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ORIGINAL ARTICLE

Preconditioning of *Caenorhabditis elegans* to anoxic insult by inactivation of cholinergic, GABAergic and muscle activity

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Abstract

For most metazoans, oxygen deprivation leads to cell dysfunction and if severe, death. Sublethal stress prior to a hypoxic or anoxic insult ("preconditioning") can protect cells from subsequent oxygen deprivation. The molecular mechanisms by which sublethal stress can buffer against a subsequent toxic insult and the role of the nervous system in the response are not well understood. We studied the role of neuronal activity preconditioning to oxygen deprivation in *Caenorhabditis elegans*. Animals expressing the histamine gated chloride channels (HisCl1) in select cell populations were used to temporally and spatially inactivate the nervous system or tissue prior to an anoxic insult. We find that inactivation of the nervous system for 3 h prior to the insult confers resistance to a 48-h anoxic insult in 4th-stage larval animals. Experiments show that this resistance can be attributed to loss of activity in cholinergic and GABAergic neurons as well as in body wall muscles. These observations indicate that the nervous system activity can mediate the organism's response to anoxia.

KEYWORDS

anoxia, *C. elegans*, cholinergic neurons, GABAergic neurons, ischemia, muscle activity, neural circuits, oxygen deprivation, preconditioning, stress

1 | INTRODUCTION

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

A transient, sub-lethal experience of hypoxia or ischemia can protect against an otherwise lethal subsequent hypoxic–ischemic insult. This phenomenon, referred to ischemic or hypoxic preconditioning,[3-7] suggests that cells have a latent adaptive capacity to combat the noxious effects of ischemia and hypoxia. If we understood the biochemical basis for the preconditional effect, it could potentially be harnessed for therapeutic purposes. Previous work in mammalian systems on ischemic preconditioning has highlighted a role for signal transduction pathways, (i.e., PI3K-AKTand ERK pathways), as well as hypoxia inducing factor (HIF).[8-10] Nonetheless, a complete understanding of the phenomenon is lacking.

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

C. elegans display the preconditioning phenomenon and genetic studies implicate classical stress response pathways, genes required for lifespan, energy homeostasis, dauer formation and genes involved cell death pathways.[15,17,19-21] Given this starting point, we wondered if the preconditioning phenomena in *C. elegans* similarly displayed cell non-autonomous features and involved the nervous system. We designed experiments to address the following questions: (a) What is the role of the nervous system in sensing and responding to anoxic stress? And (b) What are the tissues or neuronal populations underlying the preconditioning response to anoxic stress? Our results support a role for inactivity of cholinergic and GABAergic neurons, and muscle in modulating survival to a subsequent anoxic insult.

2 | MATERIAL AND METHODS

2.1 | Strains

The following strains were used in this work. N2, referred to as wild type, CX14373 *kyEx4571 [pNp403 (tag-168:HisCl1::SL2::GFP;myo-3::mCherry]* from Pokala et al., 2014. [22] CX14845 *kyEx5104[pNP424 (mec-3::HisCl1::SL2::mCherry; unc-122::GFP)]*, from Pokala et al., 2014.[22] CX15341 *kyEx5161[pNP488 (unc-4::HisCl1::SL2::mCherry; elt-2::mCherry)]*, from Pokala et al., 2014. [22] CX15457 *kyIs620[pNP472 (inx-1::HisCl1::SL2:GFP;myo-3::mCherry)]*, from Pokala et al., 2014.[22] PS6963 *syIs336 [pHW383(Pmyo-3::nls::GAL4SK::VP64::unc-54 3′UTR; Pmyo-2::nls::mCherry)]*, from Wang et al., 2017.[23] PS7160 *syIs393 [pHW504(Punc-47::nls::GAL4::VP64::let-858 3′UTR; Punc-122::RFP)]*, from Wang et al., 2017.[23] PS7199 *syIs371 [pJL046(15xUAS:: Δpes-10::HisCl1::SL2::GFP::let-858 3′UTR;Punc-122::GFP)]*, from Wang et al., 2017.[23] RK200 (*Punc-47::nls::GAL4SK::VP64::let-858 3′UTR;15xUAS:: Δpes-10::HisCl1::SL2::GFP::let-8583′UTR;Punc-122::GFP*), RK201[*(Pmyo-3::nls::GAL3SK::VP64::unc-54 3′UTR;15xUAS:: Δpes-10::HisCl1::SL2::GFP::let-858 3′UTR;Punc-122::GFP)]*, RK206 *sdEx5[pJP673(Punc-17::HisCl1;myo-2::mCherry)]*, *RK207 sdEx6[pJP673(Punc-17::HisCl1;myo-2::mCherry)]*, RK210 sdEx7[*(Ptph-1::HisCl1; Pmyo-2::mCherry]*, RK211 *sdEx8[(Ptph-1::HisCl1;Pmyo- 2::mCherry)]*, RK222 *sdEx9 [pJL033(Peat4::nls::GAL4SK::VP64::unc-543′UTR;Pmyo-2::nls::mCherry*, RK223 *sdEx10 [pJL033(Peat-4::nls::GAL4SK::VP64::unc-54 3′UTR;Pmyo-2::nls::mCherry)]*, RK225 *sdEx11[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3′UTR;Pmyo-2::nls::mCherry)]*, RK228 *sdEx12[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3′UTR;Pmyo-2::nls::mCherry)]*,RK229 *sdEx13[pJL033(Peat-4::nls::GAL4SK::VP64::unc-543′UTR;Pmyo-2::nls::mCherry;3′UTR;15xUAS::Δpes-10::HisCl1::SL2::GFP::let-8583′UTR;Punc-122::GFP)]*, RK230 *sdEX14 [pJL033(Peat-4::nls::GAL4SK::VP64::unc-543′UTR;Pmyo-2::nls::mCherry;3′UTR;15xUAS::Δpes-10::HisCl1::SL2::GFP::let-8583′UTR;Punc-122::GFP)]*, RK231 *sdEx15[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3′UTR;Pmyo-2::nls::mCherry;15xUAS::Δpes-10::HisCl1::SL2::GFP::let-8583′UTR;Punc-122::GFP)]* RK240 *sdEx16[pJL063(Pcat-2::nls::GAL4SK::VP64::unc-54 3′UTR;Pmyo-2::nls::mCherry;15xUAS::Δpes-10::HisCl1::SL2::GFP::let-8583′UTR;Punc-122::GFP)]*. All GAL4 UAS strains and plasmids were a kind gift provided by the Sternberg lab.

2.2 | *C. elegans* culture and media preparation

Strains were reared on NGM plates seeded with *Escherichia coli* OP50 as a food source under standard conditions. NGM-H+ refers to NGM plates with 10 mM Histamine dichloride added to the agar and seeded with OP50 *E. coli*. NGM-H- refers to standard NGM plates with OP50 and no histamine. Plates were prepared as described in Pokala et al., 2014.[22] Synchronous populations of nematodes were generated using a 1:1 mixture of 1 N NaOH and hypochlorite bleach solution for no more than 10 min with gravid adults. Two days later L4 stage animals were collected and assayed for survival after anoxic exposure. Protocol and details described by Theresa Stiernagle in Wormbook chapter entitled Maintenance of *C. elegans* and used in previous studies to synchronize *C. elegans* for hypoxia assays in Flibotte et al., 2014 and Doshi et al., 2019.[13,18,24]

2.3 | Anoxia exposure and assessment of survival

All experiments were performed on L4 stage animals. For anoxic insult, 30 mid L4 stage animals per genotype were picked to an NGM

plate, which was placed in a Bio-Bag (Type A anaerobic environmental system, Becton-Dickinson Company, Franklin Lakes, New Jersey), anoxic conditions were induced and maintained for 48 h at 20°C as described previously.[13,25] Bags were then opened, and animals were allowed to recover in ambient oxygen for 24 h before being scored for survival. Surviving animals were identified as those that moved spontaneously or after gentle prodding with a platinum wire. Most resumed feeding and matured into egg-laying adults.

2.4 | Preconditioning paradigm with histamine

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

2.5 | Preconditioning paradigm to starvation

Animals were fed OP50 *Escherichia coli* until the early L4 stage of development. Early L4 stage animals were transferred to either NGM plates seeded with OP50 *E. coli* or unseeded plates for a period of 3.5 h. Experimental animals were taken off NGM unseeded plates and placed onto NGM plates containing OP50 for 1.5 h. Control animals were always exposed to conditions where food was plentiful.

2.6 | Activity assays

The "WorMotel" device, a multiwell imaging platform, was used to assay *C. elegans* activity for individual animals for a 3.5 h period.[26] Device construction, *C. elegans* cultivation, and imaging setup were performed as described in Churgin and Fang-Yen 2017 [26] except images were recorded every 10 s.

2.7 | Statistical analysis

All statistical analysis and graph construction were prepared using GraphPad Prism version 8 or MATLAB. We averaged survival results from 3+ independent trials performed on different days. Experiments are done in triplicate with 30 animals per genotype or condition. Error bars indicate the standard error of the mean for all experiments. Significant differences were assessed by paired Student's *t* tests (two tailed) for differences between two groups. For groups of three or more, the survival was analyzed by one-way analysis of variance (ANOVA), followed by a Tukey's multiple comparisons post hoc test. Significance was considered if $p < 0.05$.Video recordings of *C. elegans* behavior were analyzed using a MATLAB script as previously described in Churgin and Fang-Yen 2017.[26] The relationship between statistical power and effect size (Figure 4B) was determined using MATLAB and assuming normally distributed data with variance equal to that observed in the real activity data. The Anderson-Darling test was used prior to statistical testing to determine whether data were consistent with a normal distribution.

3 | RESULTS

3.1 | Hyperpolarization of the nervous system prior to anoxic insult yields a survival benefit

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

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

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

pnHisCl1 animals, when grown on NGM-H+ plates are paralyzed and display no pharyngeal pumping (feeding) behavior. We considered the possibility that inhibition of pharyngeal pumping, which would impede food intake for 3.5 h, might activate a stress response pathway that protects animals against anoxic stress. This is suggested by prior work showing that a period of starvation, referred to as starvation induced stress response or caloric restriction, and can lead to stress resistance and increase in longevity.[27-29] To examine this issue, we reared wild type animals on NGM OP50 plates, and at the early L4 stage transferred animals to NGM plates with or without OP50 (starvation condition). Animals were then transferred to NGM plates with

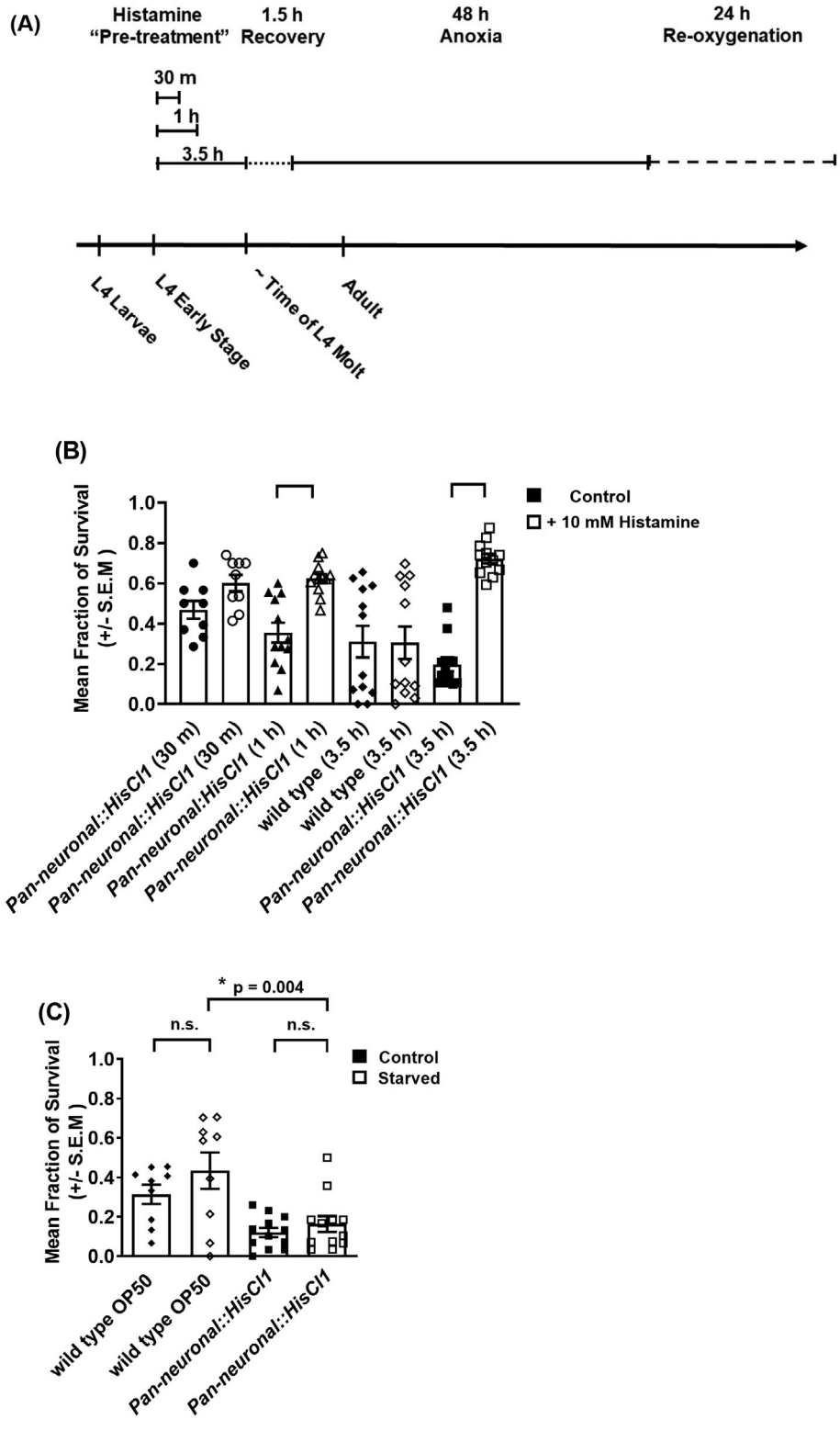

FIGURE 1 Impaired neuronal activity prior to anoxic insult increases survival (A) Preconditioning to 48 h of anoxia experimental paradigm. Thirty animals carrying the histamine gated chloride channel behind a neuronal or a tissue specific promoter were selected as early L4 animals. Animals were placed on NGM plates containing either 10 mM of histamine seeded with OP50 *E. coli* for 30 min, 1, or 3.5 h. Control animals placed to NGM OP50 *E. coli* plates lacking histamine. Animals recovered on non-histamine plates for 1.5 h and then asphyxiated for 48 h. Fraction of survival was scored for animals that developed into adults, regained movement and resumed feeding 24 h after anoxic insult. (B) Inactivation of the nervous system, prior anoxic insult has a beneficial effect. Wild type animals and animals carrying the histamine gated chloride channel 1 behind a pan-neuronal *tag-168* promoter were treated as described in panel A. Controls (black filled shapes) and experimental histamine exposed (non-filled shapes). Results are shown for four independent trials, n = 360 animals per condition (C) Starvation prior to 48 h of anoxia has no impact on survival. Wild type and animals expressing histamine gated chloride channel were selected as early L4 animals to NGM plates seeded with OP50 *E. coli* (control black filled shapes) or plates with no OP50 *E. coli* (starved non-filled shapes). Results are shown for three independent trials (n = 270 animals) wild type and four independent trials pan-neuronal histamine strain, n = 360 animals per condition

OP50 (food) for 3.5-h and subsequently subjected to anoxia. We find that 3.5 h of starvation prior to anoxia does not enhance survival after an anoxic insult (N2 + food 0.31 ± 0.05 vs. N2 starvation 0.43 ± 0.09, pnHisCl1 + food 0.13 ± 0.02 vs. pnHisCl1 starvation 0.15 ± 0.04 ANOVA, $F_{(3,38)} = 7.444$, $p = 0.924$ Figure 1C). These results show that the nervous system inactivity is unlikely to protect against anoxic injury owing to a brief period of starvation.

3.2 | Hyperpolarization of cholinergic and GABAergic signaling preconditions *C. elegans* to anoxic stress

To determine which neuronal population confers survival benefit when inactivated, we studied animals with HisCl1 in neurochemically defined classes of neurons. To study cholinergic or serotonergic

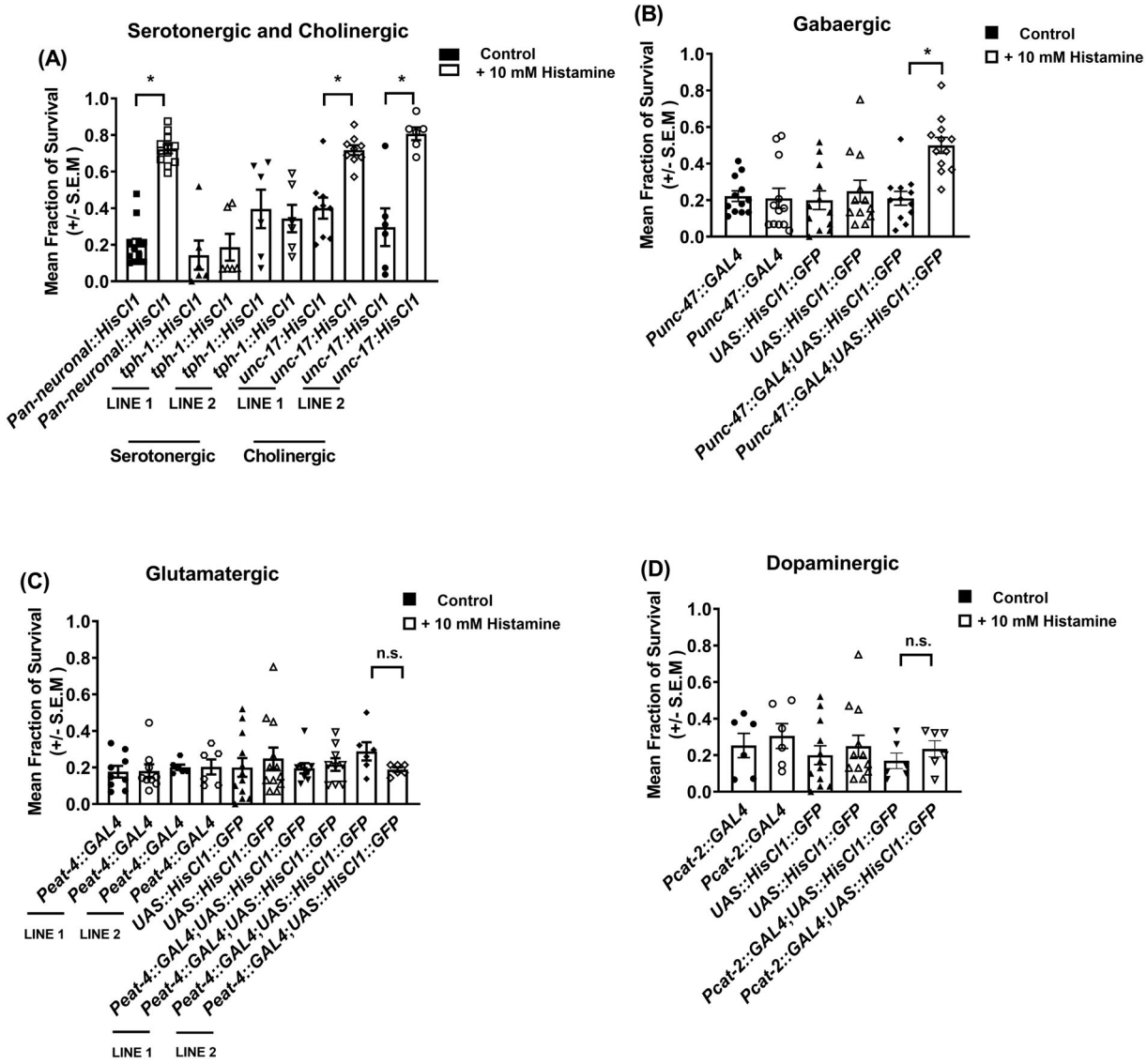

FIGURE 2 Inactivity of cholinergic and GABAergic neurons mediates the preconditioning effect. (A) Inactivation of cholinergic neurons, prior to anoxic insult has a beneficial effect. Wild type animals and animals carrying the histamine gated chloride channel under the pan-neuronal promoter *tag-103*,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

neurons we generated transgenic animals in which the *unc-17* or *tph-1* promoter (respectively) drove expression of HisCl1. Four independent extrachromosomal array lines were generated and tested. Both groups of animals (HisCl1 in cholinergic neurons: ch-HisCl1, or in serotonergic neurons: ht-HisCl1) appeared normal on NGM-H- plates. When placed on NGM-H+ plates the ch-HisCl1 animals became paralyzed, while the ht-HisCl1 displayed no overt phenotype. In our pre-conditioning paradigm, we find that inactivation of cholinergic activity, but not serotonin activity, for 3.5 h prior to an anoxic insult confers a survival benefit (ch-HisCl1 NGM-H-

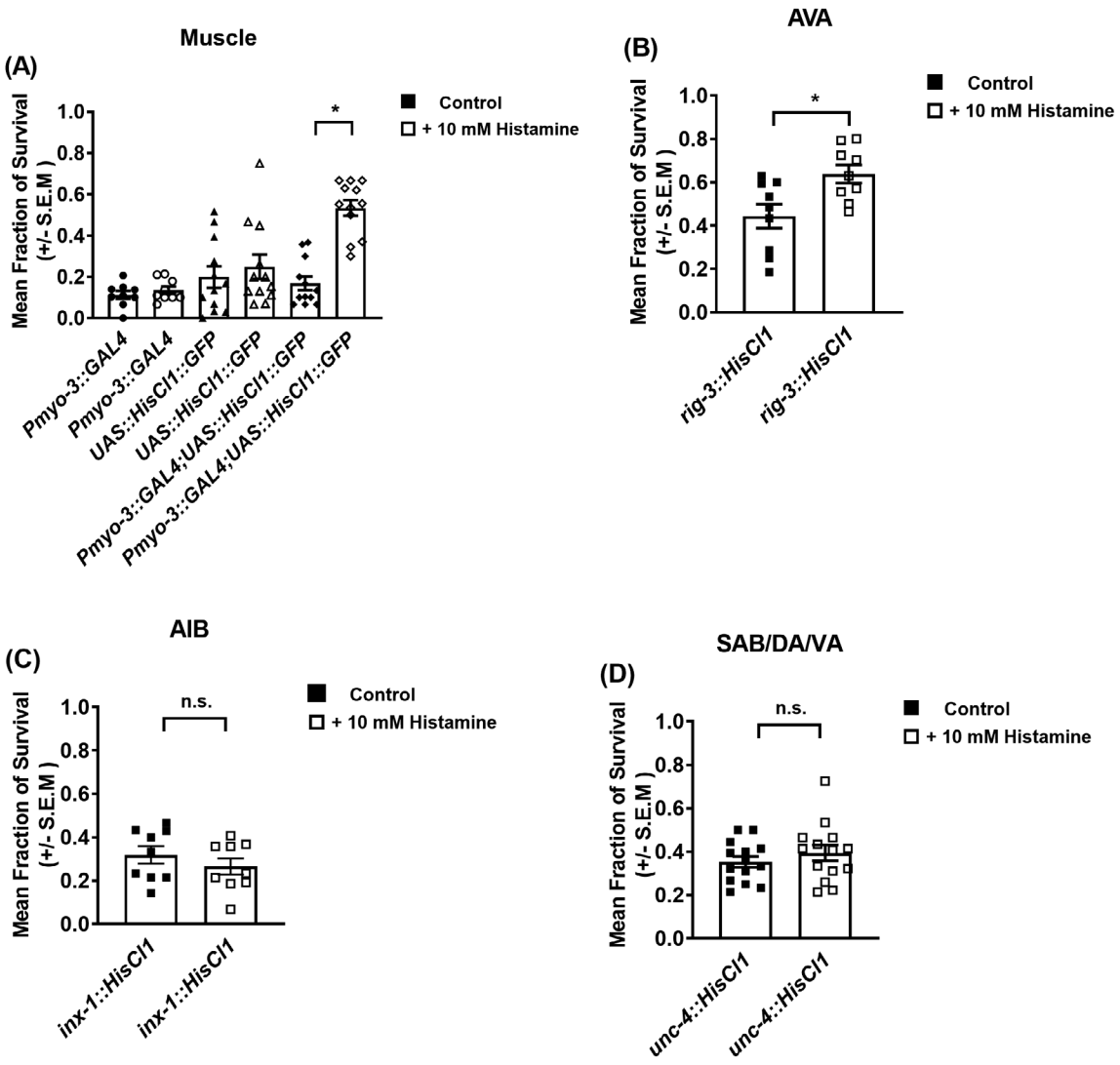

FIGURE 3 Neuromuscular activity mediates the preconditioning effect to anoxia. (A) Inactivation of the muscle prior to anoxic insult has an advantageous effect on survival. Wild type, animals expressing the muscle promoter (*Pmyo-3*) behind the GAL4 sequence, animals carrying the histamine gated chloride channel behind the UAS activated sequence, as well as animals expressing GAL4 under the muscle promoter with the UAS histamine gated chloride channel were selected to 10 mM histamine or control plates as early L4 animals 3.5 h, prior to anoxic stress. Fraction of survival was scored after 24 h. Significance between *Pmyo-3*::*GAL4*:*15xUAS*::*HisCl1*::*SL2*::*GFP* control versus experimental; error bars represent the SEM. Results are shown for four independent trials. (B) Inactivity of command interneurons AVA yields a survival advantage to anoxia. Animals carrying the histamine gated chloride channel behind the *rig-3* promoter were selected as early L4 animals. Controls animals (black filled shapes) and animals exposed to 10 mM histamine, experimental condition, (non-filled shapes) as early L4 animals, prior to 48 h of anoxic stress. Fraction of survival was scored after 24 h. Results are shown for four independent trials. (C) Inactivity of AIB interneuron prior to anoxic insult does not provide a survival advantage. Animals expressing the histamine gated chloride channel behind the *inx-1* promoter were tested, experimental paradigm as in panel C. Results are shown for four independent trials. (D) SAB-DA-VA motor neuron inactivity is dispensable for the preconditioning response to anoxic insult. Animals expressing the histamine gated chloride channel behind the *unc-4* promoter were tested for pre-conditioning response to 48 h of anoxic insult experimental paradigm as in panel C. Results are shown for four independent trials

0.40, ± 0.06 and 0.30, ± 0.10 lines 1 and 2 respectively vs. ch-HisCl1 NGM-H+ 0.72, ± 0.03 and 0.81, ± 0.03 lines 1 and 2 respectively ht-HisCl1 NGM-H- 0.20, ± 0.08 and 0.40, ± 0.10 lines 1 and 2, respectively ht-HisCl1 NGM-H+ 0.19, ± 0.07 and 0.34, ± 0.07 lines 1 and 2 respectively ANOVA $F_{(9,68)}$ = 18.83 *p* value <0.0001, Figure 2A).

Next, we used the bipartite GAL4-UAS system to study other neurochemically defined neuronal populations. [23] Animals containing UAS sequences driving HisCl1 were crossed to animals in which the *unc-47* promoter drives GAL4 to generate animals expressing the HisCl1 in GABAergic neurons (ga-HisCl1). Ga-HisCl1 animals appeared normal on NMG-H- plates, but displayed a severely uncoordinated phenotype, that is, abnormal body wall contraction or defective movement when prodded with the platinum wire pick, when placed on NGM-H+ plates. In our pre-conditional paradigm, we find that loss of GABAergic activity for 3.5h prior to 48 h of anoxia conferred a survival benefit (ga-

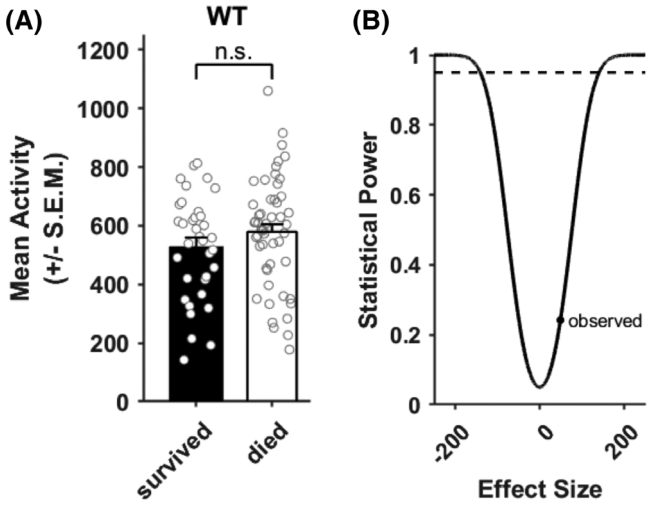

FIGURE 4 Survival is not related to locomotor activity prior to anoxic insult. (A) Activity of survivors and non-survivors prior to anoxic insult. Wild type animals were selected at the early L4 stage and loaded into the "WorMotel" device and assayed for locomotor activity for a 3.5 h period. Dots indicate activity of individual animals. Results are shown for 87 individual animals (35 survivors and 52 non-survivors) from five replicates. (B) Statistical power analysis. The statistical power (probability of rejecting the null hypothesis of no difference in activity between survivors and non-survivors if the null hypothesis were not true) of our experiment for different effect sized based on the variance and sample sizes in panel A is shown (black line) A statistical power of 0.95 is indicated by a horizontal dashed line, and the observed effect size from panel a is indicated by a dot

HisCl1-NGM-H- 0.21 ± 0.04 vs. ga-HisCl1-NGM-H+ 0.50 ± 0.04,-ANOVA $F_{(5,66)}$ = 6.089, p value = 0.0001,Figure 2B).

To generate *C. elegans* with glutamatergic or dopaminergic expression of HisCl1, constructs containing *eat-4*, and *cat-2* promoter regions driving GAL4 sequences, along with plasmids containing the UAS sequences driving the HisCl1were injected into N2 animals to generate extrachromosomal array lines. Four independent lines were generated with each glutamatergic (glu-HisCl1) and dopaminergic (dop-HisCl1) construct. Glu-HisCl1 and dop-HisCl1 animals appeared normal on standard NGM plates that lacked histamine, and displayed no overt phenotype on NGM-H+ plates. We find that loss of neither glutamatergic nor dopaminergic activity for 3.5 h prior to anoxic insult conferred a survival benefit (glu-HisCl1 NGM-H- 0.20 ± 0.04 and 0.29 ± 0.05 lines 1 and 2, respectively vs. glu-HisCl1 NGM-H+ 0.18 ± 0.04 and 0.19 ± 0.01 lines 1 and 2 respectively, ANOVA $F_{(9,74)}$ = 0.5165 p = 0.8582. dop-HisCl1 NGM-H- 0.17 ± 0.04 vs. dop-HisCl1 NGM-H+ 0.23 ± 0.04 ANOVA $F_{(7,46)}$ =*4.551* p = 0.996 Figure 2C,D. respectively). Combined, these results suggest that activity of cholinergic or GABAergic neurons regulate the pre-conditioning response to anoxia.

3.3 | Hyperpolarization of muscle activity preconditions *C. elegans* to anoxia

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

3.4 | The preconditioning response to anoxia is dependent on AVA command interneurons

To further probe the neural circuit regulating the preconditioning response to anoxia, we studied the role of select interneurons. We tested AVA command interneurons first because these neurons facilitate backward locomotion in the animal, receive acetylcholine neurotransmitter input and make connections onto motor neurons. To determine if AVA command interneurons play a role in in mediating the preconditioning response to anoxia, we studied animals expressing HisCl1 under the control of the *rig-3* promoter (AVA-HisCl1).[32] AVA-HisCl1 animals appeared normal on NGM-H– plates and displayed a mild uncoordinated (Unc) phenotype when placed on NGM-H+ plates. Animals with a loss in AVA activity for 3.5 h prior to 48-h anoxic insult had increased survival compared with controls (AVA-HisCl1 NGM-H- 0.44, ± 0.06 vs. AVA-HisCl1 NGM-H+ 0.64, ± 0.04, $t_{(8)}$=*2.971*, p = 0.01, paired t test, Figure 3B).

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

Since AVA interneurons make connections with motor neurons, we asked if inactivity of specific motor neurons prior to anoxia might lead to increased survival. We tested DA and VA motor neurons for several reasons: (a) they innervate dorsal and ventral muscles respectively,[33] (b) they receive cholinergic input,[34] (c) they receive direct input from AVA interneurons to initiate backward locomotion,[35] and (d) AVA hyperpolarization maybe mediated by

inactivation of about 2/3 of cholinergic motor neurons, including classes DA and VA motor neurons through gap junctions.[33,36,37] To determine if DA and VA motor neurons play a role in in mediating the preconditioning response to anoxia, we studied animals expressing HisCl1 under the control of the *unc-4* promoter, which also expresses in three SAB head motor neurons (SAB-DA-VA-HisCl1).[38] SAB-DA-VA-HisCl1 animals appeared normal on standard NGM-H- plates and displayed a weak phenotype when placed on NGM-H+ plates. We find that inactivity of SAB, DA and VA motor neurons in our preconditioning paradigm does not confer a survival after anoxic insult. (SAB-DA-VA NGM-H- 0.35 ± 0.03 vs. SAB-DA-VA NGM-H+ 0.39 ± 0.04, $t_{(13)}$=1.172, p = 0.262, paired t test, Figure 3D).

3.5 | Lack of locomotor activity prior to anoxic insult is not a predictor of survival

Our results show that hyperpolarization of certain population of neurons or muscle prior to anoxic insult either impaired or paralyzed animals, and led to a survival benefit. We asked whether the amount of locomotor activity prior to anoxia could predict survival after an anoxic insult in untreated wild type animals. To this end we monitored spontaneous activity of N2 animals prior to 48-h of anoxic stress using a multi-well imaging platform (WorMotel) and image analysis. Within a population of *C. elegans*, individuals display variations in locomotor activity. We compared the pre-anoxia activity of animals that survived anoxia to those that died. We found only a small, nonsignificant difference of 49.2 activity values between survivors and non-survivors (two tailed t test, p value = 0.22, Figure 4A). Based on the sample size and variance of our dataset, the smallest difference in activity we could have reliably detected (statistical power = 0.95) was 140.8 activity values (Figure 4B). Collectively, these result suggests that, while normal *C. elegans* vary in their spontaneous activity levels, they are operating above the threshold that evokes the preconditioning phenomenon.

4 | DISCUSSION

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

One salient feature of our investigations here and in prior publications (Flibotte et al., 2014 and Doshi et al., 2019)[13,18] is variability of survival after a 48-h anoxic insult. We considered potential sources of this in our isogenic population of *C. elegans* (i.e., modest differences in animal age, number of animals on a plate, prior history of starvation, distance of plate from catalyst that induce anoxic conditions, number of plates in a biobag and age of NGM plates) and none appear to account for the variability. We suspect that natural stochasticity in biological systems may be the underlying source of the variability we see. This is a topic of great interest to the *C. elegans* community [42-46] and an area of active inquiry. Regardless of the source, by undertaking many independent trials and reporting averages we have worked to control for this variability. Using this approach, we believe we can draw valid conclusions despite the unavoidable variability.

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

Early phase cardiac precondition involves the local release of factors such as reactive oxygen or nitrogen species, bradykinin and adenosine.[41,55] In parallel these agents activate several signaling cascades that include Akt, Erk1/2, protein kinase C [56] and lead to the opening of mitochondrial ATP-sensitive potassium channels (K_{ATP}).[19,57] This has been termed the reperfusion injury salvage kinase ("RISK") pathway.[55] The cardioprotective effects are thought to be related to opposition of the mitochondrial permeability transition pore opening by active K_{ATP}. Another pathway that is involved in cardio-protection ("survivor activation factor enhancement") involves TNF- and the JAK/STAT pathway.[58]

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

Several groups have studied the preconditioning phenomena in *C. elegans*. The Crowder group showed that unfolded protein response component IRE-1 (in a pathway independent XBP-1) and GCN-2 (in a pathway independent of phosphorylation of translation factor eIF2α) mediate the pre-conditioning response to hypoxia.[19,20] In addition, they implicated the apoptosis factor CED-4 (also known as *apaf-1*) in a novel mechanism that does not require any other known core apoptosis genes.[20,59] Genetic pathways that regulate energy dynamics have roles in the preconditioning response. The Padilla group showed that survival to anoxia was dependent on the energy sensor AMP regulated protein kinase (AMPK).[59] This same beneficial effect could be

mimicked by exposing animals to the dietary restriction-like state induced by metformin.[59] Work from the Miller group showed that a several hours of fasting blunted protein homeostasis defects evoked by hypoxia and that this involved the insulin/insulin-like growth factor receptor *daf-2* but not its downstream target, *daf-16*.[60] Collectively, these studies provide valuable information about the genetic underpinning of the preconditioning phenomena, however it remains to be determined if these genes work in a single pathway or multiple parallel pathways. In addition, these studies do not provide insights into the cell autonomous versus cell non-autonomous contributions to the preconditioning phenomena.

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

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

5 | CONCLUSION

The role of the nervous system in preconditioning to anoxic insult has not been extensively studied. The neurons and tissues involved in modulating the preconditioning response to anoxia are not known. Our results implicate cholinergic, GABAergic and muscle activity in mediating the preconditioning response to anoxic insult. Our observations raise several questions that should be addressed in the future. What is special about the cholinergic and GABAergic neurons (as opposed to other neurochemically defined neurons that also impact muscle function) that is particularly beneficial to *C. elegans* under standard cultivation conditions? What signals are elaborated by cholinergic and GABAergic neurons and muscle cells that heightens susceptibility of anoxia? Do all tissues respond to these signals or is there a cascade of signal transduction from tissue to tissue? Insight into these issues may bring us closer to harnessing the preconditioning phenomena for therapeutic use.

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AUTHOR CONTRIBUTIONS

Heather L. Bennett and Robert G. Kalb designed the experiments and wrote the manuscript. All authors proofread manuscript. Heather L. Bennett and Patrick D. McClanahan conducted experiments and constructed figures. Heather L. Bennett analyzed all results.

DATA AVAILABILITY STATEMENT

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

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