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1 **Preconditioning of *Caenorhabditis elegans* to Anoxic Insult by Inactivation of**
2 **Cholinergic, GABAergic, and Muscle Activity**

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14

15

16 **Abstract**

17 For most metazoans, oxygen deprivation leads to cell dysfunction and if severe,
18 death. Sublethal stress prior to a hypoxic or anoxic insult (“preconditioning”) can protect
19 cells from subsequent oxygen deprivation. The molecular mechanisms by which
20 sublethal stress can buffer against a subsequent toxic insult and the role of the nervous
21 system in the response are not well understood. We studied the role of neuronal activity
22 preconditioning to oxygen deprivation in *C. elegans*. Animals expressing the histamine

1 gated chloride channels (HisCl1) in select cell populations were used to temporally and
2 spatially inactivate the nervous system or tissue prior to an anoxic insult. We find that
3 inactivation of the nervous system for 3 hours prior to the insult confers resistance to a
4 48-hour anoxic insult in 4th-stage larval animals. Experiments show that this resistance
5 can be attributed to loss of activity in cholinergic and GABAergic neurons as well as in
6 body wall muscles. These observations indicate that the nervous system activity can
7 mediate the organism's response to anoxia.

8 **1 Introduction**

9 The function of all cells requires the constant provision of fuel and (for aerobic
10 life), oxygen. Highly prevalent human conditions such as stroke and myocardial
11 infarction result from a mismatch between fuel and oxygen delivery and tissue
12 demands. During development, hypoxic insults have devastating effects on newborn
13 infants, leading to global tissue dysfunction and disabilities.¹ Cells can survive and
14 adapt to low-oxygen (hypoxia) or zero oxygen (anoxia) conditions for a limited time
15 based on cell-type specific factors and the duration or degree of oxygen deprivation.
16 Reperfusion, the process of re-establishing blood flow to ischemic tissues, has been the
17 principal clinical method to minimize cellular damage. However, reperfusion itself can
18 contribute to cellular damage, thus there is an unmet need to develop better therapeutic
19 options.²

20 A transient, sub-lethal experience of hypoxia or ischemia can protect against an
21 otherwise lethal subsequent hypoxic-ischemic insult. This phenomenon, referred to
22 ischemic or hypoxic preconditioning,³⁻⁷ suggests that cells have a latent adaptive
23 capacity to combat the noxious effects of ischemia and hypoxia. If we understood the

1 biochemical basis for the preconditional effect, it could potentially be harnessed for
2 therapeutic purposes. Previous work in mammalian systems on ischemic
3 preconditioning has highlighted a role for signal transduction pathways, (*i.e.*, PI3K-
4 AKT and ERK pathways), as well as hypoxia inducing factor (HIF).⁸⁻¹⁰ Nonetheless, a
5 complete understanding of the phenomenon is lacking.

6 Genetically tractable organisms have proven to be a powerful platform for
7 discovery of novel genes and pathways of biological significance. The nematode *C.*
8 *elegans* prefers oxygen between 5 and 12%¹¹ and has the ability to sense and
9 respond to shifts in oxygen that fall outside the preferred range.^{12,13} *C. elegans* are
10 capable of surviving low oxygen stress and use a variety of pathways to achieve this
11 depending on the degree and duration of oxygen deprivation.¹⁴⁻¹⁷ We found that
12 ablation of the oxygen-sensing BAG (but not the URX) neurons rendered animals
13 resistant to an anoxic insult.¹³ We postulated the neural circuit in which the BAG neuron
14 was embedded secreted a factor(s) that heightened peripheral tissue sensitivity to
15 anoxia. This hypothesis was derived from the observations that: 1) inhibition of
16 neuropeptide processing (*e.g.*, *C. elegans* with *egl-3* or *egl-21* mutation) and secretion
17 (*e.g.*, *C. elegans* with *unc-31* mutation) protected against anoxia, 2) nervous system-
18 specific rescue of *egl-3* expression in *egl-3* mutant animals restored sensitivity to
19 anoxia, and 3) we identified one neuropeptide *nlp-40* and its receptor *aex-2* that are
20 likely to be involved in this process.¹⁸ These observations highlight the cell non-
21 autonomous determinants of *C. elegans* survival upon anoxia insult.

22 *C. elegans* display the preconditioning phenomenon and genetic studies
23 implicate classical stress response pathways, genes required for lifespan, energy

1 homeostasis, dauer formation, and genes involved cell death pathways.^{15,17,19-21} Given
2 this starting point, we wondered if the preconditioning phenomena in *C. elegans*
3 similarly displayed cell non-autonomous features and involved the nervous system. We
4 designed experiments to address the following questions: 1) What is the role of the
5 nervous system in sensing and responding to anoxic stress? And 2) What are the
6 tissues or neuronal populations underlying the preconditioning response to anoxic
7 stress? Our results support a role for inactivity of cholinergic and GABAergic neurons,
8 and muscle in modulating survival to a subsequent anoxic insult.

9 **2 Material and Methods**

10 **2.1 Strains**

11 The following strains were used in this work. N2, referred to as wild type, CX14373
12 *kyEx4571 [pNp403 (tag-168:HisCl1::SL2::GFP;myo-3::mCherry]* from Pokala *et al.*,
13 2014.²² CX14845 *kyEx5104[pNP424 (mec-3::HisCl1::SL2::mCherry; unc-122::GFP)]*,
14 from Pokala *et al.*, 2014.²² CX15341 *kyEx5161[pNP488 (unc-4::HisCl1::SL2::mCherry;*
15 *elt-2::mCherry)]*, from Pokala *et al.*, 2014.²² CX15457 *kyls620[pNP472 (inx-*
16 *1::HisCl1::SL2::GFP;myo-3::mCherry)]*, from Pokala *et al.*, 2014.²² PS6963 *syIs336*
17 *[pHW383(Pmyo-3::nls::GAL4SK::VP64::unc-54 3'UTR; Pmyo-2::nls::mCherry)]*, from
18 Wang *et al.*, 2017.²³ PS7160 *syIs393 [pHW504(Punc-47::nls::GAL4::VP64::let-858*
19 *3'UTR; Punc-122::RFP)]*, from Wang *et al.*, 2017.²³ PS7199 *syIs371 [pJL046(15xUAS::*
20 *Δpes-10::HisCl1::SL2::GFP::let-858 3'UTR;Punc-122::GFP)]*, from Wang *et al.*, 2017.²³
21 RK200 (*Punc-47::nls::GAL4SK::VP64::let-858 3'UTR;15xUAS::Δpes-*
22 *10::HisCl1::SL2::GFP::let-8583'UTR;Punc-122::GFP)*, RK201[(*Pmyo-*
23 *3::nls::GAL3SK::VP64::unc-54 3'UTR;15xUAS:: Δpes-10::HisCl1::SL2::GFP::let-858*

1 3'UTR;Punc-122::GFP)], RK206 sdEx5[pJP673(Punc-17::HisCl1;myo-2::mCherry)],
2 RK207 sdEx6[pJP673(Punc-17::HisCl1;myo-2::mCherry)], RK210 sdEx7[(Ptp-
3 1::HisCl1; Pmyo-2::mCherry)], RK211 sdEx8[(Ptp-1::HisCl1;Pmyo- 2::mCherry)],
4 RK222 sdEx9 [pJL033(Peat4::nls::GAL4SK::VP64::unc-543'UTR;Pmyo-2::nls::mCherry,
5 RK223 sdEx10[pJL033(Peat-4::nls::GAL4SK::VP64::unc-54 3'UTR;Pmyo-
6 2::nls::mCherry)], RK225 sdEx11[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54
7 3'UTR;Pmyo-2::nls::mCherry)], RK228 sdEx12[pJL063(Pcat-
8 2::nls::GAL4SK::VP64::unc-54 3'UTR;Pmyo-2::nls::mCherry)],RK229
9 sdEx13[pJL033(Peat-4::nls::GAL4SK::VP64::unc-543'UTR;Pmyo-
10 2::nls::mCherry;3'UTR;15xUAS:: Δ pes-10::HisCl1::SL2::GFP::let-8583'UTR;Punc-
11 122::GFP)], RK230 sdEX14[pJL033(Peat-4::nls::GAL4SK::VP64::unc-543'UTR;Pmyo-
12 2::nls::mCherry;3'UTR;15xUAS:: Δ pes-10::HisCl1::SL2::GFP::let-8583'UTR;Punc-
13 122::GFP)], RK231 sdEx15[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3'UTR;Pmyo-
14 2::nls::mCherry;15xUAS:: Δ pes-10::HisCl1::SL2::GFP::let-8583'UTR;Punc-122::GFP)]
15 RK240 sdEx16[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3'UTR;Pmyo-
16 2::nls::mCherry;15xUAS:: Δ pes-10::HisCl1::SL2::GFP::let-8583'UTR;Punc-122::GFP)].
17 All GAL4 UAS strains and plasmids were a kind gift provided by the Sternberg lab.

18 **2.2 C. elegans culture and media preparation**

19 Strains were reared on NGM plates seeded with *Escherichia coli* OP50 as a food
20 source under standard conditions. NGM-H+ refers to NGM plates with 10mM Histamine
21 dichloride added to the agar and seeded with OP50 *E. coli*. NGM-H- refers to standard
22 NGM plates with OP50 and no histamine. Plates were prepared as described in Pokala
23 *et al.*, 2014.²² Synchronous populations of nematodes were generated using a 1:1

1 mixture of 1N NaOH and hypochlorite bleach solution for no more than 10 minutes with
2 gravid adults. Two days later L4 stage animals were collected and assayed for survival
3 after anoxic exposure. Protocol and details described by Theresa Stiernagle in
4 Wormbook chapter entitled Maintenance of *C. elegans* and used in previous studies to
5 synchronize *C. elegans* for hypoxia assays in Flibotte *et al.*, 2014 and Doshi *et al.*,
6 2019.^{13,18,24}

7 **2.3 Anoxia exposure and assessment of survival**

8 All experiments were performed on L4 stage animals. For anoxic insult, 30 mid L4 stage
9 animals per genotype were picked to an NGM plate, which was placed in a Bio-Bag
10 (Type A anaerobic environmental system, Becton-Dickinson Company, Franklin Lakes,
11 New Jersey), anoxic conditions were induced and maintained for 48 hours at 20°C as
12 described previously.^{13,25} Bags were then opened, and animals were allowed to recover
13 in ambient oxygen for 24 hours before being scored for survival. Surviving animals were
14 identified as those that moved spontaneously or after gentle prodding with a platinum
15 wire. Most resumed feeding and matured into egg-laying adults.

16 **2.4 Preconditioning paradigm with histamine**

17 Early L4 stage animals, as judged by vulval morphology, were plated on NGM-H+ or
18 NGM-H- plates for either 0.5, 1 or 3.5 hours. Animals were then moved to NGM-H-
19 plates for 1.5 hours and subsequently exposed to anoxic conditions.

20 **2.5 Preconditioning paradigm to starvation**

21 Animals were fed OP50 *E. coli* until the early L4 stage of development. Early L4 stage
22 animals were transferred to either NGM plates seeded with OP50 *E. coli* or unseeded

1 plates for a period of 3.5 hours. Experimental animals were taken off NGM unseeded
2 plates and placed onto NGM plates containing OP50 for 1.5 hours. Control animals
3 were always exposed to conditions where food was plentiful.

4 **2.6 Activity Assays**

5 The “WorMotel” device, a multi-well imaging platform, was used to assay *C. elegans*
6 activity for individual animals for a 3.5 hour period.²⁶ Device construction, *C. elegans*
7 cultivation, and imaging setup were performed as described in Churgin and Fang-Yen
8 2017²⁶ except images were recorded every 10 seconds.

9 **2.7 Statistical Analysis**

10 All statistical analysis and graph construction were prepared using GraphPad Prism
11 version 8 (San Diego, California) or MATLAB (Natick, Massachusetts). We averaged
12 survival results from 3+ independent trials performed on different days. Experiments are
13 done in triplicate with 30 animals per genotype or condition. Error bars indicate the
14 standard error of the mean for all experiments. Significant differences were assessed by
15 paired Student’s t-tests (two tailed) for differences between two groups. For groups of
16 three or more, the survival was analyzed by one-way ANOVA, followed by a Tukey’s
17 multiple comparisons post hoc test. Significance was considered if $p < 0.05$. Video
18 recordings of *C. elegans* behavior were analyzed using a MATLAB script as previously
19 described in Churgin and Fang-Yen 2017.²⁶ The relationship between statistical power
20 and effect size (Figure 4b) was determined using MATLAB and assuming normally
21 distributed data with variance equal to that observed in the real activity data. The

1 Anderson-Darling test was used prior to statistical testing to determine whether data
2 were consistent with a normal distribution.

3 **2.8 Data Availability Statement**

4 The data that support the findings of this study, all primary data, including number of
5 animals scored, and number of independent replicates is available from the
6 corresponding author upon reasonable request.

7 **3 Results**

8 **3.1 Hyperpolarization of the Nervous System Prior to Anoxic Insult Yields a** 9 **Survival Benefit**

10 We hypothesized that an animal's susceptibility to an anoxic insult would be
11 influenced by nervous system activity preceding the insult. To test this idea, we used a
12 chemo-genetic approach based on the transgenic expression of histamine gated
13 chloride channels (HisCl1) in select populations of cells. Wild type *C. elegans* neither
14 express HisCl1 nor synthesize histamine. Exogenous provision of histamine to
15 transgenic *C. elegans* expressing HisCl1 in neurons leads to hyperpolarization and
16 reduced activity.²²

17 We began by studying animals expressing the HisCl1 expressed throughout the
18 nervous system via the *tag-168* promotor (*i.e.*, pan neuronal (pn) HisCl1s). In the
19 absence of histamine, the animals with pnHisCl1 appeared and behaved like wild type
20 animals, as previously reported.²² When animals with pnHisCl1 expression were placed
21 on nematode growth media agar plates supplemented with histamine (NGM-H+) they
22 became paralyzed in about 2 minutes.

1 Next we asked if a brief period of paralysis prior to anoxia influenced survival
2 after an anoxic insult. To study this, early L4 stage pnHisCl1 animals were placed on
3 NGM-H+ for either 30 minutes, 1 hour, or 3.5 hours, then transferred to standard NGM
4 plate for 1.5 hours, where they regained locomotor ability. When then subjected to 48
5 hours of anoxic insult and assessed after 24 hours of normoxic recovery, we found that
6 pnHisCl1 animals that had been exposed to histamine (NGM-H+ plates) had increased
7 survival relative to pnHisCl1 animals (that had been grown on NGM-H- plates) (Figure
8 1B). There was a trend towards increased survival at 1 hour and a statistically
9 significant beneficial effect was seen in pnHisCl1 animals with 3.5 hours of nervous
10 system inactivity prior to anoxia (pnHisCl1 NGM-H- 0.31 +/- 0.06 versus pnHisCl1 NGM-
11 H+ 0.73 +/-0.02, ANOVA $F_{(7,82)} = 12.96$, $P < 0.0001$, Figure 1B). As a result, this time
12 point is used as the pre-conditioning manipulation for all other strains. Exposure of wild
13 type animals to histamine conferred no anoxia survival benefit. (N2 NGM-H- 0.31 +/-
14 0.08 versus N2 NGM-H+ 0.30 +/- 0.08 Figure 1B). These results indicate that inactivity
15 of the entire nervous system prior to an anoxia insult protects against a subsequent
16 anoxic insult (*i.e.*, preconditioning).

17 pnHisCl1 animals, when grown on NGM-H+ plates are paralyzed and display no
18 pharyngeal pumping (feeding) behavior. We considered the possibility that inhibition of
19 pharyngeal pumping, which would impede food intake for 3.5 hours, might activate a
20 stress response pathway that protects animals against anoxic stress. This is suggested
21 by prior work showing that a period of starvation, referred to as starvation induced
22 stress response or caloric restriction, and can lead to stress resistance and increase in
23 longevity.²⁷⁻²⁹ To examine this issue, we reared wild type animals on NGM OP50

1 plates, and at the early L4 stage transferred animals to NGM plates with or without
2 OP50 (starvation condition). Animals were then transferred to NGM plates with OP50
3 (food) for 3.5-hours and subsequently subjected to anoxia. We find that 3.5 hours of
4 starvation prior to anoxia does not enhance survival after an anoxic insult (N2 +food
5 0.31 +/-0.05 versus N2 starvation 0.43 +/-0.09, pnHisCl1 +food 0.13 +/-0.02 versus
6 pnHisCl1 starvation 0.15 +/-0.04 ANOVA, $F_{(3,38)} = 7.444$, $P=0.924$ Figure 1C). These
7 results show that the nervous system inactivity is unlikely to protect against anoxic injury
8 owing to a brief period of starvation.

9 **3.2 Hyperpolarization of Cholinergic and GABAergic Signaling Preconditions** 10 ***C. elegans* to Anoxic Stress**

11 To determine which neuronal population confers survival benefit when
12 inactivated, we studied animals with HisCl1 in neurochemically defined classes of
13 neurons. To study cholinergic or serotonergic neurons we generated transgenic
14 animals in which the *unc-17* or *tph-1* promoter (respectively) drove expression of
15 HisCl1. Four independent extrachromosomal array lines were generated and tested.
16 Both groups of animals (HisCl1 in cholinergic neurons: ch-HisCl1, or in serotonergic
17 neurons: ht-HisCl1) appeared normal on NGM-H- plates. When placed on NGM-H+
18 plates the ch-HisCl1 animals became paralyzed, while the ht-HisCl1 displayed no overt
19 phenotype. In our pre-conditioning paradigm, we find that inactivation of cholinergic
20 activity, but not serotonin activity, for 3.5 hours prior to an anoxic insult confers a
21 survival benefit (ch-HisCl1 NGM-H- 0.40, +/-0.06 and 0.30, +/- 0.10 lines 1 and 2
22 respectively versus ch-HisCl1 NGM-H+ 0.72, +/- 0.03 and 0.81, +/-0.03 lines 1 and 2
23 respectively ht-HisCl1 NGM-H- 0.20, +/-0.08 and 0.40, +/-0.10 lines 1 and 2 respectively

1 ht-HisCl1 NGM-H+ 0.19, +/-0.07 and 0.34, +/-0.07 lines 1 and 2 respectively ANOVA
2 $F_{(9,68)} = 18.83$ p value < 0.0001, Figure 2A).

3 Next, we used the bipartite GAL4-UAS system to study other neurochemically
4 defined neuronal populations.²³ Animals containing UAS sequences driving HisCl1
5 were crossed to animals in which the *unc-47* promoter drives GAL4 to generate animals
6 expressing the HisCl1 in GABAergic neurons (ga-HisCl1). Ga-HisCl1 animals appeared
7 normal on NGM-H- plates, but displayed a severely uncoordinated phenotype, *i.e.*
8 abnormal body wall contraction or defective movement when prodded with the platinum
9 wire pick, when placed on NGM-H+ plates. In our pre-conditional paradigm, we find that
10 loss of GABAergic activity for 3.5 hours prior to 48 hours of anoxia conferred a survival
11 benefit (ga-HisCl1-NGM-H- 0.21 +/- 0.04 versus ga-HisCl1-NGM-H+ 0.50 +/- 0.04
12 ,ANOVA $F_{(5,66)} = 6.089$, p value= 0.0001, Figure 2B).

13 To generate *C. elegans* with glutamatergic or dopaminergic expression of HisCl1,
14 constructs containing *eat-4*, and *cat-2* promoter regions driving GAL4 sequences, along
15 with plasmids containing the UAS sequences driving the HisCl1 were injected into N2
16 animals to generate extrachromosomal array lines. Four independent lines were
17 generated with each glutamatergic (glu-HisCl1) and dopaminergic (dop-HisCl1)
18 construct. glu-HisCl1 and dop-HisCl1 animals appeared normal on standard NGM
19 plates that lacked histamine, and displayed no overt phenotype on NGM-H+ plates. We
20 find that loss of neither glutamatergic nor dopaminergic activity for 3.5 hours prior to
21 anoxic insult conferred a survival benefit (glu-HisCl1 NGM-H- 0.20 +/-0.04 and 0.29 +/-
22 0.05 lines 1 and 2 respectively versus glu-HisCl1 NGM-H+ 0.18 +/-0.04 and 0.19 +/-
23 0.01 lines 1 and 2 respectively, ANOVA $F_{(9,74)} = 0.5165$ p=0.8582. dop-HisCl1 NGM-H-

1 0.17 +/-0.04 versus dop-HisCl1 NGM-H+ 0.23 +/-0.04 ANOVA $F_{(7,46)} = 4.551$ $p=0.996$

2 Figure 2C-D respectively). Combined, these results suggest that activity of cholinergic
3 or GABAergic neurons regulate the pre-conditioning response to anoxia.

4 **3.3 Hyperpolarization of Muscle Activity Preconditions *C. elegans* to Anoxia.**

5 Given that loss in cholinergic or GABAergic activity led to paralysis and impaired
6 locomotion respectively, and conferred survival prior to anoxic stress, we asked whether
7 muscle inactivity prior to anoxia would also yield a survival benefit. To test this, we used
8 the UAS-GAL4 system to express HisCl1 in body wall and vulval muscle cells.^{30,31}
9 Animals expressing HisCl1 in muscles on NGM-H- plates were indistinguishable from
10 N2 *C. elegans*. When placed on NGM-H+ plates the muscle-HisCl1 animals became
11 paralyzed and this effect reversed when subsequently moved to NMG-H- plates. We
12 found that 3.5 hours of muscle paralysis prior to 48 hours anoxic insult confers a
13 survival benefit (muscle NGM-H- 0.17, +/- 0.03 versus muscle NGM-H+ 0.53, +/- 0.03
14 ANOVA $F_{(5,60)} = 13.79$, p value <0.0001 Figure 3A). This might indicate that active
15 muscle secretes a factor that makes the organism sensitive to anoxia. Or inactive
16 muscle secretes a factor that makes the organism resistant to anoxia. Regardless of the
17 mechanism, these results suggest that reducing muscle activity below a threshold
18 contributes to the preconditioning phenomenon.

19 **3.4 The Preconditioning Response to Anoxia is Dependent on AVA Command** 20 **Interneurons.**

21 To further probe the neural circuit regulating the preconditioning response to
22 anoxia, we studied the role of select interneurons. We tested AVA command

1 interneurons first because these neurons facilitate backward locomotion in the animal,
2 receive acetylcholine neurotransmitter input, and make connections onto motor
3 neurons. To determine if AVA command interneurons play a role in in mediating the
4 preconditioning response to anoxia, we studied animals expressing HisCl1 under the
5 control of the *rig-3* promoter (AVA-HisCl1).³² AVA-HisCl1 animals appeared normal on
6 NGM-H- plates and displayed a mild uncoordinated (Unc) phenotype when placed on
7 NGM-H+ plates. Animals with a loss in AVA activity for 3.5 hours prior to 48-hour
8 anoxic insult had increased survival compared to controls (AVA-HisCl1 NGM-H- 0.44,+/-
9 0.06 versus AVA-HisCl1 NGM-H+ 0.64, +/- 0.04, $t_{(8)}=2.971$, $p=0.01$, paired t test, Figure
10 3B).

11 Next we tested a different population of interneurons that are also part of the
12 locomotor circuit. AIB are a pair of amphid interneurons that receive input from sensory
13 neurons, make connections onto motor neurons, and also regulate locomotion in *C.*
14 *elegans*. Animals expressing HisCl1 in AIB interneuron pair on NGM-H- and NGM-H+
15 plates are indistinguishable from N2 *C. elegans*. In our preconditioning paradigm, we
16 find that impairing AIB interneuron activity prior to anoxia did not yield a survival benefit
17 (AIB-HisCl1 NGM-H 0.32 +/-0.04 versus AIB-HisCl1 NGM-H+ 0.26 +/-0.04, $t_{(8)}=1.215$,
18 $p=0.258$, paired t test Figure 3C). This suggests that the pre-conditioning phenomenon
19 involves the activity within specific neurons of the locomotor circuit.

20 Since AVA interneurons make connections with motor neurons, we asked if
21 inactivity of specific motor neurons prior to anoxia might lead to increased survival. We
22 tested DA and VA motor neurons for several reasons: 1) they innervate dorsal and
23 ventral muscles respectively,³³ 2) they receive cholinergic input,³⁴ 3) they receive

1 direct input from AVA interneurons to initiate backward locomotion,³⁵ and 4) AVA
2 hyperpolarization maybe mediated by inactivation of about 2/3 of cholinergic motor
3 neurons, including classes DA and VA motor neurons through gap junctions.^{33,36,37} To
4 determine if DA and VA motor neurons play a role in in mediating the preconditioning
5 response to anoxia, we studied animals expressing HisCl1 under the control of the *unc-*
6 *4* promoter, which also expresses in three SAB head motor neurons (SAB-DA-VA-
7 HisCl1).³⁸ SAB-DA-VA-HisCl1 animals appeared normal on standard NGM-H- plates
8 and displayed a weak phenotype when placed on NGM-H+ plates. We find that
9 inactivity of SAB, DA, and VA motor neurons in our preconditioning paradigm does not
10 confer a survival after anoxic insult. (SAB-DA-VA NGM-H- 0.35 +/-0.03 versus SAB-DA-
11 VA NGM-H+ 0.39 +/-0.04, $t_{(13)}=1.172, p=0.262$, paired t-test, Figure 3D).

12 **3.5 Lack of Locomotor Activity Prior to Anoxic Insult is Not a Predictor of** 13 **Survival**

14 Our results show that hyperpolarization of certain population of neurons or muscle
15 prior to anoxic insult either impaired or paralyzed animals, and led to a survival benefit.
16 We asked whether the amount of locomotor activity prior to anoxia could predict survival
17 after an anoxic insult in untreated wild-type animals. To this end we monitored
18 spontaneous activity of N2 animals prior to 48-hours of anoxic stress using a multi-well
19 imaging platform (WorMotel) and image analysis. Within a population of *C. elegans*,
20 individuals display variations in locomotor activity. We compared the pre-anoxia activity
21 of animals that survived anoxia to those that died. We found only a small, non-significant
22 difference of 49.2 activity values between survivors and non-survivors (two tailed t-test, p
23 value = 0.22, Figure 4A). Based on the sample size and variance of our dataset, the

1 smallest difference in activity we could have reliably detected (statistical power = 0.95)
2 was 140.8 activity values (Figure 4B). Collectively, these result suggests that, while
3 normal *C. elegans* vary in their spontaneous activity levels, they are operating above the
4 threshold that evokes the pre-conditioning phenomenon.

5 **4 Discussion**

6 The preconditioning phenomena is a physiological process that raises the
7 threshold for cellular damage evoked by environmental insults. A mechanistic
8 understanding of this process might be harnessed for therapeutic ends. Here we show
9 that activity of specific set of neurons and muscle cells have a substantial impact on the
10 susceptibility of developing nematodes to anoxic insult. Since the preconditioning
11 phenomena has been described throughout the animal kingdom, insight into this
12 physiological response in a genetically tractable organism may have broad
13 application.³⁹⁻⁴¹

14 One salient feature of our investigations here and in prior publications (Flibotte *et*
15 *al.*, 2014 and Doshi *et al.*,2019) is variability of survival after a 48-hour anoxic insult.
16 We considered potential sources of this in our isogenic population of *C. elegans* (*i.e.*,
17 modest differences in animal age, number of animals on a plate, prior history of
18 starvation, distance of plate from catalyst that induce anoxic conditions, number of
19 plates in a biobag and age of NGM plates) and none appear to account for the
20 variability. We suspect that natural stochasticity in biological systems may be the
21 underlying source of the variability we see. This is a topic of great interest to the *C.*
22 *elegans* community⁴²⁻⁴⁶ and an area of active inquiry. Regardless of the source, by
23 undertaking many independent trials and reporting averages we have worked to control

1 for this variability. Using this approach, we believe we can draw valid conclusions
2 despite the unavoidable variability.

3 A substantial amount of research into the preconditioning phenomena comes
4 from investigations of heart tissue. Two temporally distinct phases in ischemic-
5 reperfusion models have been described; early (or “first window of protection”, lasting ~
6 2-3 hours) and late (“second window of protection”, onset 12-24 post preconditioning
7 and lasting ~72-90 hours).^{41,47-49} Much of the physiology, cell biology and molecular
8 biology is studied at the level of the heart itself – for example, transient interruption of
9 coronary blood flow prior to a vessel occlusion reduces cardiac infarction size.⁵
10 Another form of cardiac preconditioning is termed “remote” because it is elicited by
11 inducing transient ischemia of distal organs such as the small intestine, kidney and
12 skeletal muscle.⁵⁰⁻⁵³ Humeral factors are posited to be released from extracardiac
13 organs in this paradigm which confer stress resistance on the heart. Neuronal
14 pathways and systemic responses may also be involved.^{40,54}

15 Early phase cardiac precondition involves the local release of factors such as
16 reactive oxygen or nitrogen species, bradykinin and adenosine.^{41,55} In parallel these
17 agents activate several signaling cascades that include Akt, Erk1/2, protein kinase C⁵⁶
18 and lead to the opening of mitochondrial ATP-sensitive potassium channels (K_{ATP}).^{19,57}
19 This has been termed the reperfusion injury salvage kinase (“RISK”) pathway.⁵⁵ The
20 cardioprotective effects are thought to be related to opposition of the mitochondrial
21 permeability transition pore opening by active K_{ATP} . Another pathway that is involved in
22 cardio- protection (“survivor activation factor enhancement”) involves TNF- and the
23 JAK/STAT pathway.⁵⁸

1 Precisely how these cardioprotective pathways are coordinated and regulated
2 remains an area of active investigation. Late phase cardiac preconditioning appears
3 contingent on early phase signals and on transcription and translation. Remote
4 preconditioning bears the signature of both early and late preconditioning (*i.e.*,
5 involvement of adenosine, bradykinin, *etc.*) but the nature of the humeral factors, the
6 putative receptors and signaling processes are unknown.^{41,55}

7 Several groups have studied the preconditioning phenomena in *C. elegans*. The
8 Crowder group showed that unfolded protein response component IRE-1 (in a pathway
9 independent XBP-1) and GCN-2 (in a pathway independent of phosphorylation of
10 translation factor eIF2 α) mediate the pre-conditioning response to hypoxia.^{19,20} In
11 addition, they implicated the apoptosis factor CED-4 (also known as *apaf-1*) in a novel
12 mechanism that does not require any other known core apoptosis genes.^{20,59} Genetic
13 pathways that regulate energy dynamics have roles in the preconditioning response.
14 The Padilla group showed that survival to anoxia was dependent on the energy sensor
15 AMP regulated protein kinase (AMPK).⁵⁹ This same beneficial effect could be mimicked
16 by exposing animals to the dietary restriction-like state induced by metformin.⁵⁹ Work
17 from the Miller group showed that a several hours of fasting blunted protein
18 homeostasis defects evoked by hypoxia and that this involved the insulin/insulin-like
19 growth factor receptor *daf-2* but not its downstream target, *daf-16*.⁶⁰ Collectively, these
20 studies provide valuable information about the genetic underpinning of the
21 preconditioning phenomena, however it remains to be determined if these genes work
22 in a single pathway or multiple parallel pathways. In addition, these studies do not

1 provide insights into the cell autonomous versus cell non-autonomous contributions to
2 the preconditioning phenomena.

3 What accounts for this heightened state of resistance? One interpretation is that
4 inactivation of neuronal populations that impair movement suspend natural development
5 in early L4 stage animals and these developmentally younger animals are inherently
6 resistant to anoxic insult. However, we think that is unlikely because previous work
7 showed no difference in survival to anoxic stress between early versus late stage
8 animals.¹⁸ We therefore consider two, not mutually exclusive, possibilities to explain
9 these observations. First, normal physiological activity of cholinergic and GABAergic
10 neurons and muscle might secrete an “anoxia sensitivity factor” which heightens
11 organismal vulnerability to anoxia. When these cells are electrically silenced, the
12 abundance of this putative factor is reduced temporarily and thus organisms display
13 increased rates of survival after an anoxic insult. Second, cholinergic, GABAergic
14 neurons and muscle that are electrically silenced might secrete an “anoxia resistance
15 factor”. This putative factor temporarily increases the resistance of the organism to an
16 anoxia insult. These considerations are aligned with well-described cell non-
17 autonomous stress signaling in *C. elegans*; such signals can originate from distinct
18 populations of neurons as well as glial cells.⁶¹⁻⁶⁵ A goal of future studies should be to
19 determine whether the preconditioning phenomenon evoked by muscle inactivity (for
20 example) is due to a sensitivity versus a resistance factor. Understanding the
21 biochemical nature of this putative factor and how its signaling affects the response to
22 an anoxic insult will be of enormous interest.

1 Finally, we note that the work described herein is unique in that the
2 preconditioning stimulus is not an abbreviated exposure to an otherwise toxic insult.
3 This differs from remote preconditioning wherein short duration ischemia to the heart or
4 distal organs influences the outcome of a subsequent coronary vessel occlusion.⁵ It
5 differs from the work of Dasgupta *et al.* in which 4 hours of hypoxia followed by a 24
6 hours recovery period afforded protection against 24 hours of hypoxia.²⁰ We retain the
7 nomenclature of preconditioning since it is a transient manipulation prior to a severe
8 insult that moderates outcome, although this designation is arguable. We believe that
9 expanding the notion of preconditioning in this way can help identify physiological states
10 of higher or lower susceptibility to insult that are dynamic and susceptible to
11 manipulation.

12 **5 Conclusion**

13 The role of the nervous system in preconditioning to anoxic insult has not been
14 extensively studied. The neurons and tissues involved in modulating the preconditioning
15 response to anoxia are not known. Our results implicate cholinergic, GABAergic, and
16 muscle activity in mediating the preconditioning response to anoxic insult. Our
17 observations raise several questions that should be addressed in the future. What is
18 special about the cholinergic and GABAergic neurons (as opposed to other
19 neurochemically defined neurons that also impact muscle function) that is particularly
20 beneficial to *C.elegans* under standard cultivation conditions? What signals are
21 elaborated by cholinergic and GABAergic neurons and muscle cells that heightens
22 susceptibility of anoxia? Do all tissues respond to these signals or is there a cascade of

1 signal transduction from tissue to tissue? Insight into these issues may bring us closer
2 to harnessing the preconditioning phenomena for therapeutic use.

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18 H.L.B analyzed all results.

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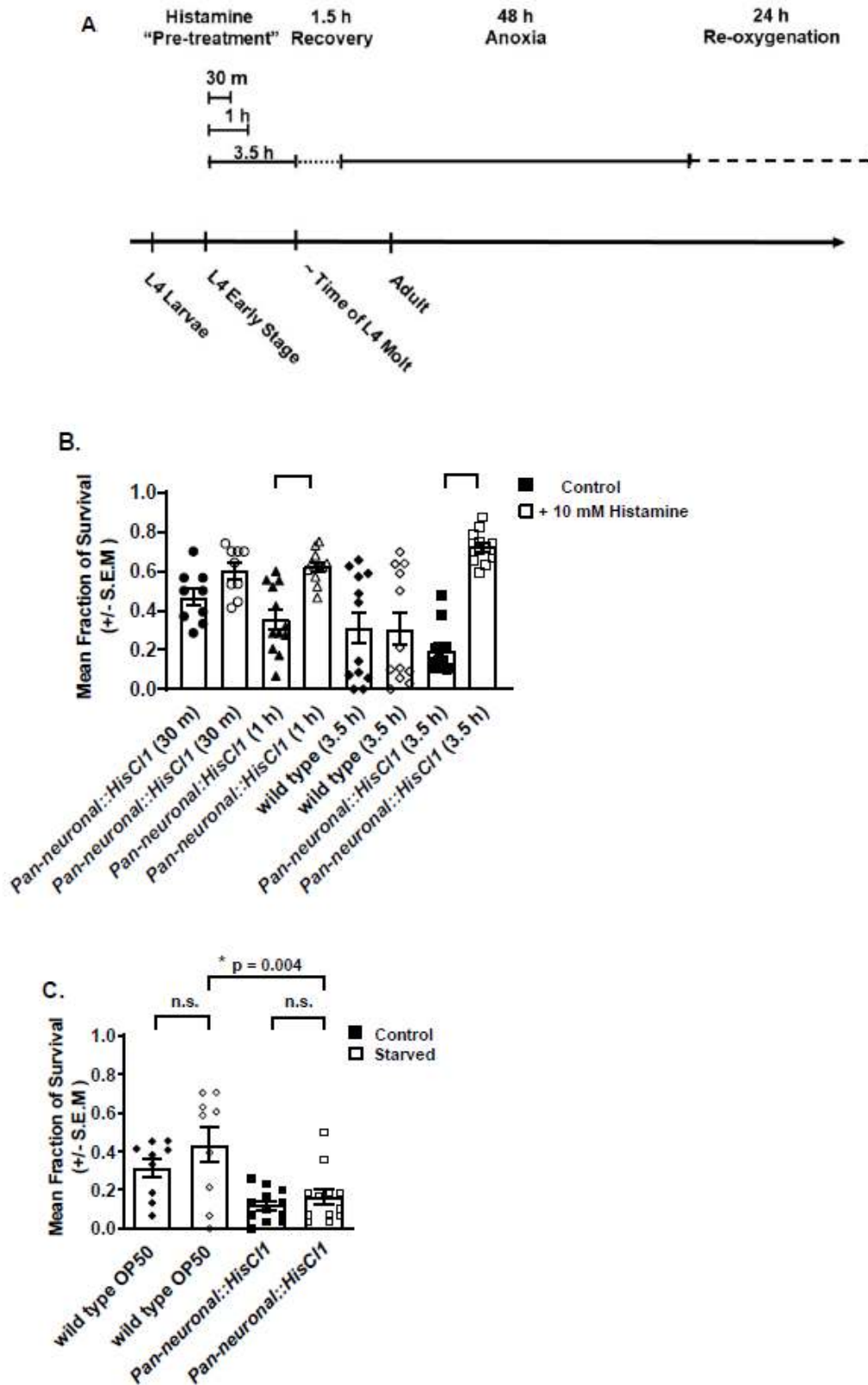
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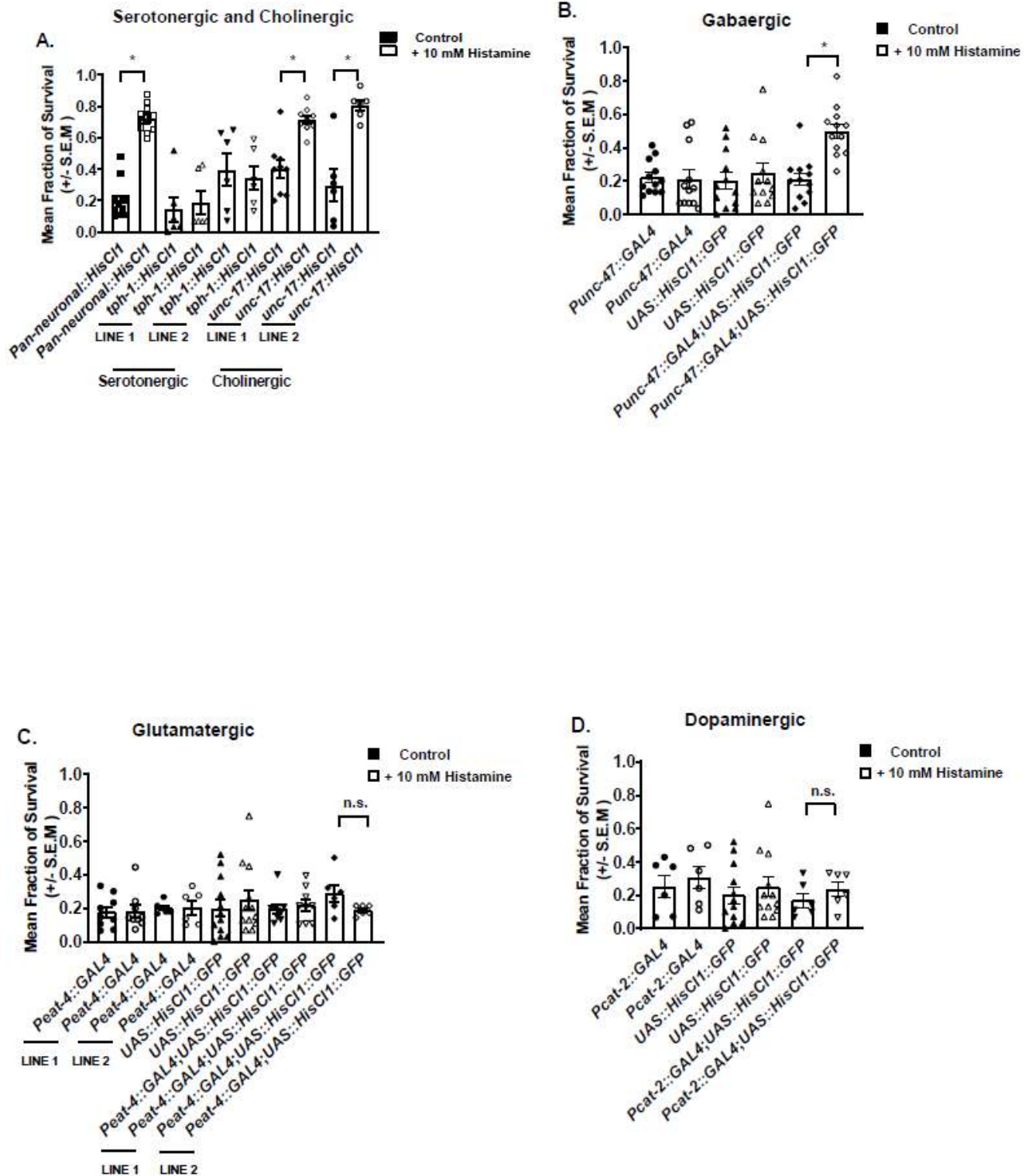
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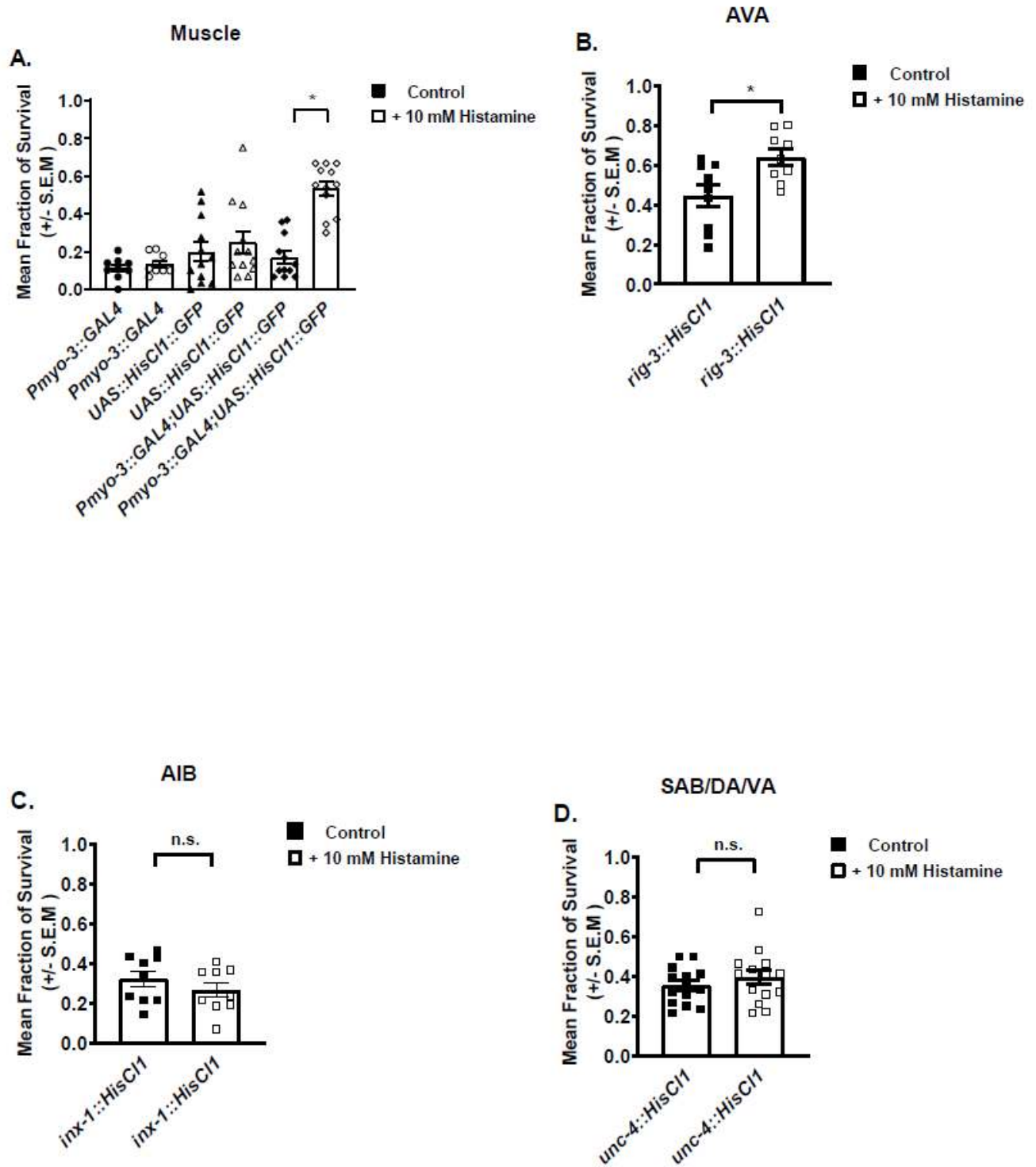
1 **Figure 1: Impaired Neuronal Activity Prior to Anoxic Insult Increases Survival**



1 **Figure 2: Inactivity of Cholinergic and GABAergic Neurons Mediates the**
 2 **Preconditioning Effect**



1 **Figure 3: Neuromuscular Activity Mediates to the Preconditioning Effect to**
2 **Anoxia.**



1 **Figure 4: Survival is Not Related to Locomotor Activity Prior to Anoxic Insult.**

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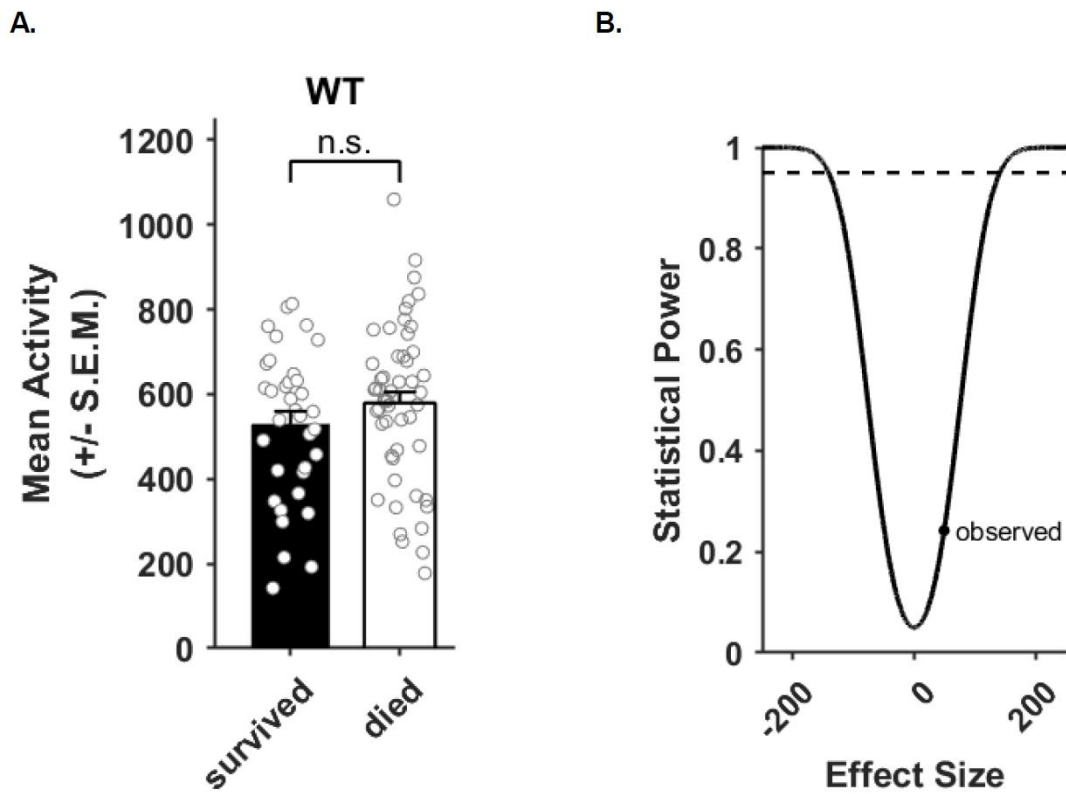
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1 **Figure Legends**

2 **Figure 1: Impaired Neuronal Activity Prior to Anoxic Insult Increases Survival**

3 **A.) Preconditioning to 48 hours of anoxia experimental paradigm.** Thirty animals

4 carrying the histamine gated chloride channel behind a neuronal or a tissue specific

5 promoter were selected as early L4 animals. Animals were placed on NGM plates

6 containing either 10mM of histamine seeded with OP50 *E. coli* for 30 minutes, 1 hour, or

7 3.5hours. Control animals placed to NGM OP50 *E. coli* plates lacking histamine.

8 Animals recovered on non-histamine plates for 1.5 hours and then asphyxiated for 48

9 hours. Fraction of survival was scored for animals that developed into adults, regained

10 movement and resumed feeding 24 hours after anoxic insult. **B.) Inactivation of the**

11 **nervous system, prior anoxic insult has a beneficial effect.** Wild type animals and

12 animals carrying the histamine gated chloride channel 1 behind a pan-neuronal *tag-168*

13 promoter were treated as described in panel A. Controls (black filled shapes) and

14 experimental histamine exposed (non-filled shapes). Results are shown for 4

15 independent trials, n=360 animals per condition **C.) Starvation prior to 48 hours of**

16 **anoxia has no impact on survival.** Wild type and animals expressing histamine gated

17 chloride channel were selected as early L4 animals to NGM plates seeded with OP50 *E.*

18 *coli* (control black filled shapes) or plates with no OP50 *E. coli* (starved non- filled

19 shapes). Results are shown for 3 independent trials (n=270 animals) wild type and 4

20 independent trials pan-neuronal histamine strain, n=360 animals per condition.

21 **Figure 2: Inactivity of Cholinergic and GABAergic Neurons Mediates the**

22 **Preconditioning Effect**

1 **A.) Inactivation of cholinergic neurons, prior to anoxic insult has a beneficial**
2 **effect.** Wild type animals and animals carrying the histamine gated chloride channel
3 under the pan-neuronal promoter *tag-103*, cholinergic promoter, *unc-17*, or
4 serotonergic promoter *tph-1* were selected to control plates (black filled shapes) or
5 10mM histamine plates (non-filled shapes) as early L4 animals, prior to anoxic
6 stress. * denotes significance. Results are shown for 4 independent trials, n=360
7 animals per condition. **B.) Loss of GABAergic signaling prior to anoxic insult**
8 **confers a survival benefit.** Wild type, animals expressing the GABAergic promoter
9 (*Punc-47*) behind the GAL4 sequence, animals carrying the histamine gated chloride
10 channel behind the UAS activated sequence, as well as animals expressing GAL4
11 under the GABAergic promoter with the UAS histamine gated chloride channel were
12 tested. * denotes significance *Punc-47::GAL4:15xUAS::HisCl1::SL2::GFP* control
13 versus experimental. **C.) Loss of glutamatergic signaling does not precondition**
14 **animals to anoxia.** Wild type animals, animals expressing the glutamatergic
15 promoter (*Peat-4*) behind the GAL4 sequence, animals carrying the histamine gated
16 chloride channel behind the UAS activated sequence, as well as animals expressing
17 GAL4 under the *eat-4* promoter with the UAS histamine gated chloride channel were
18 tested as in panel B. Results are shown for 2 independent trials. **D.) Inactivation of**
19 **dopaminergic pathway prior to anoxia does not yield a survival advantage.**
20 Wild type animals, animals expressing the dopaminergic promoter (*Pcat-2*) behind
21 the GAL4 sequence, animals carrying the histamine gated chloride channel behind
22 the UAS activated sequence, as well as animals expressing GAL4 under the *cat-2*

1 promoter with the UAS histamine gated chloride channel were tested as in panel B.
2 Results are shown for 2 independent trials.

3 **Figure 3: Neuromuscular Activity Mediates the Preconditioning Effect to Anoxia.**

4 **A.) Inactivation of the muscle prior to anoxic insult has an advantageous effect**

5 **on survival.** Wild type, animals expressing the muscle promoter (*Pmyo-3*)
6 behind the GAL4 sequence, animals carrying the histamine gated chloride
7 channel behind the UAS activated sequence, as well as animals expressing
8 GAL4 under the muscle promoter with the UAS histamine gated chloride channel
9 were selected to 10mM histamine or control plates as early L4 animals 3.5
10 hours, prior to anoxic stress. Fraction of survival was scored after 24 hours.
11 Significance between *Pmyo-3::GAL4:15xUAS::HisCl1::SL2::GFP* control versus
12 experimental; error bars represent the SEM. Results are shown for 4

13 independent trials. **B.) Inactivity of command interneurons AVA yields a**

14 **survival advantage to anoxia.** Animals carrying the histamine gated chloride
15 channel behind the *rig-3* promoter were selected as early L4 animals. Controls
16 animals (black filled shapes) and animals exposed to 10mM histamine,
17 experimental condition, (non-filled shapes) as early L4 animals, prior to 48 hours
18 of anoxic stress. Fraction of survival was scored after 24 hours. Results are

19 shown for 4 independent trials. **C.) Inactivity of AIB interneuron prior to**

20 **anoxic insult does not provide a survival advantage.** Animals expressing the
21 histamine gated chloride channel behind the *inx-1* promoter were tested,
22 experimental paradigm as in panel C. Results are shown for 4 independent trials.

23 **D.) SAB-DA-VA motor neuron inactivity is dispensable for the**

1 **preconditioning response to anoxic insult.** Animals expressing the histamine
2 gated chloride channel behind the *unc-4* promoter were tested for pre-
3 conditioning response to 48hours of anoxic insult experimental paradigm as in
4 panel C. Results are shown for 4 independent trials.

5 **Figure 4: Survival is Not Related to Locomotor Activity Prior to Anoxic Insult.**

6 **A.) Activity of survivors and non-survivors prior to anoxic insult.** Wild type
7 animals were selected at the early L4 stage and loaded into the “WorMotel” device
8 and assayed for locomotor activity for a 3.5 hour period. Dots indicate activity of
9 individual animals. Results are shown for 87 individual animals (35 survivors and
10 52 non-survivors) from five replicates. **B.) Statistical power analysis.** The
11 statistical power (probability of rejecting the null hypothesis of no difference in
12 activity between survivors and non-survivors if the null hypothesis were not true)
13 of our experiment for different effect sized based on the variance and sample sizes
14 in panel A is shown (black line) A statistical power of 0.95 is indicated by a
15 horizontal dashed line, and the observed effect size from panel a is indicated by a
16 dot.

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