Inactivation of GABA_A receptor is related to heat shock stress response in organism model *Caenorhabditis elegans*

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Abstract The mechanisms underlying oxidative stress (OS) resistance are not completely clear. *Caenorhabditis elegans* (*C. elegans*) is a good organism model to study OS because it displays stress responses similar to those in mammals. Among these mechanisms, the insulin/IGF-1 signaling (IIS) pathway is thought to affect GABAergic neurotransmission. The aim of this study was to determine the influence of heat shock stress (HS) on GABAergic activity in *C. elegans*. For this purpose, we tested the effect of exposure to picrotoxin (PTX), gamma-aminobutyric acid (GABA), hydrogen peroxide, and HS on the occurrence of a shrinking response (SR) after nose touch stimulus in N2 (WT) worms. Moreover, the effect of HS on the expression of UNC-49 (GABA_A receptor ortholog) in the EG1653 strain and the effect of GABA and PTX exposure on HSP-16.2 expression in the TJ375 strain were analyzed. PTX 1 mM- or H₂O₂ 0.7 mM-exposed worms displayed a SR in

about 80 % of trials. GABA exposure did not cause a SR. HS prompted the occurrence of a SR as did PTX 1 mM or H₂O₂ 0.7 mM exposure. In addition, HS increased UNC-49 expression, and PTX augmented HSP-16.2 expression. Thus, the results of the present study suggest that oxidative stress, through either H₂O₂ exposure or application of heat shock, inactivates the GABAergic system, which subsequently would affect the oxidative stress response, perhaps by enhancing the activity of transcription factors DAF-16 and HSF-1, both regulated by the IIS pathway and related to *hsp-16.2* expression.

Keywords Heat shock stress · GABA_A receptor · HSP-16.2 · *C. elegans*

Introduction

The occurrence of reactive oxygen species (ROS) is a normal feature of aerobic life. However, environmental contaminants, such as sunlight (i.e., UV exposure), smoke, ozone, herbicides, etc., are additional sources of ROS as well. Although these compounds have an important role in several biological systems, their excessive production could result in oxidative stress (OS) which is an imbalance between pro-oxidant and antioxidant compounds, always in favor of the former. OS is associated with a vast series of pathologies which have a subtle beginning, become chronic, and end by being incapacitating and extremely costly, such as premature aging, atherosclerosis, chronic degenerative diseases (Parkinson's and Alzheimer's disease, amyotrophic lateral sclerosis, diabetes), and cancer. The ability of the organism to cope with oxidative stress depends on a wide range of sophisticated stress response mechanisms through which cells and tissues adapt to, repair, and eliminate injured molecules. At the cellular level, responses depend on cell type, the oxidizing agent, dosage, and/or duration of exposure and can range from

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proliferation to growth arrest, to senescence, or to cell death. Despite the range of responses, they reveal a balance among the number of intracellular stress signaling pathways, whose activation modulates transcription factors and consequently the pattern of gene expression (Martindale and Holbrook 2002).

Several studies on oxidative stress have been carried out in animal models, including the nematode *Caenorhabditis elegans* (Kahn et al. 2008; Koon and Kubiseski 2010; Morimoto 2006), the fruit fly *Drosophila Melanogaster* (Ahamed et al. 2010; Bahadorani et al. 2010), and mice (Straadt et al. 2010). *C. elegans* in particular exhibits defense mechanisms against oxidative stress similar to those in human beings, such as antioxidant enzymes, glutathione system, and stress response signaling pathways. In fact, assays conducted on the nematode *C. elegans* have shown the existence of genes whose expression results in nuclear receptors and proteins of the insulin/IGF-1 signaling (IIS) pathway that are involved in environmental stress response and life span. Certainly, a decrease in the activity of the IIS pathway modifies development, raises stress resistance, protects against proteotoxicity, and extends life span in worms, flies, and mice (Morimoto 2006). The IIS pathway comprises a receptor that signals to a PI3 kinase/AKT conserved pathway which, finally, downregulates to DAF-16 activity by inhibiting its translocation into the nucleus. Thus, the major downstream target of IIS is DAF-16, the sole *C. elegans* ortholog of the FOXO family of transcription factors, and its translocation induces the expression of cytoprotective proteins such as heat shock proteins (HSPs), some antioxidant enzymes (superoxide dismutase and catalase), and other proteins against stress (de Pomerai et al. 2008; Hsu et al. 2003). HSPs are evolutionarily conserved chaperones present in all cellular compartments and classified according to their molecular weight. Some of the major chaperones (Hsp70, Hsp90, small HSPs) are present at high concentrations in non-stressed cells reaching 1–5 % of total cellular protein (Soti and Csermely 2007).

Indeed, after a stressful stimulus in *C. elegans*, cellular response is based on induction of stress proteins or heat shock proteins, through inhibition of the IIS pathway (Morimoto 2006). Genetic manipulations to overexpress individual chaperones, like Hsp90 and Hsp70, resulted in a subtle extension of life span (Morley and Morimoto 2004); however, the small chaperone Hsp16 seemed to be more efficient in worms (Soti and Csermely 2007), particularly HSP-16.2 that is often used as an indicator of increases in thermal and oxidative stress (Hartwig et al. 2009; Rea et al. 2005; Strayer et al. 2003; Wiegant et al. 2009; Wu et al. 2006; Xiao et al. 2009).

Other IIS-regulated transcription factors participate in DAF-16 target genes. Thus, a second key regulator of the stress response is HSF-1, which is required to activate small *hsp* (*shsp*) expression after heat shock, just like DAF-16. DAF-16 seems to act independently of HSF-1; however, HSF-1 is necessary for a greater expression of the gene *shsp* (Honda and Honda 2005). Therefore, in *C. elegans*, the stress

response is almost entirely due to the activity of transcription factors DAF-16 and HSF-1.

Molecular, structural, and functional changes also take place in the nervous system, as a result of oxidative stress or aging. Thus, neurons can give way to neurodegenerative processes or adapt to these changes, through multiple mechanisms for maintaining the integrity of neuronal circuitry, facilitating the responses to environmental impacts, and promoting functional recovery after injury. In this sense, it has been reported that DAF proteins impinge on the nervous system (Wolkow et al. 2000). Moreover, it has been reported that IIS reduces glutamate-induced gamma-aminobutyric acid (GABA) release in mammals (Castro-Alamancos et al. 1996; Castro-Alamancos and Torres-Aleman 1993). Interestingly, GABA is the primary inhibitory neurotransmitter in vertebrate and invertebrate nervous systems. In fact, GABA neurotransmission abnormalities cause neurological diseases such as anxiety disorders and epilepsy in humans (Kleppner and Tobin 2001; Sieghart 1995).

Presynaptic and postsynaptic mechanisms of GABA neurotransmission are both well-characterized and conserved between *C. elegans* and humans. Predominantly, *C. elegans* GABA_A receptors are expressed in muscles, and their activation promotes receptor conformational change, allowing an intracellular chloride influx and cell hyperpolarization. Consequently, during a bend of the body, the 19 GABAergic D-type neurons inhibit contraction of the ventral or dorsal body wall muscles, whereas the cholinergic neurons contract muscles on the opposite side, which gives the worm its distinctive sinusoidal body posture (McIntire et al. 1993a). Thus, worms display a characteristic locomotory deficiency when their GABA neurotransmission fails (McIntire et al. 1993b). Hence, locomotion depends on the interaction between GABA_A receptor (GABA_AR) and GABA-releasing neurons (Jorgensen 2005).

As yet, the cellular mechanisms underlying in vivo oxidative and/or thermic stress are not fully understood. Therefore, studies aimed to increase knowledge of these mechanisms are important in order to devise new pharmacological and genetic approaches for preventing oxidative stress by environmental agents or aging.

Insofar as *C. elegans* offers an integrated vision of the response to different types of environmental stresses in correlation with its cellular and molecular mechanisms, this model organism allows us to study aging and neurodegeneration processes. Thus, the aim of this work is to determine the influence of GABAergic activity on heat shock stress response in the nematode *C. elegans*.

Methods

Strains and culture conditions

The strains used in this study included the following: Bristol N2 (wild type), TJ375 [*hsp-16-2::GFP(gpIs1)*], and EG1653

oxIs22 [*Punc-49::UNC-49::GFP, lin-15(+)*] II, which were provided by the Caenorhabditis Genetics Center (Minneapolis, MN, USA). EG1653 and TJ375 strains have been regularly used, respectively, to quantify the expression of UNC-49 (Abraham et al. 2011; Byrd et al. 2001; Garafalo et al. 2015; Knobel et al. 2001; Rowland et al. 2006) and HSP-16.2 (Abbas and Wink 2010; Lamark and Johansen 2012; Leroy et al. 2012; Rea et al. 2005; Shore et al. 2012; Strayer et al. 2003; Wang et al. 2012; Wu et al. 2006) based on the quantitation of their GFP fluorescence intensities.

In the case of EG1653, this encloses the *C. elegans* GABA_A receptor UNC-49 tagged with GFP. This strain contains an integrated transgene of the functional UNC-49B::GFP marker with GFP inserted, in-frame, into the intracellular loop of the UNC-49B subunit. This UNC-49B-GFP translational fusion protein fully rescues the uncoordinated phenotype of *unc-49* (e407) and localizes to synapses. Also, GABA_A receptors in *C. elegans* muscle cells are simple and uniform, in contrast to the structurally complex mammalian GABA_A receptors (Bamber et al. 1999; Gally and Bessereau 2003; Rowland et al. 2006). UNC-49 receptors normally form clusters opposite inhibitory presynaptic terminals and, if this is not the case, are internalized and traffic to autophagosomes for degradation. Thus, the fluorescence intensity of GFP-tagged UNC-49 correlates with the abundance of GABAR subunit UNC-49 at the neuromuscular junction, and this asset has been exploited in several works (Garafalo et al. 2015; Kullyev et al. 2010; Schuske et al. 2004, 2007).

For its part, TJ375 is a chromosomally integrated transgenic strain containing the 400-bp *hsp-16.2* promoter coupled to the gene encoding GFP. The reporter encodes no HSP-16.2 product itself, and GFP protein expressed via this construct has the same expression pattern as HSP-16.2, so that those worms with high GFP expression have a high HSP-16 expression, and worms with low GFP expression have a low HSP-16 expression (Link et al. 1999). Consequently, the levels of GFP fluorescence and HSP-16.2 expression are strongly correlated in TJ375. Therefore, this strain provides an accurate assessment of the total amount of native HSP-16.2 protein (Rea et al. 2005; Wu et al. 2006).

All experiments were conducted on age-synchronized day 1 adult worms, obtained according to standard methods (Hope 2000). Nematodes were cultivated at 20 °C in petri dishes on nematode growth medium (NGM), with *Escherichia coli* OP50 bacteria as a food source, as described by Brenner (Brenner 1974).

Reagents

Picrotoxin (PTX) hydrogen peroxide and GABA were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Heat shock stress

For heat shock stress protocol, 30–40 adult worms (4 days) were transferred to 35-mm petri dishes seeded with *E. coli* (OP50) bacteria and subsequently shifted to 34 °C for 1 h. All animals that underwent heat shock conditions were allowed to rest for a period of 4 h at 20 °C before being moved to the next experimental stage.

Hydrogen peroxide assay

Adult worms from a WT strain were transferred into a 24-well plate with an average of 20 animals per well. At time zero, serially M9 buffer-diluted hydrogen peroxide was added to the wells. A set consisted of four concentrations from 0 to 0.7 mM hydrogen peroxide in a final volume of 2 ml. After 30 min, the total number of animals and the number of animals presenting shrinking response were scored for each well.

Drug exposure

In order to test the effects of several doses (picrotoxin, GABA), the drugs used in this study were diluted in M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 85 mM NaCl, 1 mM MgSO₄) (Brenner 1974; Jiang et al. 2011) and the assays were conducted in 24-well plates. The final volume in each well was 2 ml of different dilutions of drugs or 2 ml of M9 buffer (control). Nearly 50 1-day adult worms were transferred to each well. Exposure time was 4 h at 20 °C and without food source (Bamber et al. 2003, 2005).

Motor behavioral test

Shrinking response (SR). This response was evoked by employing the “nose touch response” or *Not* Assay, which induces a reversal coupled with the suppression of foraging head movements followed by a deep ventral bend (omega turn) and an 180° change in the direction of locomotion (Pirri and Alkema 2012). This assay was tested by stroking an animal with an eyebrow at the posterior bulb of the pharynx of an animal moving forward, as described by Chalfie and Sulston (1981). Normally, a trial is scored as a success when animals either halted forward locomotion or initiated backward movement following the stimulus. However, in our case, we were looking for a failure in the GABA system, so it was scored as a success when we found a “shrinking” response. Assays were performed by scoring the number of shrinking responses to ten nose touch trials (% response) as previously described (Kaplan and Horvitz 1993). Worms were video-recorded in a Motic DM143 stereoscopic microscope equipped with a digital camera (Motic Instrument, Inc., Richmond, BC, Canada), and subsequently, videos were analyzed for shrinking response with NIH Image Tracker software.

Fluorescence microscopy

Our purpose in using the transgenic strains was to detect UNC-49 and HSP-16.2 proteins without killing the worms, taking into account the optical transparency of *C. elegans*, which allows for non-invasive visual assessment of the GFP-fused HSP-16.2 and UNC-49 proteins through the quantification of fluorescence intensity. In addition, in the series of experiments that we conducted, we exposed the different study groups to the same stressor exposure time and they were visualized at a similar point time after exposure, in order to measure in a reproducible and comparable manner. All epi-fluorescence images were captured using a 20 $\times$ objective of an Olympus IX71 inverted microscope with a digital camera (Olympus Corporation, Tokyo, Japan). Every image was taken at constant exposure times. As green fluorescent protein (GFP) is excited in blue light range, a triple band filter that allows for that excitation range was employed. Fluorescent images were processed with software Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) (Strayer et al. 2003). Briefly, images were converted to grayscale at 16 bits, and afterwards, optical density calibration was undertaken. The latter procedure consists in an image segmentation, in which each pixel is assigned to one group or segment, in this case, pixels with or without fluorescence, in order to find an appropriate threshold value for fluorescence derived from the GFP-fused protein of interest. Thereafter, head and body areas in *C. elegans* were outlined, and then, integrated optical density (IOD) was measured. IOD is a parameter describing the average fluorescence intensity per area or volume of fluorescence ($\text{IOD} = \text{area} \times \text{fluorescence intensity}$) (Belelli and Lambert 2005; Peters et al. 2007). All values obtained were normalized to the untreated control mean and expressed as a percent of change.

Data analysis and statistics

Data are indicated as means $\pm$ SEM. The number of determinations, n , is indicated in parentheses. One-way ANOVA and Student's t test were used to evaluate the statistical significance of unpaired averages. $P < 0.05$ was considered significant. For statistical analysis, Sigma Plot 11.0 software was used.

Results

Picrotoxin and H_2O_2 exposure increase the shrinking response in a dose-dependent fashion

As expected, the CTL group responded normally to nose touch stimulus in 92.9 ± 2.1 % of trials, a similar result to those formerly reported (Hart et al. 1999; Kaplan and Horvitz 1993). However, among CTL animals with a defective response, we

could not observe shrinking after stimulus. Therefore, in CTL worms, SR was not considered to be present. Figure 1a shows a progressive increment of SR, which is a picrotoxin dose-dependent effect, using concentrations from 0.1 to 1000 μM . This dose-response curve showed an EC_{50} of 283.9 μM , but since 100 μM PTX, SR was present in 32 ± 5.3 % of trials. Even more, the last dose, 1000 μM , showed that in 82 ± 6.3 % of trials, SR was present. Likewise, hydrogen peroxide (H_2O_2) exposure also increased the presence of SR in a dose-dependent manner, with an EC_{50} of 0.543 mM, and at a concentration of 0.7 mM, exposed worms display SR in 80 % of trials (Fig. 1b).

Heat shock provoked an increment in shrinking response, comparable to those induced by 1 mM picrotoxin or 0.7 mM H_2O_2 , and GABA reduced SR presented in H_2O_2 -exposed worms

N2 specimens underwent a heat shock treatment (HS group) according to a heat shock stress protocol. Later, SR was assayed and recorded in both groups as described above. The shrinking response was present in 83.6 ± 4.4 % of trials in heat shock-treated N2 worms. This result was compared to those in worms exposed to 1 mM PTX (82 ± 6.3 % of trials) or 0.7 mM H_2O_2 (80 ± 6.5 % of trials), but they were not significantly different statistically (one-way ANOVA, $P > 0.7$, Fig. 2a).

Furthermore, we tried the effect of administration of exogenous GABA (10, 100, and 1000 μM) in the nose touch assay, where exposed worms showed a normal backward locomotion (omega turn) by more than 90 % of the trials and SR was not observed; nevertheless, in 10 % of trials, some response to nose touch was not detected at 10 and 100 μM GABA, although the responsiveness was not significantly different from that exhibited by control worms (one-way ANOVA, $P > 0.6$; Fig. 2b).

On the other hand, N2 worms that were co-exposed to 0.5 mM H_2O_2 and 100 μM GABA showed a low appearance of SR (17.7 ± 5 % of trials) as compared with those exposed solely to 0.5 mM H_2O_2 (39.1 ± 7.6 % of trials). This implies that GABA reduced by 45.2 % the number of SR events induced by oxidation, a statistically significant change (Student's t test, $P < 0.02$; Fig. 2c).

Heat shock increased UNC-49 protein expression in ventral body wall muscle cells

Worms of the EG1653 strain of *C. elegans* expressing GFP-tagged GABA $_A$ receptor protein UNC-49 (Bamber et al. 1999) were subjected to a heat shock stress protocol. After a recovery period of 4 h, at 20 $^{\circ}\text{C}$, epi-fluorescent microphotographs were captured. These microphotographs were taken and analyzed according to the fluorescence microscopy protocol. Results of animals exposed to heat shock (HS group) were contrasted with those of non-exposed animals (CTL group). Graphs in Fig. 3 depict these comparisons. The head

Fig. 1 Picrotoxin and hydrogen peroxide increase the shrinking response after a nose touch in *C. elegans*. **a** Percent of shrinking response in *C. elegans* exposed to different doses of picrotoxin and **b** worm exposed to different concentration of hydrogen peroxide ($n = 90$ assays per group, $*P < 0.05$ vs non-exposed worms)

and body areas were explored. With regard to the head area, HS animals showed a decrease of 13.4 ± 2.6 % in UNC-49 protein expression, although this change was not statistically significant (Fig. 3a). On the other hand, body wall muscle cells showed that expression of UNC-49 in the ventral side was significantly more abundant than the dorsal side in both CTL (39.3 ± 5.6 , $P < 0.001$, Student's t test, $n = 12$, Fig. 3b) and HS groups (67.2 ± 18 , $P < 0.005$, Student's t test, $n = 14$, Fig. 3c). Taking this fact into account, we prioritized the comparison of populations of UNC-49 receptor between HS and CTL group in the ventral body wall muscle cells. Thus, we found that heat shock-treated worms displayed a greater expression of the UNC-49 protein in relation to non-treated worms, and this difference was statistically significant (an increase of 51 ± 16.3 % in relation to CTL group, $P < 0.01$, Student's t test, $n = 14$, Fig. 3d).

Picrotoxin exposure enhanced HSP-16.2 protein expression

TJ375 worms expressing GFP fused to heat shock protein HSP-16.2 were divided into four groups: One group of worms was not subjected to heat shock stress protocol or exposed to picrotoxin (CTL group); one group of animals underwent heat shock for 1 h at 34 °C (HS group); one group of worms was subjected to 1 h of exposition to PTX (PTX group), and finally, one group of worms was both exposed to picrotoxin and underwent heat shock stress (PTX + HS group). After recovery (4 h at 20 °C), epi-fluorescence microphotographs were captured and analyzed in accordance with the fluorescence microscopy protocol mentioned in the Methods section, but with the singularity that in this case, we only outlined the pharynx, because in this area, the HSP-16.2::GFP fluorescence intensity

Fig. 2 Shrinking response results from an impairment of GABAergic system. **a** Comparison of effects of treatments of heat shock, PTX 1 mM, and GABA 0.7 mM on shrinking response of *C. elegans*. **b**

Effect of GABA on the nose touch response. **c** Percent of shrinking response in GABA non-treated and treated worms exposed to H₂O₂ ($n = 90$ assays per group, $*P < 0.05$)

Fig. 3 Heat shock modifies the UNC-49 expression in *C. elegans*. **a** Effect of heat shock on UNC-49 expression in EG1653 worms. **b**, **c** Comparison between heat shocked and control worms in UNC-49::GFP fluorescence intensity in head; differences in UNC-49::GFP fluorescence

intensity between the dorsal side and ventral side of **b** control and **c** heat-shocked animals. **d** Differences in UNC-49::GFP fluorescence intensity in ventral side of control and heat-shocked worms ($n = 10$ worms per group, $*P < 0.05$)

is more perceptible, although usually it can be seen in the entire worm (Link et al. 1999).

The findings of these experiments are shown in the graph in Fig. 4a. As was expected, the HS group showed an increase of $67.9 \pm 9.2\%$ in HSP-16.2::GFP expression in relation to the CTL group ($P < 0.0001$, Student's *t* test). Moreover, we observed a remarkable increase in the expression of HSP-16.2::GFP in the PTX group ($217.5 \pm 12.4\%$) and the PTX + HS group ($197.2 \pm 11.3\%$) with regard to the CTL and HS groups. In such cases, these differences were statistically significant (one-way ANOVA, $P < 0.001$), but at the same time, the PTX and PTX + HS groups were not significantly different

from each other ($P > 0.22$, Student's *t* test). Representative epi-fluorescence microphotographs of all the study groups (CTL, HS, PTX, and PTX + HS) are shown in Fig. 4b.

Discussion

Damage induced by thermal stress has been associated with the accumulation of ROS and consequently with oxidative stress (Finkel and Holbrook 2000; Kampkotter et al. 2007). This is because hyperthermia increases the production and flow of cellular free radicals by uncoupling mitochondria

Fig. 4 Picrotoxin increases HSP-16.2::GFP expression measured via intensity fluorescence in TJ375 worms. **a** Graph of HSP-16.2::GFP intensity fluorescence in control worms (CTL), worms submitted to heat shock protocol (HS), picrotoxin-exposed worms (PTX), and worms exposed to picrotoxin and submitted to heat shock protocol (PTX + HS) ($n = 20$ worms per group, $*P < 0.001$ vs CTL group and $\#P < 0.001$ vs HS

group). **b** Fluorescent microphotographs (20x) of TJ375 worms: control worms (CTL), worms submitted to heat shock protocol (HS), picrotoxin-exposed worms (PTX), and worms exposed to picrotoxin and submitted to heat shock protocol (PTX + HS). Scale bar represents 100 μm . The strongest expression of HSP-16.2::GFP in TJ375 strain is in the pharynx area

and activating enzymes involved in the formation of free radicals (Flanagan et al. 1998), such as superoxide and H₂O₂, and additionally by impairing cellular antioxidant defenses against H₂O₂ and enhancing its cytotoxicity (Lord-Fontaine and Averill-Bates 2002; Lord-Fontaine and Averill 1999). In particular, hydrogen peroxide, a non-radical molecule, commonly produced by the action of the superoxide dismutase, although considered a weak oxidant, can indirectly cause damage in most of the biological macromolecules by prompting hydroxyl radical generation that is a potent oxidant (Lord-Fontaine and Averill 1999). Consequently, in this work, heat shock stress (HS [34 °C]) and H₂O₂ were utilized as ROS inducers in order to better understand the effects of oxidation on the GABAergic system.

Disruption of the GABAergic system causes a shrinking response after a nose touch in *C. elegans* due to the loss of inhibitory input to body wall muscles, which induces a simultaneous contraction of opposing muscle groups (Bamber et al. 1999; McIntire et al. 1993b; Reiner and Thomas 1995; Schuske et al. 2004). Thus, the shrinking response was augmented after 1 mM picrotoxin exposure, a situation that was also observed after heat shock (34 °C), even after 4 h of rest, suggesting that this treatment induces an inhibition or deactivation of the UNC-49 receptor, probably mediated by oxidant activity of ROS derived from thermal stress. The latter is supported by the fact that the shrinking response was increased after 0.7 mM H₂O₂ exposure as well.

On the other hand, the administration of exogenous GABA or GABA_AR agonists could not have any effect on shrinking response, because in the worst case scenario, this would cause a reduction of responsiveness to nose touch due to excessive muscle relaxation. We tested this effect on N2 worms exposed to GABA (10, 100, and 1000 µM), and SR was not observed; although a slight reduction in the response to nose touch was detected, responsiveness was not significantly different from that exhibited by control worms. This is consistent with a recent report in which the administration of muscimol, a GABA_AR agonist, did not induce any shortening of the entire animal in adult N2 wild-type *C. elegans* (Han et al. 2015).

Nonetheless, in the case of an impaired GABAergic neurotransmission, exogenous GABA would restore its inhibitory action depending on GABA_AR functionality. Indeed, in our experiments on worms exposed to H₂O₂, the addition of exogenous GABA restored the normal nose touch response by decreasing the frequency of the shrinking response, but only partially; therefore, this would indicate an important damage in GABA_AR.

Furthermore, over the last several years, researchers have reported a greater susceptibility of GABA_A receptors to oxidant agents (Amato et al. 1999; Castel et al. 2000; Robello et al. 1996; Wexler et al. 1998), including the *C. elegans* UNC-49 receptor (Dabbish and Raizen 2011). In all of the cases, oxidation induced an inhibition of GABA_A receptors. Sigmoid

movement depends on acetylcholine (ACh) neurotransmission as well but in our experiments appears unaffected by heat shock treatment. These results concurred with reports that ROS actions do not deactivate nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction (Ben-Haim et al. 1973; Giniatullin et al. 2005). On the contrary, ROS and heat shock inhibit or deactivate enzyme acetylcholinesterase (Eichler and Silman 1995), keeping the ACh action longer in the synaptic cleft.

As a result, we sought to determine if thermal stress induced any change in GABA_AR expression. We observed that heat shock induced a statistically significant increase in the expression of UNC-49 protein in worms exposed to thermal stress in relation to the non-exposed group. The effect was more apparent in body muscle cells than in head muscle cells and could be a result of a compensatory response to oxidative damage on UNC-49 as reported in other models (Fritschy et al. 1999; Yamanaka et al. 2000; Zeller et al. 2008).

On the other side, picrotoxin induced a statistically significant increase in the HSP-16.2 protein level in the presence of heat shock stress as well as by itself. The expression of the HSP-16.2 small chaperone protein needs joint activity of transcription factors HSF-1 and DAF-16 (Hartwig et al. 2009; Hsu et al. 2003; Soti and Csermely 2007; Walker and Lithgow 2003; Wiegant et al. 2009), both regulated, in turn, by the IIS pathway (Baumeister et al. 2006; Chiang et al. 2012; Murphy and Hu 2013; Yen et al. 2011). This suggests that GABA_A inactivation influences the IIS pathway and consequently the stress response.

How deactivation of GABA_AR is linked to HSF-1 and/or DAF-16 activation could be speculated from recent findings. It has been postulated that the cytotoxic effect of heat shock and oxidative stress is mainly based on denaturation and misfolding of cytoplasmic and membrane proteins via thermal disruption of free energy states and oxidation/glutathione conjugation of oxidized proteins (Hildebrandt and Wust 2007; Moronetti Mazzeo et al. 2012).

Additionally, several reports have shown that UNC-49 inactivation provokes an imbalance between ACh and GABA in favor of ACh, causing a defective protein folding and aggregation and therefore inducing a protein homeostasis breakdown in postsynaptic muscle cells (Garcia et al. 2007; Gidalevitz et al. 2011; Prahlad and Morimoto 2009), which triggers a heat shock response, including a transcription rapid of *hsp* genes dependent on HSF-1. For this to happen, the misfolding protein accumulation could interact with a complex of monomeric HSF-1 and HSPs to induce its cleavage. Thus, HSF-1 monomers would be translocated into the nucleus, which would result in activation of transcription and synthesis of the heat shock protein (Prahlad and Morimoto 2009; Santoro 2000).

Since the enhanced synthesis and release of heat shock proteins, particularly HSP-16.2, take place largely in order to counteract the accumulation of misfolded and damaged proteins, via protein folding and clearance mechanisms

(Lamark and Johansen 2012), we assume that both heat shock and picrotoxin induce an increase in protein aggregation. In particular, picrotoxin could cause an imbalance in protein homeostasis in postsynaptic muscle cells through its effect as a GABA_AR blocker, consistent with a study by Garcia et al. (2007) which stated that modifications that alter the balance of ACh and GABA at the neuromuscular junction (NMJ) cause an increase in polyQ aggregation on *C. elegans* strains expressing chimeric polyQ fusion proteins to yellow fluorescent protein (YFP).

Additional mechanisms would involve DAF-16 or IIS directly. Initially, some enzymes are involved in the DAF-16 translocation. In particular, AKT-1 and AKT-2, the downstream serine/threonine kinases of the IIS pathway (Gardner et al. 2012), ultimately phosphorylate and negatively regulate DAF-16. Thus, if this phosphorylation cascade was detained upstream by a protein, it would facilitate the translocation and activation of DAF-16. Two possible proteins involved in this process could be DAF-18 and SHC-1. Both of them are implied in mechanisms of stress response and aging by opposing IIS pathway (Brisbin et al. 2009; Mihaylova et al. 1999; Neumann-Haefelin et al. 2008). DAF-18, a phosphatase ortholog of PTEN, which acts in opposition to PI3 kinase AGE-1, mediates phosphatidylinositol 3,4,5-trisphosphate dissociation and, therefore, reduces AKT activation (Mihaylova et al. 1999; Ogg and Ruvkun 1998). Moreover, the adaptor protein SHC-1, the *C. elegans* homolog of human p52Shc, disables the IIS signaling pathway by interacting with DAF-2, the *C. elegans* insulin/IGF receptor (Neumann-Haefelin et al. 2008) through a mechanism that has not yet been explained. Also, deacetylation of DAF-16 allows its translocation into the nucleus and sirtuins, a class of lysine deacetylases, that have been related to stress response and aging (Cohen et al. 2009; Jeong et al. 2012; Tang 2006; Wilson 2004). Accordingly, in *C. elegans* sirtuins, SIR-2.1 and SIR-2.4 have been correlated with DAF-16-dependent stress response and life span extension.

In this sense, further research about GABA_AR and the proteins mentioned above will generate new information which could contribute to elucidating new mechanisms involved in the relationship between the GABAergic system and DAF-16.

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