TOXIC STRESS-INDUCED ADAPTIVE CELLULAR AND BEHAVIORAL RESPONSES IN *C. ELEGANS*

PhD thesis

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I. Introduction

Challenging environmental alterations, i.e. stresses require adequate protective responses in multicellular organisms. The sensory system is responsible for orchestrating perception of environmental cues representing safety or danger as well as internal, physiological state in order to generate optimal adaptive response. Hence, the "fight-or-flight" response is a result of complex, complementary interplay between cellular and behavioral adaptations coordinated by neuroendocrine signals. Avoidance can be evoked by association of prior stressful experiences, such as physiological toxicity with sensory cues representing important resources. Such maladaptive repulsive behavior is characteristic in phobias, eating disorders and anxiety disorders which are challenging problems of our modern society.

The *Caenorhabditis elegans* soil nematode is a versatile model organism due to fully mapped genome, neuronal network of its 302 neurons as well as highly conserved cellular stress pathways. To combat stresses, each cell possesses evolutionary conserved molecular surveillance mechanisms, mainly transcription factors or upstream kinases are activated by several signal transduction pathways. Induction of cellular stress in *C. elegans* are easy to monitor in vivo due to its transparency.

Elimination and neutralization of harmful agents, toxins, pathogens to maintain core cellular processes (i. e. gene expression, synthesis and degradation of primary and secondary metabolites, organelle-specific processes and bioenergetics reactions), are priorities of cellular immune and stress pathways. In stress biology, the process of

“hormesis” is the elevation physiological stress tolerance by mild stresses via the efficient activation of cellular adaptive “fight” stress responses. In contrast, inadequate adaptive cellular responses lead to the phenomenon of “distress”, a deficient capacity of the organism to combat the stress.

C. elegans exhibits behavioral plasticity based on prior experiences, indicating efficient learning and memory processes. Conventionally, *C. elegans* can be trained to associate the presence or absence of food or toxins (unconditioned stimulus, US) with simultaneously presented gustatory or olfactory cues as well as temperature (conditioned stimulus, CS) to alter behavior. In *C. elegans,* a simple simultaneous exposure of CS and US initiates associative learning, but newly acquired plastic behavior can be retrievable only in the short-term. Repeated training sessions using intertrial intervals (ITI) called “spaced training” generates the formation of long-term memories. A specific, conserved form of lasting memory acquisition is the phenomenon of sensory imprinting, which forms in the critical perinatal period. In *C. elegans*, it is acquired in the L1 larval stage and is retrieved in adult stage.

Although stress associated cellular and behavioral mechanisms are extensively studied, the contribution of cytoprotection to behavioral regulation is largely unknown.

II. Objectives

The main objectives of my PhD studies was to investigate the expression, relationship and learning of cytoprotective and behavioral defenses in response to toxic stress. The specific aims were as follows:

1A. To establish and characterize a nematode model of volatile-induced toxic stress

1B. To investigate how cytoprotective molecular defenses affect stress-induced aversive behavior and learned behavioral decisions.

1C. To investigate whether toxic volatile stress in adulthood forms a cytoprotective memory in non-neuronal cells

2. To assess how the retrieval of an imprinted cytoprotective memory interferes with toxic stress tolerance in adulthood.

III. Methods

Reagents

All reagents used in this study were purchased from Sigma-Aldrich.

C. elegans strains and maintenance

The strains used in this study were provided by the Caenorhabditis Genetics Center, except LD001 *Is007[skn-1::gfp]* (Tibor Vellai, Eötvös Loránd University, Budapest, Hungary), MJCU017 *kIs17[gst-4::gfp, pDP#MM016B]X* (Johji Miwa, Chubu University, Kasugai, Japan).

Strains were grown, maintained and synchronized by standard procedures. All experiments were performed using day 1 adults, except monitoring of SKN-1: in L3 larvae.

Food avoidance assay

50 to 80 animals were washed twice with M9 buffer, dropped onto 200 µl of OP50 E. coli containing 6cm NGM-plates, and allowed to settle after odorant preconditioning (PC) and spaced training (ST) protocols. A drop of the given odorant was placed on a piece of parafilm in the middle of the OP50 lawn. Animals on or off the lawn were counted at the indicated times. Worms incapable to move or crawled off the agar surface were censored. The aversion index was calculated as N_{off} /N_{total}.

Odor hanging-drop treatments

Preconditioning of synchronous populations with 1 µl ccBA or 4 µl ccDA were performed on the lid of 6 cm OP50-containing NGM

plates to prevent direct contact of undiluted volatiles with worms in the presence of a large bacterial lawn. The plate was sealed with parafilm for 4 h or for the times indicated in the figure legends.

Spaced training protocol was designed by employing four sequential one-hour exposures to hanging drops of 2 µl ccBA or 4 µl ccDA with inter-trial 10-min "rest" periods allowing the animals to settle during gentle washes in M9 buffer.

Odor preference assay

80–100 naive and preconditioned worms were washed twice in M9 buffer and dropped into the middle of a CTX assay plate containing 1–1 µl of the odorants at the opposite sides. Animals were counted after 50 minutes in the 1 cm drawn circle around the respective odorants. Data are expressed as the choice index (N_o of worms at odorant #1) – (N_o of worms at odorant #2) / N_{total}

Paralysis and survival assay

Evaluation of paralysis and survival was carried out by using approximately 25–40 worms per 3 cm NGM plates. ccBA and ccDA doses were used and expressed proportionally to the volumes used in the 6-cm plates. Paralyzed worms were scored by lack of movement in response to a gentle drop of the plate to the surface. Survival of worms were scored 14 h after the end of toxic odorant exposures by tapping with a platinum worm pick. Animals that crawled off the agar surface were censored.

RNA interference treatment

HT115(DE3) *E. coli* dsRNA strains targeting *daf-16, skn-1, wdr-23* and *hsp-90* were grown overnight in LB medium containing 100 μg/ml ampicillin. Briefly, worms were grown on plates seeded with *E. coli* HT115 strains harboring the L4440 empty vector (EV) control and specific RNAi vectors, respectively, from hatching.

Stereo and fluorescence microscopy

Images of worms on plates were carried out by an Olympus SZ61-Tr stereomicroscope under dark-field illumination with 0.67–3.5× magnifications and a CAM-EP50 5Mpx Camera. Examination of fluorescence was carried out on a NIKON Eclipse E400 type fluorescence microscope linked to a Diagnostic Instruments SPOT 500 camera and a OLYMPUS CKX53 Fluorescence microscope, OLYMPUS DP74 Cooled color camera, using GFP filter cubes.

Statistics

Kaplan–Meier log-rank tests were carried out to evaluate paralysis and thermotolerance assays. Food avoidance assays were examined by one-way ANOVA with Fisher's LSD post hoc test. Odor preference assays were analyzed by two-way ANOVA with Fisher's LSD post hoc test after evaluation of normal distribution significance by the Shapiro-Wilk test. Significance in fluorescence intensity was calculated by unpaired Student's t test following evaluation of normal distribution significance by the Kolmogorov-Smirnov test. Data were expressed as mean ± standard error of the mean (SEM).

IV. Results

Distinct adaptive responses elicited by toxic odorant exposure

The discovery that food bacteria-secreted diacetyl and benzaldehyde odors when undiluted, induce repulsion, led us to hypothesize a direct toxicity of these compounds. To test this, we investigated physiological and behavioural effects of undiluted ("concentratus") benzaldehyde (ccBA) and undiluted diacetyl (ccDA).

We found that both ccBA and ccDA elicited concentration-dependent food aversion phenotypes. Further, we observed a time-dependent development of food aversion for both volatiles, in the presence of food showed a faster kinetics, than that in the kinetic chemotaxis experiments. Taken together, giving up the advantage of nutrition is a consequence of a defensive behavioral decision to avoid a harmful stimulus.

To address if animals avoided ccBA and ccDA because of toxic effects, we evaluated the heat stress tolerance, paralysis and survival rate of worms subjected to different undiluted odor doses. We found that longer ccBA and ccDA exposures to higher doses induced heat sensitivity, extensive paralysis and death in a dose- and time-dependent manner, representing a progressive disruption of physiological homeostasis. Based on these findings, we hypothesized that the behavioral avoidance of the undiluted odorants may be a consequence of their toxic effect.

Odorant preconditioning evokes distinct modes of adaptation

We observed that transient exposure to higher doses of ccBA and ccDA increased motility which returned to baseline after removing

ccBA but showed a sustained elevation after the removal of ccDA. Moreover, we found that after an extended 2-h exposure to ccBA, animals started to return to the bacterial lawn, whereas the same exposure to ccDA further increased aversion. Thus, the adverse physiological effects of ccBA might be eliminated faster than those of ccDA. We reasoned that a preconditioning to the same doses of odor exposure might differentially affect the defensive behavior to ccBA and to ccDA. We found that preconditioning with ccBA (BA PC) largely diminished ccBA-induced aversion for the entire duration of the experiment. In contrast, preconditioning with ccDA (DA PC) robustly increased the speed of ccDA lawn avoidance. To investigate whether the contrasting behavioral responses evoked by the two volatiles were accompanied by similar outcomes in physiological stress tolerance, we preconditioned the worms with odor doses used in the food leaving assays, then subjected them to lethal odor doses. With increasing preconditioning time, we observed a robust survival increase on ccBA and a complete survival decline on ccDA, representing a protective (hormetic) effect of ccBA and a debilitating (distressing) effect of ccDA preconditioning, suggesting efficient or insufficient physiological responses to the stress induced by ccBA or ccDA exposures, respectively. These results suggest that nematodes can mount efficient physiological protection against ccBA, but can only engage more alert behavioral defense through sensitization against ccDA.

Adaptive cellular responses induced by odor preconditioning

Next, we asked if the efficient *vs.* insufficient physiological protection against ccBA and ccDA exposure might be reflected in the

differential mobilization of cellular defense responses to the respective toxic stresses. Using the TJ356 strain expressing GFP-tagged DAF-16, we observed that the same ccBA dose used for preconditioning induced a strong nuclear translocation of DAF-16 after 30 min, however, DAF-16 remained cytosolic in response to ccDA. Translocation of the oxidative-xenobiotic stress master regulator SKN-1::GFP in the LD001 strain was induced by a 30-min exposure to ccBA, but not by that of ccDA. Further, ccBA, but not ccDA, induced DAF-16 and SKN-1 dependent expression of xenobiotic-metabolizing reporters: the phase I oxidative cytochrome P450 enzyme cyp-35B1 and the phase II conjugating enzyme gst-4. Next we asked whether the cytoprotective responses activated by ccBA which are known to induce physiological tolerance to various stresses might play a role in the generation of "fight-or-flight" (staying on or leaving the lawn) behavioral decisions. We found that *daf-16* and *hsp-90* mutants as well as silencing *skn-1* failed to decrease their aversion in response to preconditioning, whereas the hyperactivation of SKN-1 by knocking down the protein responsible for its degradation augmented behavioral tolerance towards ccBA. In sharp contrast, after ccDA preconditioning, neither *skn-1* silencing nor hyperactivation altered the behavioral sensitization towards ccDA exposure. These results demonstrate that specific cytoprotective responses induced by toxic ccBA exposure in non-neuronal cells confer physiological protection and actively participate in the development of behavioral tolerance.

JNK-like MAP kinases and the NPR-1 neuropeptide Y receptor connect behavioral and physiological stress tolerance

In *C. elegans*, neuroendocrine signaling is almost exclusively responsible for "top-down" inter-tissue communications, mainly via neurotransmitter release, the FMRFamide-type neuropeptide and the conserved stress-activated protein kinase (SAPK) pathways. Hence, we tested the involvement of the major downstream MAP kinases including the p38 ortholog PMK-1 as well as the JNK orthologs JNK-1 and KGB-1 in ccBA aversion by subjecting naive and ccBA-preconditioned worms to the ccBA lawn leaving assay. Our findings suggest a physiological protection of vital importance conferred by *pmk-1* against ccBA toxicity, a requirement of JNK-like kinases to favor behavioral defense vs. ccBA-specific physiological defenses, and *jnk-1* (and *kgb*-1) to elicit avoidance as the sole available protective measure against ccDA. We investigated the behavioral response of the conserved neuropeptide Y receptor ortholog NPR-1 and observed a complete suppression of behavioral tolerance in ccBA-preconditioned *npr-1* mutants but unaffected survival upon lethal ccBA exposure, suggesting that NPR-1 does not engage physiological defenses, rather appears to integrate the internal signals of physiological homeostasis into the aversive response against ccBA. We tested this prediction by boosting SKN-1 activity in *npr-1* mutants and found the lack of improved behavioral tolerance in preconditioned animals, indicating the disconnection in physiological and behavioral defenses in the absence of *npr-1*. Altogether, these results suggest that SAPK-s and NPR-1 exert opposite effects and cooperate in fine-tuning physiological and behavioral "fight-or-flight" responses to protect homeostasis in toxic stress conditions.

Cytoprotective defenses during stress determine future decisions to stress-associated olfactory cues

Previously we asked whether the prior experience of odor toxicity and the different efficiency of physiological defenses influence nematodes to make optimal choices upon encounters with the olfactory cues present at the time of stress. My colleague Eszter Gecse found the generation of distinct, avoidant, or tolerant learned behaviors to stress-associated olfactory cues of ccDA or ccBA, respectively. The elicitation of learned stress-reactive behaviors by olfactory cues raises the possibility that the learned experiences form distinct memories to cope with anticipated future insults. Hence, we tested whether spaced training induced memory consolidation might increase the persistence of the acquired behavioral tolerance to ccBA after the recovery. We found that immediately after pre-exposures, both the single preconditioning and the spaced training resulted in a similar suppression of ccBA avoidance, however, this behavioral tolerance was retained after a 2-h recovery in spaced-trained but not in single preconditioned nematodes. We also examined whether repetitive encounters with ccDA might influence food avoidance behavior in the presence of 1% DA and observed that spaced-trained worms exhibited robustly increased and extended food leaving compared to that elicited by a single preconditioning. Thus, spaced training with ccBA or ccDA leads to the stabilization of respective stress-associated memories over 2 h, which upon retrieval give rise to either tolerant, coping “fight,” or avoidant “flight” behavioral responses.

Finally, we asked how the coping memory affects the choice between the stress-associated and a natural attractive odor olfactory cue. Spaced training with ccBA almost entirely shifted the preference towards DA which was retained after a 2-h recovery resulting in stable storage and retrieval of the acquired memory. Nonetheless, the memory of physiological protection not only provides the ability to cope with real or anticipated toxicity for food, but also allows a flexible decision to spare resources when the organism also perceives the olfactory cue of a potentially toxin-free food. Taken together, learned behaviors originating from adequate or inadequate physiological responses to stress generate acquisition of distinct coping or avoidant memories.

Cellular defense memories to cope with anticipated stress

Some pathogens, such as the Gram-negative *Pseudomonas aeruginosa* secretes naturally attractive odorants developed during successful co-evolution, resulting in the typical phenomenon of initial attraction and delayed aversion of worms as a learned aversive association. The hypothesis that cytoprotective memories would be retrievable by conditioned cues might influence decision making and might confer a successful defense strategy for *C. elegans* against impending adverse events. For this reason, we carried out spaced training with stress-inducing ccBA (BA ST), and monitored DAF-16 translocation upon re-exposure to conditioned, 1% odor cues. Interestingly, approximately two-fold elevation of nuclear DAF-16 was observed in animals exposed to 1% BA after BA ST, indicating that retrieval of toxic stress memory by associated sensory cues re-

activates a stress-specific regulator to ensure efficient cellular protection.

My colleague Eszter Gecse discovered that the L1 larval stage memory of toxin-induced cytoprotection can be retrieved by toxin associated *E: coli* OP50 olfactory cues in adults. Consistently, we hypothesized that a stress inducing dose of paraquat (PQ PC) or antimycin (AM PC) treatment during the time window of imprinting might affect not only physiological AM or PQ tolerance of adults, but the memory retrieval of stress by associated olfactory cues might enhance stress tolerance. Toxin exposure during the L1 stage induced an approximately twofold increase in survival of adults, however, the re-encounter with the OP50 sensory cues before lethal stress neither altered survival rates in naive nor in toxin-imprinted worms. Thus, early life toxin exposure at the doses employed is hormetic and induces a lasting and robust stress tolerance in adulthood, which is not further enhanced by retrieval of the imprinted memory.

V. Conclusions

Our novel findings on the role of cytoprotective responses in adult and early life stress models are the following:

- We have established a nematode model of volatile-induced toxic stress:
- We characterized the impact of stress-induced cytoprotective responses on memories to cope with future anticipated stress:
    1. Toxic benzaldehyde, but not diacetyl induces activation of evolutionary conserved major stress regulators.
    2. Toxic benzaldehyde preconditioning forms a memory of behavioral tolerance through the activation of specific cytoprotective responses.
    3. Lack of apparent cytoprotection by toxic diacetyl enhances behavioral sensitization and associative aversive memory.
- We found the nuclear translocation of DAF-16 in non-neuronal cells evoked by the stress-associated olfactory cue.
- We found that early life toxic stress by antimycin A or paraquat induced adult stress tolerance, which was not further enhanced by stress-associated olfactory cues.

VI. Bibliography of the candidate's publications

Hajdú, G., Gecse, E., Taisz, I. et al. Toxic stress-specific cytoprotective responses regulate learned behavioral decisions in C. elegans. BMC Biol 19, 26 (2021). IF: 7.431
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