuclei.
 (B and C) HSF1::GFP localization at 20°C (B) and following heat shock at 34°C for 10 min (C) (arrows indicate HSF1 nuclear stress granules/puncta). The scale bar represents 5 μm .
 (D and E) HSF1::GFP in control (-ATR) animals at 0 min (D) and 20 min (E) after the start of optogenetic stimulation. The scale bar represents 5 μm .
 (F and G) HSF1::GFP in experimental (+ATR) animals at 0 min (F) and 20 min (G) after the start of optogenetic stimulation. The scale bar represents 5 μm .
 (H) Percentage of control (-ATR) and experimental (+ATR) animals showing HSF1 nuclear puncta after stimulation of AFD neurons. n = 25–37 animals.
 (I) Percentage of nuclei with HSF1 puncta in control (-ATR) and experimental (+ATR) animals following optogenetic stimulation. n = 25–37 animals; 1,503–1,746 nuclei. Values indicate mean \pm SEM, t test; **p < 0.01.
 (J) Pharyngeal pumping rates in control (-ATR) and experimental (+ATR) animals following optogenetic stimulation and pharyngeal pumping in (+ATR) experimental animals treated with 100 μM gramine and optogenetically stimulated. n = 22–70 animals. Values indicate mean \pm SEM, t test; **p < 0.01.
 See also [Figure S1](#).

Serotonin was also released in an AFD-dependent manner upon increases in temperature. Animals were subjected to a controlled increase in temperature of 1°C/min (Figure S2B), a rate known to activate the AFD thermosensory neurons [17, 32], and pharyngeal pumping was measured [29]. At 20°C–22°C, wild-type animals exhibited a pumping rate of 204 ± 24 pumps per minute, similar to what has been previously reported (Figures S2A and S2C) [29]. As early as 5 min upon temperature increase to 26.7°C, there was a dramatic increase in pumping rates to an average rate of 304 ± 50 (Figures 2A, S2A, and S2C), representing an approximately 50% increase in the number of pumps per minute. This increase was transient but occurred in all animals (Figures S2A and S2C). Animals deficient in serotonin synthesis because of mutations in the only *C. elegans* tryptophan hydroxylase gene, *tph-1* [33], did not increase their pumping rates upon temperature increase, confirming that the temperature-dependent increases in pumping occurred as a consequence of serotonergic signaling (Figures 2A, S2A, and S2D). Disrupting the function of the AFD thermosensory neurons by mutations in the guanylyl cyclase genes *gcy-8*, *gcy-18*, and *gcy-23* [32, 34] greatly diminished the increase in pharyngeal pumping upon temperature upshift (Figures 2A, S2A, S2F, and S2G), confirming the role of the AFD neurons in eliciting temperature-dependent serotonin release. On the other hand, bacteria that serve as food for *C. elegans* and are known to modulate serotonin release [28, 29] were not responsible for the temperature-enhanced pumping as animals grown on dead bacteria showed a comparable increase (from 167 ± 20, 20°C to 265 ± 39, 26.7°C; Figures 2A, S2A, and S2E). These data show that serotonin was released in response to sensed increases in temperature in an AFD-dependent manner.

We confirmed that serotonin was indeed released by visualizing endogenous serotonin localization in animals at 20°C and upon increasing temperature. Specifically, we examined animals exposed to the temperature ramp rate of 1°C/min for 5 and 10 min (26.7°C and 28.9°C, where 26.7°C corresponds to the peak of pharyngeal pumping) and upon heat shock at 34°C for 15 min. In wild-type *C. elegans* raised at 20°C, antibodies against serotonin stained cell bodies of a pair of neurosecretory motor neurons (NSMs), a pair of ADF chemosensory neurons (Figure 2D), the HSN neurons that innervate vulval muscle cells, and, less consistently, a single RIH neuron and a pair of AIM interneurons [28, 29, 35]. When animals were exposed to increases in temperature, serotonin staining expanded into areas outside serotonergic neuronal cell bodies (Figures 2E, 2F, and 2H). This was evidenced by immunolocalizing serotonin in animals expressing GFP under the *tph-1* promoter (*Ptph-1::GFP*), where GFP marked the boundaries of serotonergic neuronal cell bodies. In these animals, following heat shock, serotonin staining could be detected outside the confines of GFP (Figures 2B and 2C). We examined whether changes in *tph-1* expression itself could account for the change in localization of serotonin with increased temperatures [27, 28], as *tph-1* can be expressed in other tissues, such as pharyngeal muscle cells of the procorpus and metacarpus during embryogenesis [36]. However, neither *Ptph-1::GFP* localization (Figures 3A and 3B) nor GFP mRNA levels changed following heat shock (Figure 3C). The altered localization of serotonin following heat shock was transient, as many animals showed control immunolocalization patterns by 1 hr following recovery at 20°C after exposure to 34°C for 15 min (Figures 2F, 2G, 2H, and 2M). We confirmed that the immunolocalization pattern was specific for serotonin by using *tph-*

1(mg280) II mutant animals that do not make serotonin (Figures 2I and 2J). In addition, a loss-of-function mutation in *unc-31* that disrupts dense core vesicle-dependent neurosecretion required for serotonin secretion [35] diminished serotonin relocation upon heat shock (88% versus 33% animals showed localization outside serotonergic cell bodies; Figures 2K, 2L, and 2N).

We then investigated which of the serotonergic neurons were responsible for the temperature-dependent serotonin release by genetically ablating specific serotonergic neurons. The cell bodies of the NSM neurons are buried in cavities inside the pharyngeal muscle cells, whereas the ADF neuronal cell bodies lie just outside the pharynx, and both were possible candidates to contribute to the observed serotonin staining pattern [36, 37]. Genetic ablation of the NSM and ADF neurons only, achieved by directing the expression of a split caspase to these cells (Figures 3D–3K; [38]), resulted in the loss of serotonin relocation following heat shock (Figure 3F and 3G). As with temperature-enhanced pharyngeal pumping, the heat shock-induced immunolocalization of serotonin outside the neuronal cell bodies required AFD thermosensory neuronal function: only 27% of the *gcy-8* thermosensory mutant animals compared with 88% of wild-type animals showed serotonin release following exposure to increased temperature (Figures 3L–3N). These studies together support the conclusion that, as with optogenetic activation, temperature-induced AFD thermosensory activity also elicits the release of serotonin. This occurs from serotonergic NSM and/or ADF neurons.

Serotonin Is Necessary for the HSR

We next tested whether serotonin was necessary for heat shock-dependent induction of *hsp70* mRNA. Animals deficient in serotonin signaling and wild-type animals with intact serotonin signaling systems were subjected to a brief heat shock, corresponding to the regimen that induced the relocation of serotonin as described above. *hsp70* mRNA accumulation was then quantified using quantitative RT-PCR (qRT-PCR). Both the *tph-1* mutants (Figure 4A) and animals lacking serotonergic ADF and NSM neurons (Figure 4B) showed significant decreases in *hsp70* mRNA accumulation upon heat shock compared to wild-type animals. In *C. elegans*, four metabotropic G protein-coupled receptors (GPCRs), SER-1, SER-4, SER-5 and SER-7, and one serotonin-gated chloride channel, MOD-1, are known to bind serotonin [39, 40]. Among these receptors, loss-of-function mutations in SER-1 decreased *hsp70* upregulation upon heat shock in a manner similar to the loss of *tph-1* (Figures 3C and S3), suggesting that the serotonin signal may be transmitted through this ortholog of the mammalian metabotropic 5-HT₂ receptor to regulate the HSR. These experiments collectively indicate that serotonergic signaling is necessary for *hsp70* mRNA expression upon heat shock and could therefore serve as a signaling mechanism for HSR activation.

Optogenetic Excitation of Serotonergic ADF and NSM Neurons Activates the HSR

If serotonin release triggered by thermosensory activity was indeed a signal to nonautonomously activate HSF1 in other cells, we would predict that stimulating serotonin release directly from the NSM and ADF neurons in wild-type animals without exciting thermosensory neurons should be sufficient to activate HSF1 in distant tissues. To test this, we again used optogenetics [18] to stimulate only the NSM and ADF neurons while maintaining animals at 20°C (Figure 5A). This

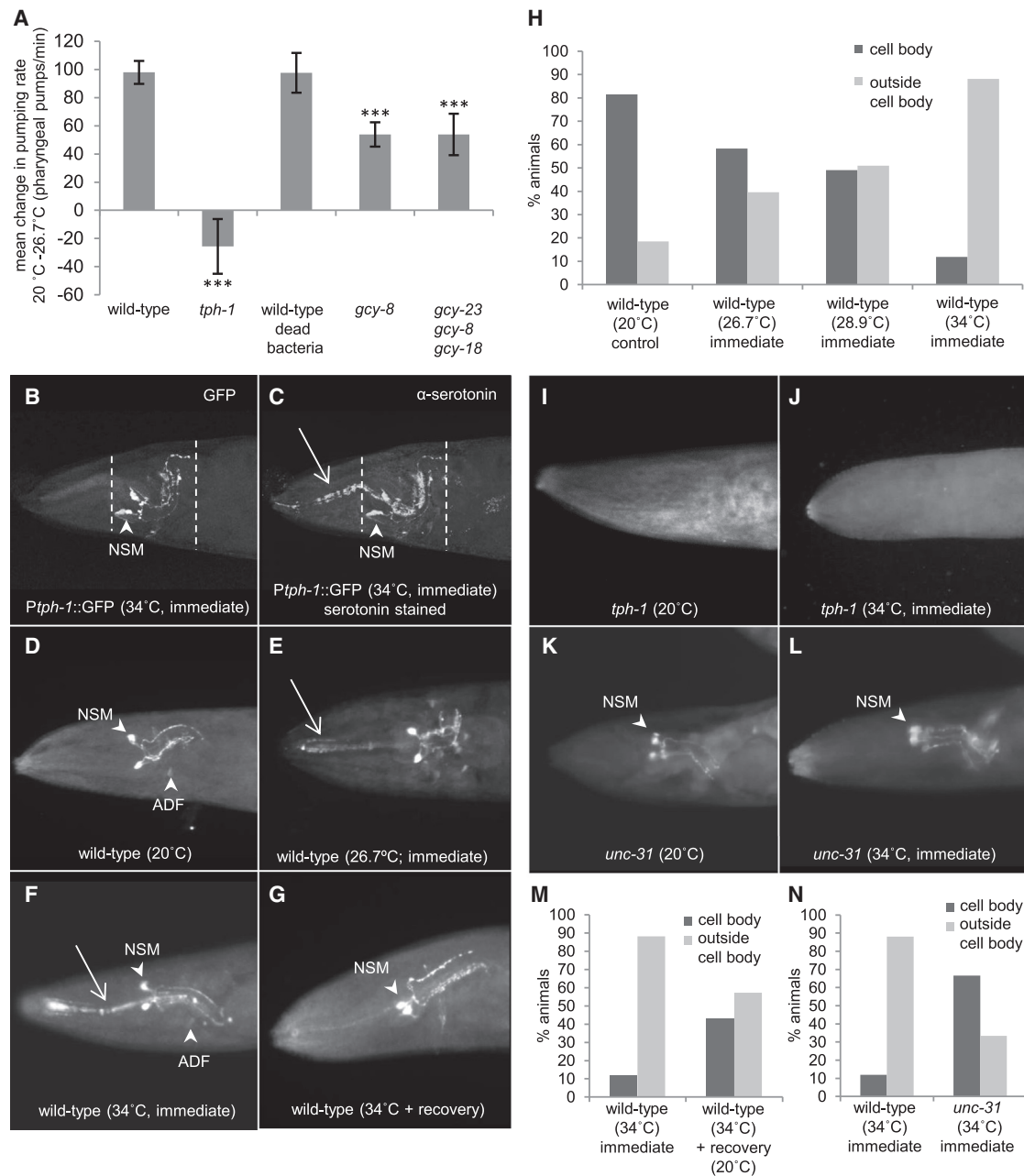


Figure 2. Serotonin Is Released upon Temperature Increase

(A) The change in mean pumping rates when temperature increases from ambient (20°C–22°C) to 26.7°C in the following animals: wild-type animals under normal growth conditions, *tph-1(mg280) II*, wild-type animals transferred to dead bacteria, *gcy-8(oy44) IV*, and *gcy-23(nj37) gcy-8(oy44) gcy-18(nj38) IV*. $n = 9$ for wild-type on dead bacteria; $n = 20$ –25 for all others. Values indicate mean \pm SEM, t test; *** $p < 0.001$.

(B and C) Collapsed confocal z stack image showing serotonin staining in *mglg42* animals expressing GFP in serotonergic neurons under a *tph-1* promoter. Animals were fixed immediately following a 15 min exposure to 34°C.

(B) *Ptp-1::GFP* marks serotonergic neuronal cell bodies (arrowhead), present within areas confined by dashed lines.

(C) Serotonin staining is detected both within the neuronal cell bodies and in areas outside the dashed lines (arrow).

(D–G) Serotonin immunolocalization in wild-type animals at 20°C ($n = 119$) (D), exposed for 5 min to temperature ramp rate of 1°C/min (corresponding to 26.7°C; $n = 96$) (E), exposed to 34°C for 15 min ($n = 119$) (F), and exposed to 34°C for 15 min and recovered at 20°C for 1 hr ($n = 7$) (G).

(H) Percentage of wild-type animals, scored blinded (see [Experimental Procedures](#)), showing serotonin localization within or outside neuronal cell bodies at 20°C, 26.7°C, 28.9°C, and 34°C.

(I–L) Serotonin immunolocalization in *tph-1(mg280) II* at 20°C ($n = 15$) (I), *tph-1(mg280) II* at 34°C for 15 min ($n = 9$) (J), *unc-31(e928) IV; C12C8.1p::mCherry* at 20°C ($n = 13$) (K), and *unc-31(e928) IV; C12C8.1p::mCherry* at 34°C for 15 min ($n = 15$) (L). Arrowheads indicate cell bodies; arrows indicate serotonin localization outside cell bodies.

(M) Percentage of wild-type animals exposed to 34°C for 15 min and recovered at 20°C for 1 hr scored blinded for serotonin localization.

(N) Percentage of *unc-31; C12C8.1p::mCherry* animals exposed to 34°C for 15 min scored blinded for serotonin localization compared to wild-type.

See also [Figure S2](#).

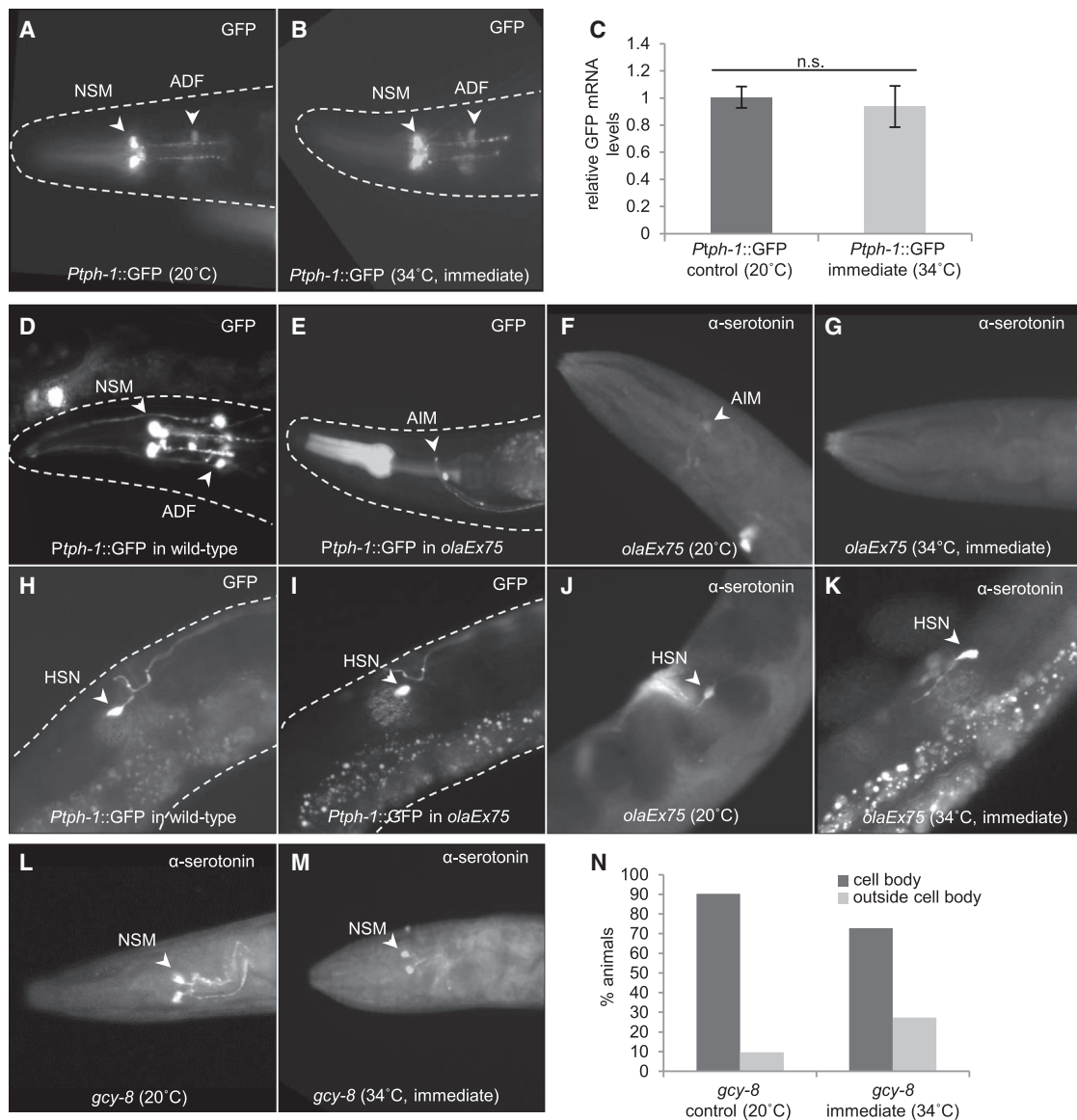


Figure 3. Serotonin Is Released from Serotonergic ADF and NSM Neurons upon Temperature Increase in an AFD-Dependent Manner

(A and B) Localization of *Ptp-1::GFP* at 20°C (A) and following exposure to 34°C for 15 min (B).

(C) GFP mRNA levels measured by qRT-PCR in *Ptp-1::GFP* animals before and immediately after 15 min heat exposure (34°C). Values indicate mean ± SEM.

(D and E) The expression of a split caspase under a minimal *tp-1* promoter ablates the ADF and NSM neurons in *olaEx75*-expressing animals; *Ptp-1::GFP* localization in serotonergic neurons in heads of wild-type animals (D) and *olaEx75*-expressing animals (E).

(F and G) Serotonin immunolocalization in *olaEx75*-expressing animals at 20°C (n = 11) (F) and immediately following a 15 min exposure to 34°C (n = 15) (G).

(H and I) *Ptp-1::GFP* expression in wild-type (H) and *olaEx75*-expressing (I) animals showing the presence of HSN neurons.

(J and K) Serotonin immunolocalization in HSN neurons is preserved in *olaEx75*-expressing animals at both 20°C (n = 11) (J) and following 15 min exposure to 34°C (n = 15) (K).

(L and M) Serotonin immunolocalization in *gcy-8(oy44) IV* animals at 20°C (n = 31) (L) and following 15 min exposure to 34°C (n = 11) (M).

(N) Percentage of *gcy-8(oy44)* animals scored for serotonin localization (20°C and 34°C, 15 min). Arrowheads indicate cell bodies.

was done by locally illuminating the NSM and ADF neurons in a previously characterized transgenic strain *lite-1(ce314) X; ljls102*, which expresses Chr2 only in these neurons [40]. We confirmed that the optogenetic activation of these serotonergic neurons caused the relocalization of serotonin into areas outside serotonergic neuronal cell bodies, as observed upon heat shock (Figures S4A–S4C). We also confirmed that, as previously reported [40], optogenetic excitation of serotonergic neurons decreased locomotory rates (number of body

bends) and increased pharyngeal pumping rates [29, 30] (Figures S4D and S4E). As described above, in all animals, prior to the light-induced excitation of the NSM and ADF neurons, HSF1 was present diffusely throughout nuclei (Figure S1C). This diffuse localization persisted in the control mock-activated animals grown in the absence of ATR (Figure 5B), and none (n = 22) of these animals displayed the relocalization of HSF1::GFP to nuclear stress granules/puncta (Figures 5D and 5E). In contrast, as seen upon excitation of the AFD

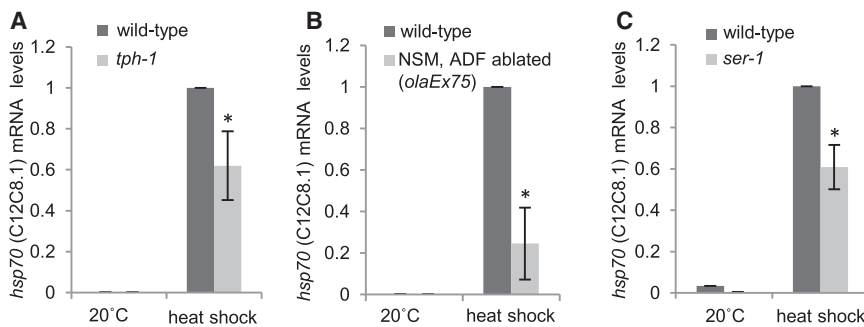


Figure 4. Serotonergic Signaling Is Necessary for *hsp70* Induction upon Heat Shock

(A–C) Quantification of *hsp70* (C12C8.1) mRNA levels by qRT-PCR following heat shock (34°C, 15 min) of wild-type and *tph-1*(*mg280*) // animals (A), wild-type and *olaEx75*-expressing animals (B), and wild-type and *ser-1*(*ok345*)X animals (C). RNA was extracted 30 min following recovery after heat shock. Values indicate mean fold change \pm SEM, t test; * $p < 0.05$. See also Figure S3.

thermosensory neurons, optogenetic activation of the ADF and NSM neurons caused 64% ($n = 44$) of the experimental animals grown on ATR to exhibit relocalization of HSF1::GFP into nuclear stress granules within 10–20 min postlight activation (Figures 5C and 5D). The number of nuclei with nuclear stress granules varied between animals and ranged from 6% to 90% per gonad ($n = 506$ nuclei), with an average of $35.9\% \pm 6.8\%$ (Figure 5E). Thus, optogenetic stimulation of the serotonergic neurons alone, even without exciting the AFD thermosensory neurons, is sufficient to activate HSF1 in distant tissues. Furthermore, optogenetic stimulation of either the ADF or the NSM neurons was effective in activating HSF1, although the ADF neurons appeared more effective (75% versus 33.3%; Figure 5D).

Although HSF1 nuclear stress granules form concomitant with the induction of heat shock gene transcription, HSF1 stress granules do not typically localize to *hsp70* promoters [19–21]. We therefore tested whether, along with the activation of HSF1 evidenced by nuclear stress granule formation, optogenetic stimulation of the ADF and NSM neurons also increased *hsp70* mRNA levels. To allow for harvesting of animals for mRNA extraction in these experiments, we performed optogenetic activation on free-behaving animals on small lawns of OP50 [39]. In experimental (+ATR), but not in control (–ATR), animals, stimulation of the ADF and NSM neurons resulted in a 5- to 8-fold induction of *hsp70* mRNA (Figures 5F and 5G). However, *hsp70* mRNA upregulation was transient, and mRNA levels declined after 20 min postoptogenetic stimulation (Figures 5F and 5G). These results taken together indicate that serotonin release from the ADF and/or NSM neurons is sufficient to activate HSF1 and initiate *hsp70* mRNA transcription. In subsequent experiments, we focused on activating only the ADF neurons.

SER-1 Is Necessary for HSF1 Activation

Serotonergic neurons not only release serotonin but also are responsible for the secretion of other small molecules, such as INS-1 [41]. Therefore, to confirm that serotonin rather than another signaling molecule was the trigger for the nonautonomous activation of HSF1, we examined whether the activation of HSF1 upon optogenetic stimulation of the ADF neurons could be abolished by mutations in the metabotropic serotonin receptor, SER-1. Indeed, in animals carrying a deletion in *ser-1*, both the numbers of animals activating HSF1 and the extent of HSF1 activation were markedly reduced (Figures 5H and 5I). Thus, 47.5% of nuclei in the wild-type animals showed the formation of HSF1 nuclear stress granules within 20 min following optogenetic activation of ADF serotonergic neurons ($n = 757$ nuclei); however, only 11.4% of nuclei in the *ser-1*(*ok345*)

deletion mutant animals showed any sign of HSF1 puncta ($n = 1,239$ nuclei) (Figures 5J and 5K). These data confirm that cell nonautonomous neuronal control of HSF1 occurs through serotonin secretion, although they do not preclude the involvement of other collaborating ligands.

Optogenetic Activation of Serotonergic Neurons Protects Protein Homeostasis

HSF1 transcriptional activation protects organisms against lethal stress and the accumulation of misfolded and aggregated proteins [6, 7]. Consistent with this, we found that optogenetic stimulation of serotonergic neurons in experimental (+ATR) animals increased organismal survival upon subsequent exposure to a lethal dose of heat stress when compared to controls (–ATR) (Figures 6A and 6B). We next examined whether optogenetic activation of the ADF neurons was sufficient to cell nonautonomously suppress misfolding and aggregation of Q35::YFP in body wall muscle cells of *C. elegans* [13, 23]. Remarkably, a single pulse of optogenetic stimulation of the ADF neurons alone was sufficient to reduce the age-dependent accumulation of Q35 aggregates (Figures 6A, 6C, and 6D). Although all animals started with a similar number of aggregates, control animals (–ATR) and experimental animals (+ATR) that had not been optogenetically activated (Figures 6D and 6E) accumulated an average of 26.2 aggregates by day 3 of adulthood. In contrast, optogenetic activation of ADF neurons reduced aggregation by 41% to an average of 15.5 aggregates per animal (Figures 6C and 6D), and aggregate accumulation remained lower with age. This reduction in the number of Q35 aggregates was HSF1 dependent as knockdown of HSF1 with RNA interference restored the number to control levels (Figure 6F).

Discussion

To date, the signaling mechanisms underlying nonautonomous control of the HSR and its biological relevance were unknown. Our experiments identify the enhanced release of serotonin resulting from thermosensory neuronal activity as a signaling mechanism sufficient to initiate the protective activation of HSF1 in remote tissues. Because in our optogenetic experiments this activation occurs in the absence of any detectable indications of stress, we hypothesize that, as in numerous psychophysical stress paradigms where serotonin release allows the animal to preemptively adapt its physiology to threatening conditions [1–3], serotonin release upon neurosensory perception of temperature change allows organisms to initiate protective protein quality control mechanisms prior to the oncoming onslaught of proteotoxic damage (Figure 7).

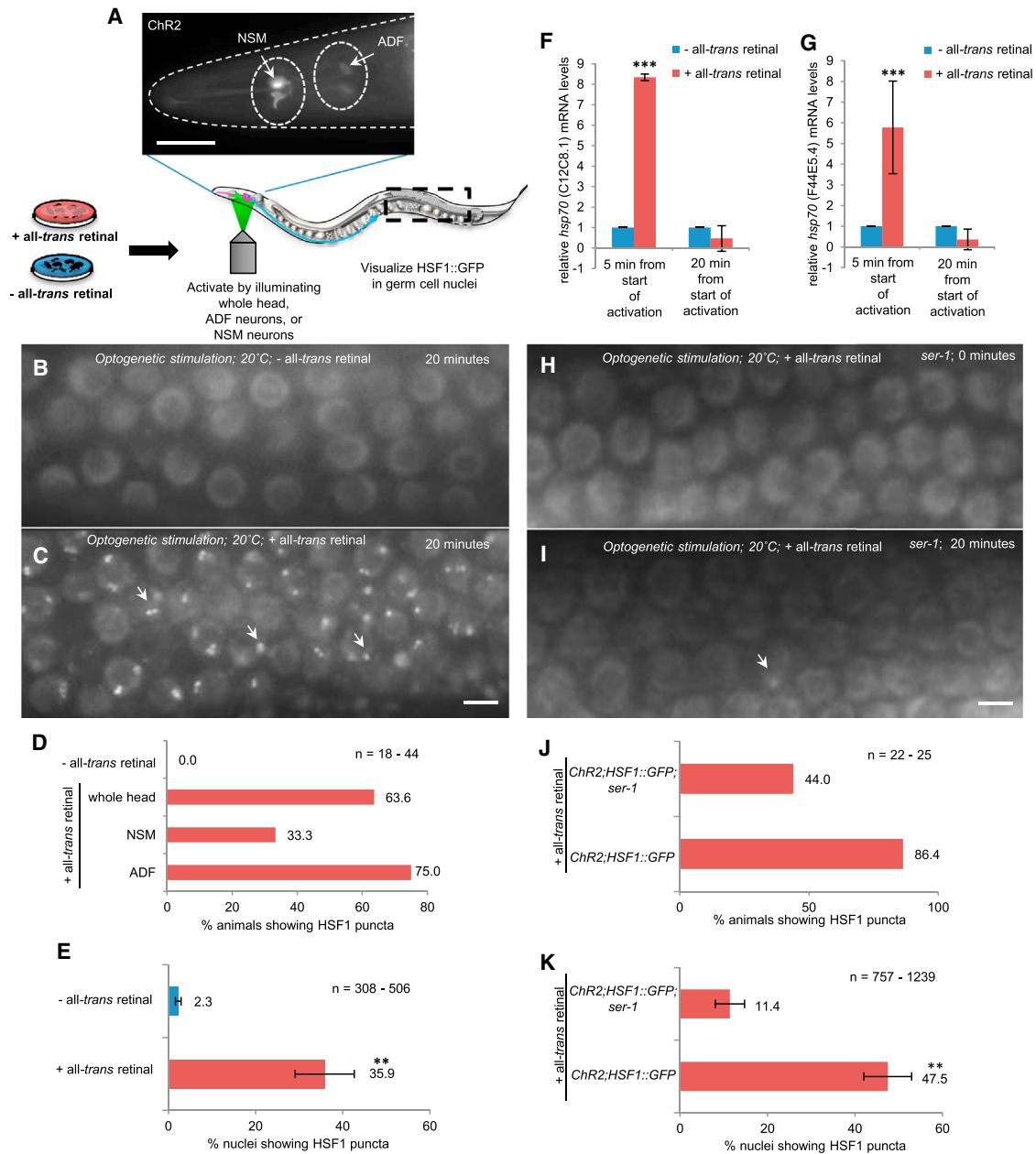


Figure 5. Optogenetic Stimulation of ADF and NSM Neurons Is Sufficient to Activate the HSR in Other Cells through the Serotonin Receptor SER-1

(A) Experimental setup: localized illumination of the whole head, serotonergic ADF neurons, or NSM neurons in individual *C. elegans* expressing ChR2 in these neurons (the scale bar represents 50 μm). Wavelength = 490 nm. Experimental animals were grown in the presence of +ATR; control animals were grown in the absence of -ATR. HSF1::GFP was monitored in germ cell nuclei.

(B and C) Germ cell nuclei showing HSF1::GFP localization in control (-ATR) (B) and experimental (+ATR) (C) animals at 20 min after the start of optogenetic stimulation (the scale bar represents 5 μm ; arrows indicate HSF1 puncta).

(D) Percentage of animals showing HSF1 nuclear puncta after stimulation of whole head (+ATR and -ATR) or only the ADF neurons or NSM neurons (+ATR). n = 18–44 animals per condition.

(E) Percentage of nuclei with HSF1 nuclear puncta in control (-ATR) and experimental (+ATR) animals following optogenetic stimulation. n = 308–506 nuclei. Values indicate mean \pm SEM, t test; **p < 0.01.

(F and G) *hsp70* (C12C8.1) (F) and *hsp70* (F44E5.4) (G) mRNA levels measured using qRT-PCR in experimental (+ATR) animals relative to control (-ATR) animals. Animals were harvested 5 min and 20 min from the start of optogenetic stimulation. Values indicate mean \pm SD, t test; ***p < 0.001.

(H and I) Germ cell nuclei showing HSF1::GFP localization in experimental (+ATR) *ser-1(ok345)*X animals at 0 min (H) and 20 min (I) after the start of optogenetic stimulation (the scale bar represents 5 μm).

(J) Percentage of animals showing HSF1 nuclear puncta after stimulation of the ADF serotonergic neurons in experimental (+ATR) wild-type and *ser-1(ok345)*X animals. n = 22–25 animals per condition.

(K) Percentage of nuclei with HSF1 puncta in experimental (+ATR) wild-type and *ser-1(ok345)*X animals. n = 757–1,239 nuclei. Values indicate mean \pm SEM, **p < 0.01.

See also Figure S4.

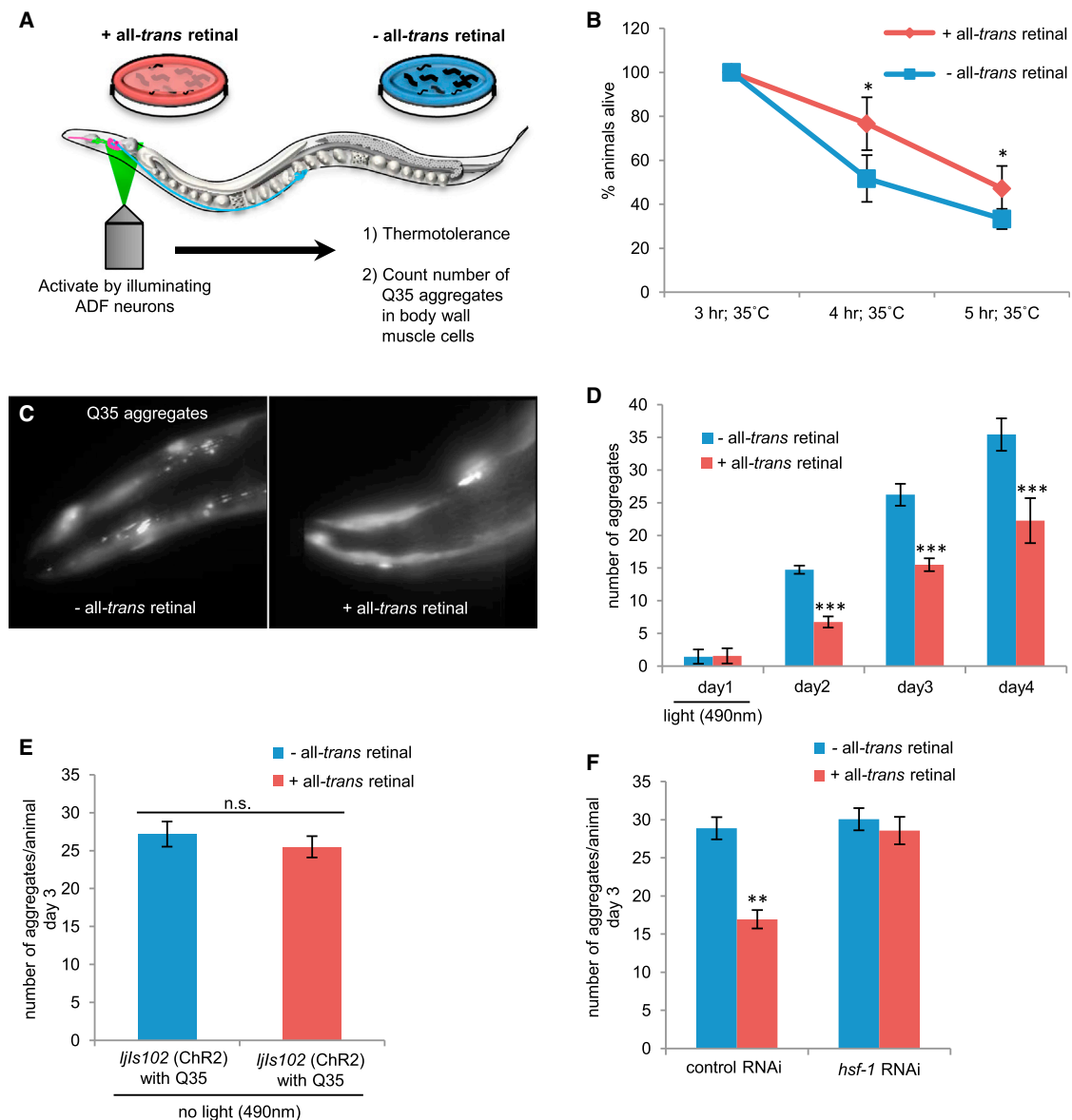


Figure 6. Optogenetic Stimulation of ADF and NSM Neurons Increases Thermotolerance and Suppresses polyQ Aggregation in Body Wall Muscle Cells

(A) Experimental set up (as described in Figure 5 legend).

(B) Thermotolerance of experimental (+ATR) versus control (–ATR) animals following optogenetic stimulation. $n = 80\text{--}83$ animals per condition; two-way ANOVA; * $p < 0.05$.

(C) Q35::YFP aggregates visualized in head-muscle cells of control (–ATR) and experimental (+ATR) animals 3 days following optogenetic stimulation.

(D) The number of Q35::YFP aggregates in control (–ATR) and experimental (+ATR) animals over time. $n = 7\text{--}10$ animals per time point per experimental manipulation, t test; *** $p < 0.001$.

(E) The number of Q35::YFP aggregates in control (–ATR) and experimental (+ATR) animals in the absence of optogenetic stimulation.

(F) The number of Q35::YFP aggregates following RNAi-mediated knockdown of HSF1 in experimental (+ATR) animals following optogenetic stimulation. $n = 26$, t test; ** $p < 0.01$.

Notably, *hsp70* accumulation upon optogenetic activation of neuronal circuitry is orders of magnitude less than what is seen following actual exposure to high temperatures or other proteotoxic conditions. Given the instability of *hsp70* mRNA under normal, nonproteotoxic conditions, this is perhaps not too surprising [42, 43]. These data support our hypothesis that serotonin release may be an initiating event in *hsp70* induction important for the adaptation of the organism to the subsequent protein damage that could be inflicted if noxious conditions persist.

Our data show that the AFD thermosensory neurons communicate with the ADF and NSM serotonergic neurons. How this occurs is not known. There is evidence for crosstalk between serotonergic ADF and thermosensory AFD neurons of *C. elegans* involving conserved signaling pathways, and these may play a role [12, 44–46]. For instance, while the lack of serotonin does not disrupt thermotaxis, serotonin is required for the adaptive plasticity of *C. elegans* thermotaxis behavior through activation of the protein kinase C homolog [46]. AFD neurons are themselves not serotonergic, nor do

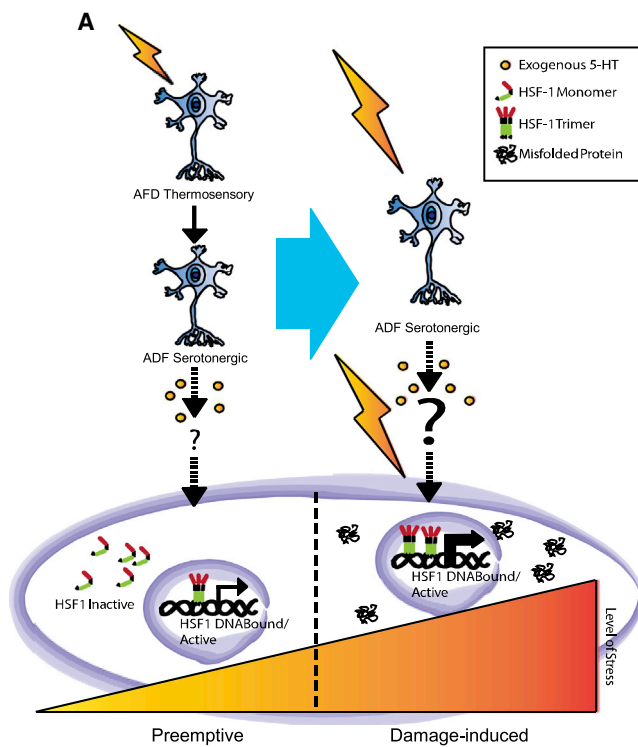


Figure 7. Model for HSF1 Activation upon Neurosensory Release of Serotonin

The HSR of a cell can be activated in *C. elegans* in two distinct phases. An initial preemptive neurosensory phase involves the excitation of AFD thermosensory neurons that typically respond to innocuous temperature shifts. This elicits serotonin release, directly or indirectly activates HSF1 to transiently increase *hsp70* mRNA levels, and occurs in the absence of protein misfolding. Subsequently, prolonged exposure to heat can cause protein misfolding, and the resulting damage induces HSF1-dependent *hsp70* transcription as has been well described. The role of serotonin in this latter phase is not known.

they synapse directly onto serotonergic neurons. Thus, AFD neurons could act through downstream interneurons, such as the AIY, to communicate with serotonergic neurons via synaptic mechanisms or electrical/chemical coupling across gap junctions. Alternatively, the excitation of AFD neurons may cause local paracrine release of neuroendocrine factors, which act on the serotonergic neurons to elicit serotonin release. These mechanisms are not mutually exclusive and remain to be investigated. We also do not know where serotonin is localized following its enhanced release or whether its effects on germ cell nuclei are direct or indirect. The NSM neurons have sensory endings in the pharyngeal lumen and are well poised to communicate with the rest of the worm's body via secretion into the pseudocoelomic fluid [37, 39], and SER-1 is expressed in many tissues, including pharyngeal muscle, neuronal processes in the nerve ring, neurons in the tail, ventral cord motor neurons in vulval muscle, uterine cells, and perhaps in the posterior intestine [47, 48], where it could play a role in the secondary transmission of the stress signal to germ cell nuclei. In addition, the AIM and RIH neurons express the serotonin reuptake channel MOD-5 and are known to uptake extrasynaptic serotonin [35]; however, since deletion of *mod-5* does not cause a decrease in the HSR, this pathway may not be integral to the signaling mechanism.

An intriguing possibility is that enhanced serotonin release could act as a node for the nonautonomous regulation of HSF1 in response to multiple noxious environmental stimuli. Serotonergic signaling is involved in the innate immune response to pathogens and response to hypoxia [27, 28]. It would be of interest to see whether activation of the sensory neurons involved in pathogen recognition or oxygen tension alone can also preemptively activate HSF1 through serotonin release.

The decrease in polyQ aggregation following stimulation of serotonergic neurons is also striking. The most dramatic decrease occurs within the first 2 days following optogenetic stimulation, presumably due to the transient activation of HSF1. However, aggregation continues to occur at apparently lower rates. At the molecular level, we do not know whether this corresponds to decreasing the pool of oligomers, increasing degradation rates, or both. Although technically challenging in our current assays, it would be interesting to determine whether a more-patterned regimen of optogenetic stimulation could completely prevent exacerbation of aggregation altogether. Serotonergic signaling pathways are highly conserved between *C. elegans* and mammals, suggesting that the nonautonomous regulation of protein homeostasis seen here may also be conserved. Indeed, serotonin is integral to thermoregulation in mammals [49, 50], and serotonergic syndrome, a life-threatening human condition associated with excessive serotonergic activity, causes fever, a stimulus for HSF1 activation [51, 52]. Indeed, modulating serotonin levels by administration of SSRIs can provide enhanced protection against neurodegeneration in vertebrate models of protein conformational diseases [53]. Conversely, dysregulation of serotonin signaling alters aging rates in *C. elegans* and is associated with Alzheimer's disease and ALS [53, 54]. In summary, if protein quality control mechanisms in multicellular organisms can be activated cell nonautonomously and preemptively through the regulation of sensory neuronal modalities and serotonin release as shown here, another treatment of neurodegenerative diseases could involve modulation of neurosensory systems: a method that can be used alone or in combination with small molecules to effectively target protein misfolding.

Experimental Procedures

Detailed experimental procedures are described in [Supplemental Experimental Procedures](#).

Growth Conditions

All strains were grown and maintained at 20°C. Extreme care was taken to maintain ambient temperature in the room very close to 20°C. Two transgenic strains were generated for these experiments: (1) a strain, AM1061, that expressed a *hsf-1* minigene fused to GFP integrated into the *ttT15605* // locus and (2) a strain, DCR186, that expressed the *olaEx75* [*Ptph-1::caspace-3(p12)::nz*; *Ptph-1::cz::caspace-3(p17)*; *Punc-122::mCherry*] transgene, which induced the caspase-mediated ablation of NSM and ADF neurons. Strains expressing Chr2 in the AFD thermosensory neurons and ADF and NSM serotonergic neurons were kind gifts from Paul Sternberg and Michael R. Koelle, respectively. The remaining strains were obtained from the Caenorhabditis Genetics Center (CGC).

Measuring Pumping Rates

The pumping rate of each worm was recorded under a MZ10F stereo microscope (Leica) at 8× magnification or under the Axio Observer A1 inverted microscope (Zeiss) at 40× magnification, as needed.

Serotonin Whole-Worm Immunostaining Protocol

Anti-serotonin staining was performed following a modified protocol developed by the Loer laboratory (<http://home.sandiego.edu/~cloer/loerlab/anti5htshort.html>). Serotonin localization patterns were scored blinded

and quantified using *Ptph-1::GFP* expression to define serotonergic neuronal cell bodies. Specifically, collapsed confocal z stack images were taken for each worm to visualize both GFP (*Ptph-1::GFP*) and serotonin. Colocalization of serotonin and GFP expression was seen within neuronal cell bodies, but only anti-serotonin staining was observed outside the neuronal cell bodies. Each worm was divided into the following three areas: area 1 (from metacarpus to buccal cavity), area 2 (serotonergic neuronal cell bodies), and area 3 (from pharyngeal terminal bulb toward posterior body). Images were classified in a binary manner, based on the following criteria: (1) location (presence of staining in area 1); (2) continuity of staining in area 1; (3) intensity higher than that of background. For criteria (1) and (3), the background fluorescence in area 1 was quantified as mean pixel intensity. Animals with continuous staining in area 1, which had an average intensity of 150 mean pixel intensity over background, were scored as positive for staining outside serotonergic neuronal cell bodies.

Heat Shock Protocol

The animals were subjected to temperature increase in two ways: (1) heat shock on a Peltier stage (PeCon GmbH Temp Controller) and (2) heat shock in a water bath.

RNA Extraction and qRT-PCR

RNA extraction was conducted according to previously published methods [1]. The relative amounts of *hsp* mRNA were determined using the delta delta C_T ($\Delta\Delta C_T$) method. Actin mRNA was used as an internal control. All relative changes of *hsp* mRNA were normalized to that of wild-type heat-shocked animals, except where otherwise noted. Technical duplicates or triplicates were used to obtain C_T values. Experiments were repeated at least three times. Values shown indicate mean \pm SEM. All quantitative PCR (qPCR) reactions were assessed by melt-curve analysis performed at the end of the reaction and included no reverse transcriptase (no-RT) controls.

Optogenetic Experiments

Optogenetic activation was conducted according to previously published methods as per the requirements of the experiment. The main concerns were to minimize stress from the methodology itself and to obtain enough sample material. Thus, for HSF1 activation and Q35 protection assays, optogenetic stimulation was conducted on immobilized animals under the Axio Observer A1 inverted microscope (Zeiss) using Andor iQ 2.9.1 software (Andor) and X-Cite XLED1 1.1.0.2 software (Lumen Dynamics) to control the light source. For *hsp70* mRNA measurements and thermotolerance assays, free-behaving *iJls102*; *lite-1(ce314)* animals were activated at 6.3 \times magnification using a MZ10 F microscope with an EL6000 light source (Leica). Optogenetic serotonin release following light stimulation was confirmed by measuring pharyngeal pumping rates or body bends per minute.

Thermotolerance Experiments

All experiments following exposure to lethal heat stress were scored blinded.

Statistical Analysis

Where applicable, significance was tested using Student's *t* tests or, for the thermotolerance assay, a two-way ANOVA. *p* values are indicated as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001. A Fisher's exact test in conjunction with a post hoc power analysis was used for determining the appropriateness of our smaller sample sizes to support our conclusions.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2014.11.040>.

Author Contributions

M.C.T., M.R.C., F.K.O., and V.P. designed and performed the experiments and wrote the manuscript. L.C. and R.I.M. made and contributed the AM1061 strain. L.A.M.-V. made the DCR186 strain. H.W.M.S. contributed the anti-serotonin antibodies. All authors commented on the manuscript.

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