Short communication

Impaired EAT-4 Vesicular Glutamate Transporter Leads to Defective Nocifensive Response of *Caenorhabditis elegans* to Noxious Heat

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Summary

In mammal, glutamate is an important excitatory neurotransmitter. Glutamate and glutamate receptors are found in areas of the periphery, spinal cord and brain specifically involved in pain sensation, transmission and transduction. In *C. elegans*, several studies have suggested glutamate pathways are associated with withdrawal responses to mechanical stimuli and to chemical repellents. However, it has not been demonstrated that glutamate pathways are important to mediate nocifensive response to noxious heat. The data presented in this manuscript reveals for the first time that glutamate signaling pathways are essential to elicit a nocifensive response to noxious heat in *C. elegans.*

In mammal, glutamate is a major excitatory neurotransmitter used by primary afferent synapses and neurons located in the spinal cord [Pereira and Goudet, 2019; Reiner and Levitz, 2018; Osikowicz *et al*. 2013]. Additionally, glutamate and glutamate receptors are found in areas of the periphery, spinal cord and brain specifically involved in pain sensation, transmission and transduction [Petralia *et al*. 1998]. Thus, glutamate receptors were investigated as a target for the development of new analgesics, but the widespread distribution and physiological functions resulted in drugs targeting the glutamate receptors exhibited some undesirable effects. *Caenorhabditis elegans* (*C. elegans*) consists of 959 cells including 302 neurons, which make this model attractive to study neuronal communication at the physiological levels [Wittenburg and Baumeister, 1999]. *C. elegans* is particularly useful for the study of nociception as it exhibits a well-defined and reproducible nocifensive behavior, involving a reversal and change in direction away from the noxious stimulus [Wittenburg and Baumeister, 1999; Carr and Zachariou, 2014; Nkambeu *et al*. 2019]. Consequently, *C. elegans* is a commonly used model organism to examine heat avoidance [Nkambeu et al. 2019; Kotera *et al*. 2016; Thies *et al*. 2016; Wittenburg and Baumeister, 1999]. Recently, we demonstrated *C. elegans* ability to avoid noxious heat is strongly associated with the FLP-18/FLP-21/NPR-1 neuropeptide signaling pathways but results also suggested that other neuropeptides signaling pathways or classical neurotransmitters are most likely playing an important role [Nkambau et al. 2019]. The analysis of glutamate receptor function in *C. elegans* has been investigated and reported [Zou *et al*. 2018; Vangindertael *et al*. 2015; Brockie and Maricq, 2006]. Neuronal communications through chemical synapses involve the activation of several neurotransmitter receptors on postsynaptic interneurons and neurons, including glutamate

receptors in *C. elegans*. Studies have suggested glutamate pathways are associated with withdrawal responses to mechanical stimuli and chemical repellents [Mellem *et al*. 2002; Hart *et al*. 1995]. However, it has not been demonstrated that glutamate pathways are important to mediate nocifensive responses to noxious heat in *C. elegans*. The glutamate needs a specific transporter to move across membranes and participate in chemical synapses. *C. elegans* vesicular glutamate transporter (i.e. *eat-4*) is therefore necessary for glutamatergic synaptic transmission [Lee *et al*. 2008].

All chemicals and reagents were obtained from Fisher Scientific (Fair Lawn, NJ, USA) or MilliporeSigma (St-Louis, MO, USA). For mass spectrometry analysis, formic acid, water (HPLC-MS Optima grade), acetonitrile (HPLC-MS Optima grade), trifluoroacetic acid (TFA), were purchased from Fisher Scientific. The N2 (Bristol) isolate of *C. elegans* was used as a reference strain. Mutant strains used in this work included: *grl-1* (KP4); *glr-2* (RB1808); *nmr-1* (VM487); *nmr-2* (VC2623); *flp-18* (AX1410); *flp-21* (RB982); *npr-1* (CX4148); *eat-4* (IK600); *eat-4* (IK602); *eat-4* (MT6308); *eat-4* (MT6318). N2 (Bistrol) and other strains were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA). Strains were maintained and manipulated under standard conditions as described [Brenner, 1974; Margie et al., 2013]. Worms were grown and kept on Nematode Growth Medium (NGM) agar at 22˚C in a Thermo Scientific Heratherm refrigerated incubators. Analyses were performed at temperature ranging from 22 to 25 °C unless otherwise noted.

The principle behind evaluating the *C. elegans* response to a stimulus (i.e thermal or

chemical) is to observe and quantify the movement evoked in response to a specific stimulus. The method proposed in this manuscript for the evaluation of thermal avoidance behavior is adapted from the two and four quadrants strategies previously described [Margie *et al*., 2013; Porta-de-la-Riva *et al*. 2012]. We have previously published the experimental details for the behavioral assay used in this manuscript [Nkambeu *et al*. 2019]. The selection of quadrant temperature was based on previous experiments [Wittenburg and Baumeister, 1999].

Mutant strains used for the proneuropeptide analyses work included: N2 (Bristol); *eat-4* (MT6308); *eat-4* (MT6318); *eat-4* (IK600); *eat-4* (IK602). Strains were cultured in liquid media standard as described [Brenner, 1974; Margie et al., 2013]. The liquid media was centrifuged at 1,000 *g* for 10 min and nematodes were collected and aliquoted to re-enforced 1.5 mL homogenizer tubes containing 500 µm glass beads. A solution of 8M urea in 100mM TRIS-HCL buffer (pH 8) containing cOmplet protease inhibitor cocktail (Roche) was added at a ratio of 1:5 (w:v). Lysing and homogenization was performed with a Fisher Bead Mill Homogenizer set at 5 m/s for 60 seconds and repeated 3 times with a 30 second delay. The homogenates were centrifuged at 9,000 g for 10 min. The protein concentration for each homogenate was determined using a Bradford assay and all samples were normalized to avoid any bias. Two hundred µg of proteins were aliquoted for each sample and proteins were extracted using ice-cold acetone precipitation (1:5 v/v). The protein pellet was dissolved in 100 µL of 50 mM TRIS-HCl buffer (pH 8) and the solution was mixed with a Disruptor Genie used at maximum speed (2,800 rpm) for 15 minutes and sonicated to improve protein dissolution yield. The proteins were denatured by heating at

120˚C for 10 min using a heated reaction block. The solution was allowed to cool down 15 minutes. Proteins were reduced with 20mM DTT and the reaction was performed at 90 ˚C for 15 minutes. Then proteins were alkylated with 40 mM IAA and the reaction was performed at room temperature for 30 min. Five µg of proteomic-grade trypsin was added and the reaction was performed at 37˚C for 24h. The protein digestion was quenched by adding 10 µL of a 1% TFA solution. Samples were centrifuged at 12,000 g for 10 min and 100 µL of the supernatant was transferred into injection vials for analysis. The HPLC system was a Thermo Scientific Vanquish FLEX UHPLC system (San Jose, CA, USA). The chromatography was performed using gradient elution along with a microbore column Thermo Biobasic C18 100 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 2 min, the ratio was maintained at 5:95. From 2 to 92 min, a linear gradient was applied up to a ratio of 40:60 and maintained for 3 min. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 20 min. The flow rate was fixed at 50 µL/min and 5 µL of sample were injected. A Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced the UHPLC system using a pneumatic assisted heated electrospray ion source. Nitrogen was used for sheath and auxiliary gases and they were set at 10 and 5 arbitrary units. Auxiliary gas was heated to 200˚C. The heated ESI probe was set to 4000 V and the ion transfer tube temperature was set to 300°C. MS detection was performed in positive ion mode and operating in TOP-10 Data Dependent Acquisition (DDA). A DDA cycle entailed one MS^1 survey scan (*m/z* 400-1500) acquired at 70,000 resolution (FWHM) and precursors ions meeting user defined criteria for charge state (i.e. z = 2,3 or 4),

monoisotopic precursor intensity (dynamic acquisition of MS^2 based TOP-10 most intense ions with a minimum $1x10^5$ intensity threshold). Precursor ions were isolated using the quadrupole (1.5 Da isolation width) and activated by HCD (28 NCE) and fragment ions were detected in the Orbitrap at 17,500 resolution (FWHM). MS Data were analyzed using Proteome Discovered 2.2, a targeted database containing FLP-18 and FLP-21 protein sequences and a label free quantification strategy. All data were analyzed using a one-way ANOVA followed by Dunnett multiple comparison test (e.g. WT(N2) was the control group used). Significance was set a priori to $p < 0.05$. Additionally, only for proteomic data, the threshold is set to 2 fold-change for significance. The statistical analyses were performed using PRISM (version 8.1.0).

The first experiment included an assessment of the mobility and bias for WT (N2) and all mutant nematodes used for this study. Nematodes were placed in the center of plates divided into quadrants conserved at constant temperature (i.e. 22˚C) and no stimulus was applied (negative control). As revealed in Figure 1, there was no quadrant selection bias observed for all *C. elegans* genotypes tested. The nematodes were not preferably choosing any quadrant and were uniformly distributed after 30 minutes following the initial placement at the center of the marked petri dish. The thermal avoidance behavior of *C. elegans* was studied on petri dishes in which two opposite quadrants had a surface temperature of 33˚C to 35˚C and the other two were preserved at room temperature. The results illustrated in Figure 2 suggest that mutants associated with glutamate receptor *glr-1*, *glr-2*, *nmr-1*, *nmr-2* thermal avoidance was not affected. However, as previously demonstrated, *flp-18*, *flp-21* and *npr-1* mutants displayed a hampered thermal avoidance behavior [Nkambeu et al. 2019]. These results are not surprising due to the interplay of the

glutamate system. As it was previously established, heat avoidance relies partly on functional NPR-1 receptors located in the RMG interneuron and both, FLP-18 and FLP-21 mature neuropeptides are ligands of NPR-1 [Choi *et al*. 2013]. The results showed in Figure 3 suggest that all strains of *eat-4* mutant tested displayed defective thermal avoidance behavior. These results are coherent with our initial hypothesis and reveal for the first time an important role of glutamate pathway to trigger a nocifensive response to noxious heat. We wanted to verify if these results were also an outcome of a differential expression of the FLP-18/FLP-21/NPR-1 pathways. We therefore analyzed the effectors FLP-18 and FLP-21 at the protein levels and as shown in Figure 4, except for strain IK602, we have not observed biologically significant differences compared to N2 (WT) strain (fold-change < 2). Interestingly, the upregulation of FLP-18 in strain IK602 did not compensate the absence of glutamate transporter *eat-4*, impeding glutamate signaling pathways as shown in Figure 3. Collectively, these results reveal that FLP-18/FLP-21/NPR-1 pathways and glutamate signaling pathways are essential to elicit a nocifensive response to noxious heat in *C. elegans*.

Acknowledgements

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References

Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1977. 77:71–94.

Brockie PJ, Maricq AV. WormBook. 2006. 1-16.

Carr FB, Zachariou V, 2014. Nociception and pain: lessons from optogenetics. Front. Behav. Neurosci. 2014. 8 (69);1–6.

Choi S, Chatzigeorgiou M, Taylor KP, Schafer WR, Kaplan JM. Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in C. elegans. Neuron. 2013. 78(5):869–880.

Hart AC, Sims S, Kaplan JM. Synaptic code for sensory modalities revealed by C. elegans GLR-1 glutamate receptor. Nature. 1995. 378(6552):82-85.

Kotera I, Tran NA, Fu D, Kim JH, Byrne Rodgers J, Ryu WS. Pan-neuronal screening in Caenorhabditis elegans reveals asymmetric dynamics of AWC neurons is critical for thermal avoidance behavior. Elife. 2016. 16;5.

Lee D, Jung S, Ryu J, Ahnn J, Ha I. Human vesicular glutamate transporters functionally complement EAT-4 in C. elegans. Mol Cells. 2008. 25(1):50-54.

Margie O, Palmer C, Chin-Sang I. C. elegans chemotaxis assay. J. Vis. Exp. 2013. 74;1–6.

Mellem JE, Brockie PJ, Zheng Y, Madsen DM, Maricq AV. Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in C. elegans. Neuron. 2002. 36(5):933-944.

Nkambeu B, Salem JB, Leonelli S, Marashi FA, Beaudry F. EGL-3 and EGL-21 are required to trigger nocifensive response of Caenorhabditis elegans to noxious heat. Neuropeptides. 2019. 73:41-48.

Osikowicz M, Mika J, Przewlocka B. The glutamatergic system as a target for neuropathic pain relief. Exp Physiol. 2013. 98(2):372-384

Pereira V, Goudet C. Emerging Trends in Pain Modulation by Metabotropic Glutamate Receptors. Front Mol Neurosci. 2019. 11:464.

Petralia RS, Rubio ME, Wenthold RJ. Selectivity in the distribution of glutamate receptors in neurons. Cell Biol Int. 1998. 22(9-10):603-608.

Porta-De-La-Riva M, Fontrodona L, Villanueva A, Cerón J. Basic Caenorhabditis elegans methods: synchronization and observation. J. Vis. Exp. 2012. 64;1–9.

Reiner A, Levitz J. Glutamatergic Signaling in the Central Nervous System: Ionotropic and Metabotropic Receptors in Concert. Neuron. 2018. 98(6):1080-1098

Thies J, Neutzler V, O'Leary F, Liu H. Differential Effects of TRPA and TRPV Channels on Behaviors of Caenorhabditis elegans. J Exp Neurosci. 2016. 10:71-5

Vangindertael J, Beets I, Rocha S, Dedecker P, Schoofs L, Vanhoorelbeke K, Hofkens J, Mizuno H. Super-resolution mapping of glutamate receptors in C. elegans by confocal correlated PALM. Sci Rep. 2015. 5:13532.

Wittenburg, N., and Baumeister, R. Thermal avoidance in *Caenorhabditis elegans*: An approach to the study of nociception. PNAS 1999. 96(18):10477-10482.

Zou W, Fu J, Zhang H, Du K, Huang W, Yu J, Li S, Fan Y, Baylis HA, Gao S, Xiao R, Ji W, Kang L, Xu T. Decoding the intensity of sensory input by two glutamate receptors in one C. elegans interneuron. Nat Commun. 2018. 9(1):4311.

List of Figures

Figure 1. Comparison of the mobility and bias for WT (N2) and mutant *glr-1*, *glr-2*, *nmr-1*, *nmr-2*, *flp-18, flp-21, npr-1* and *eat-4* nematodes in plates divided into quadrants conserved at constant temperature and no stimulus were applied (negative control). No quadrant selection bias was observed for all *C. elegans* genotypes tested.

Figure 2. Thermotaxis index and avoidance were evaluated for WT (N2) and mutant nematodes. Display values (mean ± SD) were calculated from at least 6 independent experiments (n > 300 nematodes) for each genotype. Heat avoidance is not affected in *glr-1*, *glr-2*, *nmr-1* and *nmr-2* but hampered in *flp-18, flp-21* and *npr-1* mutant nematodes.
** $p < 0.01$; **** $p < 0.0001$

Figure 3. Thermotaxis index and avoidance were evaluated for WT (N2) and Vesicular glutamate transporter *eat-4* mutant nematodes. Display values (mean ± SD) were calculated from at least 6 independent experiments (n > 300 nematodes) for each genotype. Heat avoidance is impaired for *eat-4* mutant nematodes.
**** $p < 0.0001$

Figure 4. Relative concentration of FLP-18 and FLP-21 FMRF-Like peptides. The concentration of FLP-18 or FLP-21 is not significantly different (fold change < 2) for three *eat-4* mutant strains. FLP-18 is marginally different for the strain IK602.
** $p < 0.01$

Figure 1.

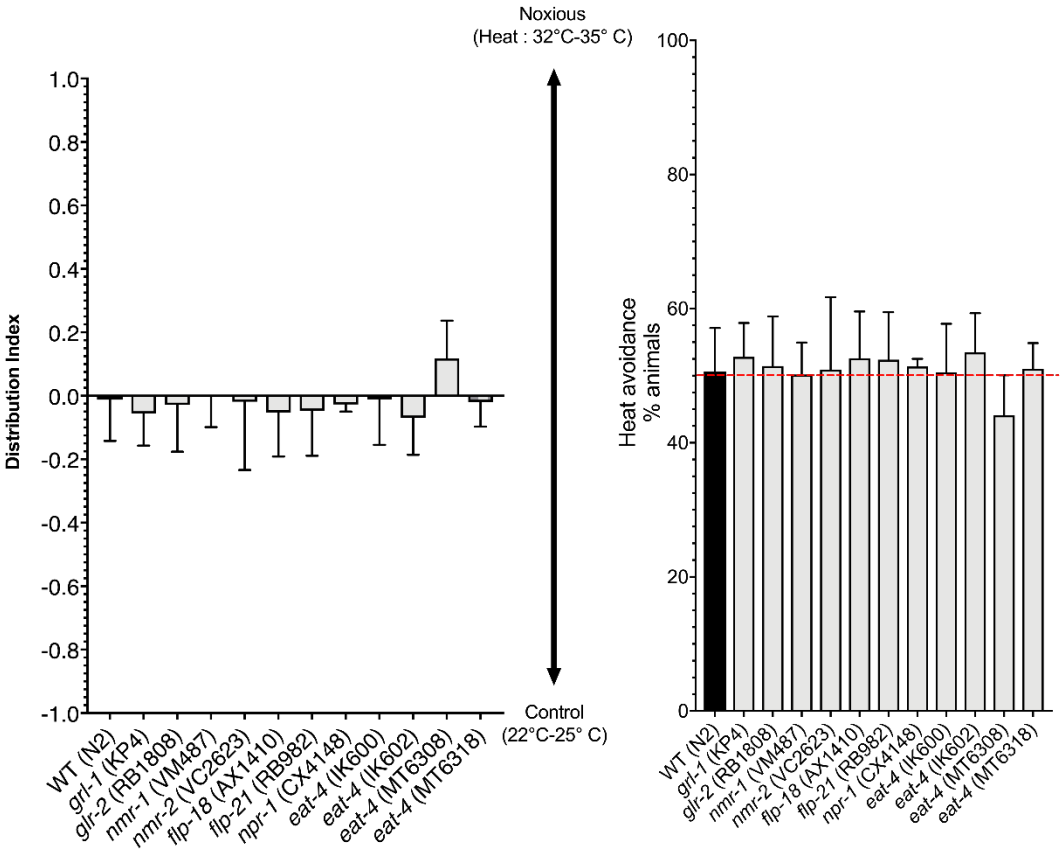

Noxious
(Heat : 32°C-35° C)
Control
(22°C-25° C)
Distribution Index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
WT (N2)
grl-1 (KP4)
glr-2 (RB1808)
nmr-1 (VM487)
nmr-2 (VC2623)
flp-18 (AX1410)
flp-21 (RB982)
npr-1 (CX4148)
eat-4 (IK600)
eat-4 (IK602)
eat-4 (MT6308)
eat-4 (MT6318)
Heat avoidance
% animals
100
80
60
40
20
0

Figure 2.

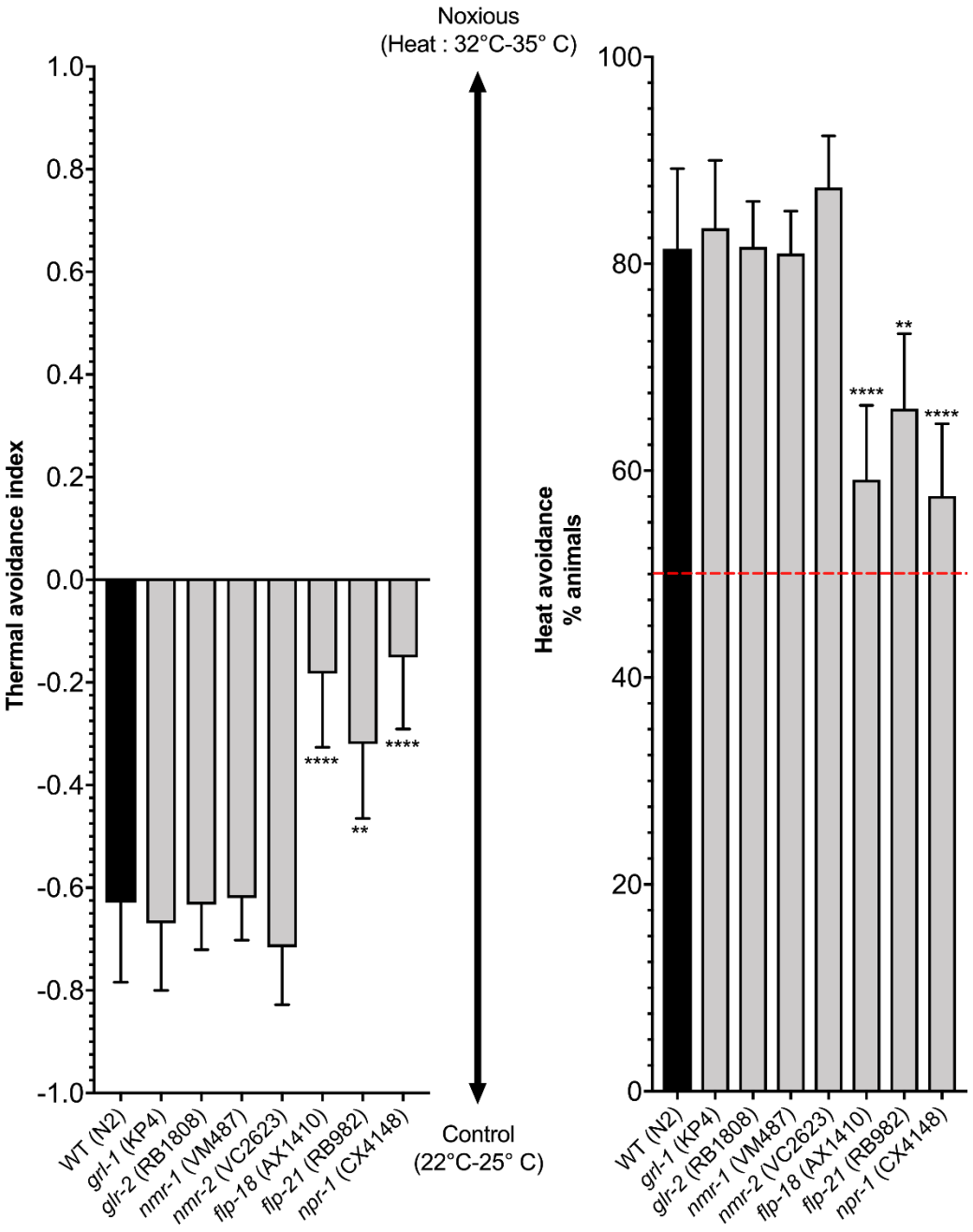

Noxious
(Heat : 32°C-35° C)
Control
(22°C-25° C)
Thermal avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
****
**
****
WT (N2)
grl-1 (KP4)
glr-2 (RB1808)
nmr-1 (VM487)
nmr-2 (VC2623)
flp-18 (AX1410)
flp-21 (RB982)
npr-1 (CX4148)
Heat avoidance
% animals
100
80
60
40
20
0
****
**
****
WT (N2)
grl-1 (KP4)
glr-2 (RB1808)
nmr-1 (VM487)
nmr-2 (VC2623)
flp-18 (AX1410)
flp-21 (RB982)
npr-1 (CX4148)

Figure 3.

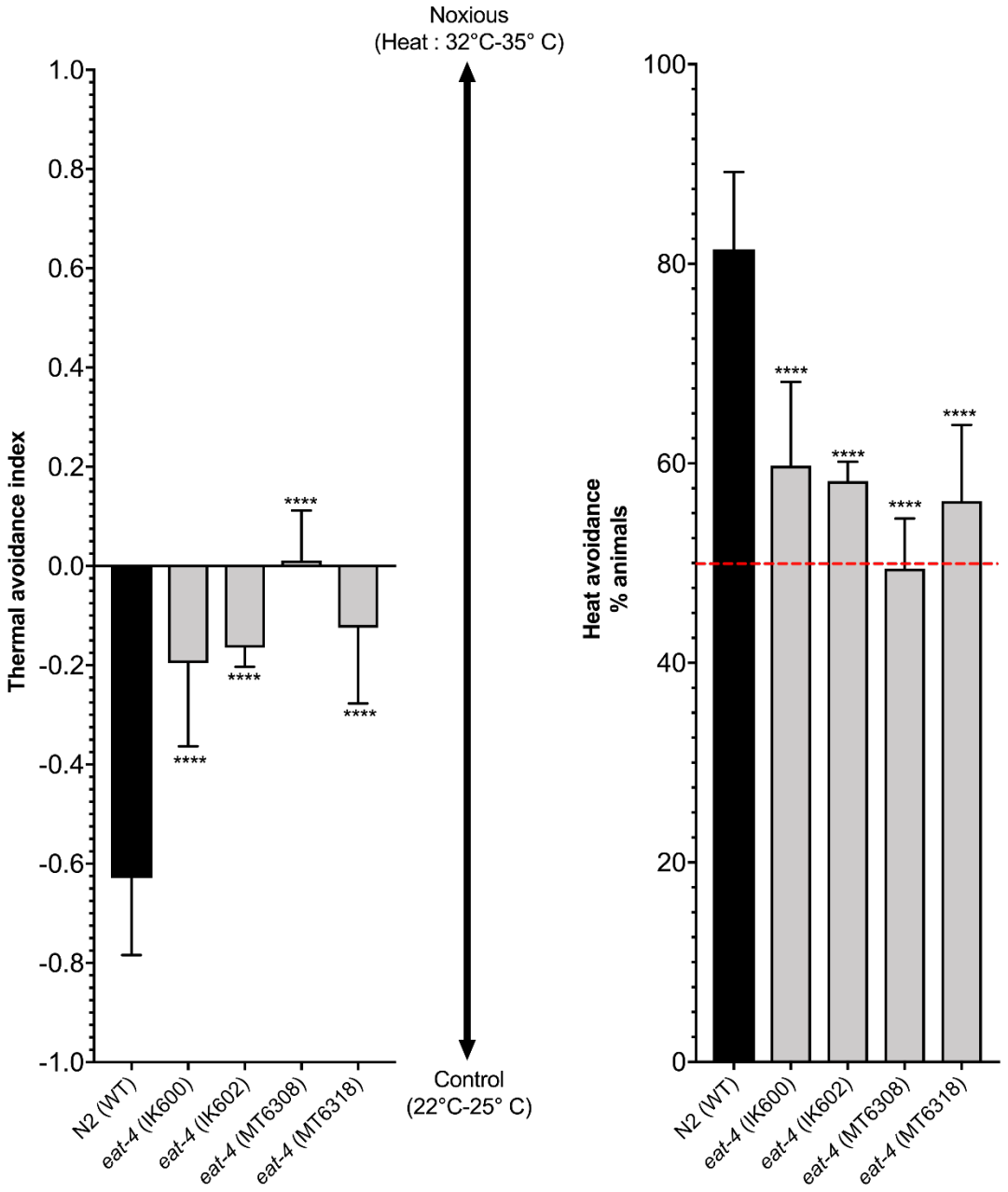
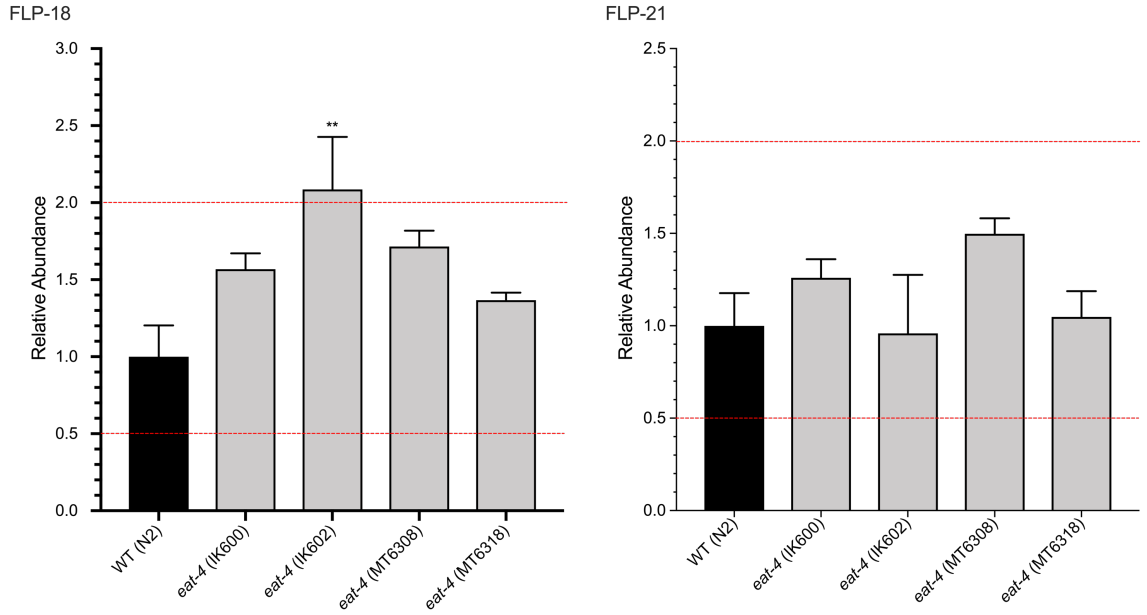

Noxious
(Heat : 32°C-35° C)
Control
(22°C-25° C)
Thermal avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
****
N2 (WT)
eat-4 (IK600)
eat-4 (IK602)
eat-4 (MT6308)
eat-4 (MT6318)
Heat avoidance
% animals
100
80
60
40
20
0

Figure 4.


FLP-18
Relative Abundance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
WT (N2)
eat-4 (IK600)
eat-4 (IK602)
eat-4 (MT6308)
eat-4 (MT6318)
FLP-21
Relative Abundance
2.5
2.0
1.5
1.0
0.5
0.0
WT (N2)
eat-4 (IK600)
eat-4 (IK602)
eat-4 (MT6308)
eat-4 (MT6318)