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Caenorhabditis elegans as a model for studies on quinolinic acid-induced NMDAR-dependent glutamatergic disorders

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Abstract

Quinolinic acid (QUIN) is an agonist of the neurotransmitter glutamate (Glu) capable of binding to N-methyl-D-aspartate receptors (NMDAR) increasing glutamatergic signaling. QUIN is known for being an endogenous neurotoxin, able to induce neurodegeneration. In *Caenorhabditis elegans*, the mechanism by which QUIN induces behavioral and metabolic toxicity has not been fully elucidated. The effects of QUIN on behavioral and metabolic parameters in *nmr-1* and *nmr-2* NMDA receptors in transgenic and wild-type (WT) worms were performed to decipher the pathway by which QUIN exerts its toxicity. QUIN increased locomotion parameters such as wavelength and movement amplitude medium, as well as speed and displacement, without modifying the number of body bends in an NMDAR-dependent-manner. QUIN increased the response time to the chemical stimulant 1-octanol, which is modulated by glutamatergic neurotransmission in the ASH neuron. Brood size increased after exposure to QUIN, dependent

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Tássia Limana da Silveira: conceptualization, methodology, data curation. investigation and formal analysis.
Marina Lopes Machado: methodology.
Fabiane Bicca Obetine Baptista: methodology.
Débora Farina Gonçalves: methodology and validation.
Diane Duarte Hartmann: methodology.
Larissa Marafiga Cordeiro: methodology.
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Cristiane Lenz Dalla Corte; validation.
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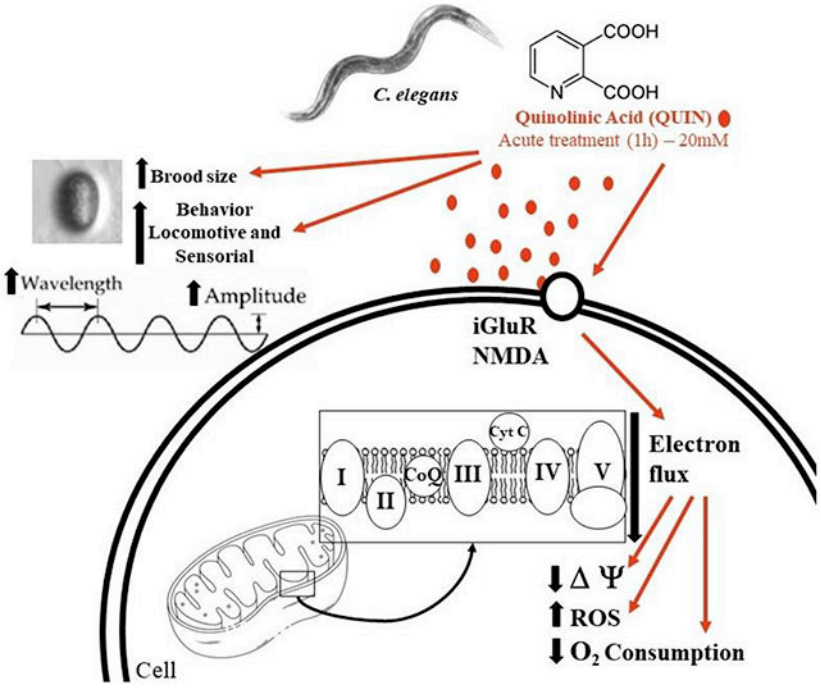
Conflict of Interest

The authors declare that they have no conflict of interest.

upon *nmr-2*/NMDA-receptor, with no change in lifespan. Oxygen consumption, mitochondrial membrane potential, and the flow of coupled and unbound electrons to ATP production were reduced by QUIN in wild-type animals, but did not alter citrate synthase activity, altering the functionality but the mitochondrial viability. Notably, QUIN modified fine locomotor and chemosensory behavioral parameters, as well as metabolic parameters, analogous to previously reported effects in mammals. Our results indicate that QUIN can be used as a neurotoxin to elicit glutamatergic dysfunction in *C. elegans* in a way analogous to other animal models.

Graphical abstract



Keywords

kynurenine pathway; glutamatergic system; neurotoxin; excitotoxicity; NMDA

1. Introduction

Quinolinic acid (QUIN) is a metabolite of tryptophan degradation in the kynurenine pathway (KP) and is responsible for numerous biological processes, primarily in neurons. QUIN is an intermediary metabolite to NAD^+ production [1, 2], an agonist of the N-methyl-D-aspartate receptors (NMDAR) [3], and is production in cellular signaling in microglia and macrophages in the initial defense stage against pathogens. In addition, at high concentrations, QUIN can elicit neurotoxicity inherent to several neuropathologies, such as Alzheimer's disease [4], Huntington's disease [5], and seizures [6].

The mechanisms by which high concentrations of QUIN cause toxicity are multiple. Among them is hyperstimulation of NMD A receptors [7], increased release of glutamate (Glu) by

neurons, as well as the reduction of its uptake by astrocytes [8, 9]. In addition, QUIN can deregulate the glutamate-glutamine cycle by inhibiting the activity of glutamine synthase, leading to increased concentration of Glu in astrocytes, as well as the synaptic cleft [10]. High levels of Glu can increase cytosolic Ca^{2+} and induce free radicals generation, such as reactive oxygen species (ROS) [11]. Higher levels of ROS may inhibit complex II of the electron transport chain and consequently deplete ATP [12], activating the caspase pathway [13, 14] and inducing cell death [15].

QUIN-induced toxicity has been studied for decades in several models, such as cell cultures [16], rodents [12, 17, 18], and more recently in Zebrafish [19] and *C. elegans* [20–22], but many knowledge gaps remain. In our recent study in *C. elegans*, we found that QUIN at high concentrations induced mortality, yet at non-lethal concentrations, it triggered behavioral changes associated with glutamatergic excitotoxicity, as well as increased ROS generation [20].

Nonetheless, little is known about QUIN toxicity in *C. elegans*, as well as the mechanisms by which QUIN mediates toxicity. In mammals, hyperstimulation of NMDA receptors has been documented through activation of the glutamatergic pathway, specifically the NR2A and NR2B receptor subunits [3, 23, 24]. Although *C. elegans* has a relatively simple nervous system, the nematode expresses three Glu metabotropic receptors (mGluRs) and ten Glu ionotropic receptor subunits (iGluRs) with Glu-controlled chloride ion channels [25, 26]. Among the *C. elegans* iGluRs, two NMDA-type subunits are NMR-1 and NMR-2, which are homologous to mammalian NR1 and NR2A subfamilies, respectively [27, 28]. Furthermore, we reported that QUIN-induced ROS generation in *C. elegans* is dependent on the NMDA receptor [20] and described that the SKN-1 pathway plays an important role in QUIN detoxification [29]. As in mammals, glutamatergic neurotransmission in *C. elegans* modulates diverse behaviors as the response to sensory, chemical, and mechanosensory stimuli [30, 31], as well as locomotor parameters, such as spontaneous locomotion [32] and forward and reversal movements [28], among others.

Behavioral changes may reflect alterations in animal metabolism, like in rats with mitochondrial dysfunction that showed alterations in a locomotion standard [33]. Still, these modifications can also be related to the physiology and function of the neurons that control them [34, 35]. In addition, it has been reported that the increased intracellular calcium influx generated by 300 nmol QUIN in rats alters mitochondrial metabolism by decreasing the activity of mitochondrial complexes [18, 36], leading to neurotoxicity and cell death [37]. Additionally, a recent study showed that QUIN increased brood size by increasing nucleotide synthesis [22], likely by increasing intracellular availability QUIN.

Accordingly, in the present study, we addressed the effects of QUIN on the glutamatergic system and worms' behavior, such as sensory and fine locomotive patterns, egg production, lifespan, and the effects on the mitochondrial metabolism of worms, after only QUIN insult, like what occurs in convulsive events in mammals [38].

2. Materials and Methods

2.1. *C. elegans* strains, maintenance, and treatment

The WT strain N2 and mutant strains VM487 [*nmr-1*(ak4)] and VC2623[*nmr-2*(ok3324)] were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA).

For all worms, pregnant hermaphrodite animals were synchronized through a bleaching protocol using bleaching solution (1% NaOCl, 0.25 M NaOH) that consists of breaking the animals cuticle, releasing the eggs in the middle by isolating embryos from gravid hermaphrodites. Eggs were left in M9 buffer for overnight to allow all viable eggs to hatch and reach the first larval stage L1. After, the L1 worms was transferred to NGM (Nematode Growth Medium) seeded with *Escherichia coli* OP50 as a food source, at 20°C until the worms achieve the young adulthood stage at a temperature of 20° C [39].

The exposition acutely to 20 mM QUIN or vehicle (M9 buffer) was performed using worms at the young adult stage for 1 hour in plates containing NGM and OP50 according to the protocol established in our group [20]. After this, all assays were performed.

2.2. Behavior analysis

2.2.1. Wormlab® behavioral evaluation—Well-fed worms were transferred to a food-free NGM plate and allowed to freely move. After 30 s adaptation, the worms were recorded using HiROCAM MA88-500 5.0 Megapixels 2592 x 1944 resolution camera, containing 1.5 frames/s, during 1 minute in grayscale. The videos were later analyzed using the Wormlab® software (Version 2.0.1, MBF Bioscience, Williston, VT) by the skeletonized capture, where more than one point in the worm is evaluated.

Wormlab® is a software that allows image tracking and analysis to collect animal data from videos and images, enabling automatization of the data search. The analyses performed focused on morphological parameters such as mean length, mean width, mean area, wavelength, mean amplitude, and max amplitude of the in one wavelength and parameters of locomotion as the displacement (track length), speed, traveled straight-line distance, distance traveled forward, distance traveled in reverse, the number of reversals, body bends, omega, cumulative reversal time and cumulative forward time. Approximately 35 worms were analyzed individually in three independently performed experiments.

2.2.2. Octanol response—Response to 1-Octanol was carried out as previously described with minor modifications [40, 41]. After QUIN exposure, worms were transferred to non-OP50-seeded NGM plates and allowed to adapt for 5 min. After 5 min, 1-Octanol was presented to a forward-moving animal via a glass capillary that was dipped in 30% 1-octanol solution (dissolved in 100% ethanol, v/v), and the latency time to backward movement was counted [42]. Five worms were used in each experiment, and the assays were independently performed a minimum of four times.

2.3. Brood size and laying

After QUIN-exposure, the worms were picked up one by one from the treatment plate and transferred to new NGM plates seeded with *E. coli* in the center plate in absence of QUIN. Worms were individually kept on plates and transferred each day to new NGM plates seeded with *E. coli* in the center of the plate until the 5th day of adulthood when egg production is dramatically reduced. At least 10 worms were used in each experiment, and the assays independently performed a minimum of three times [43].

2.4. Oxygen consumption

The oxygen consumption was performed as previously described with few modifications [44]. *C. elegans* were collected from QUIN-exposure or control plates and washed three times with M9 buffer to remove all bacteria. Approximately 300 alive worms were transferred to the Oroboros Oxygraph-2K chamber and the volume was completed with M9 buffer to 2 mL. The oxygen consumption was measured over 40 minutes. Approximately 10 analyzes were performed at 4 different times.

2.5. Mitochondrial membrane potential determination

The mitochondrial membrane potential assays were performed according to previously described methods [45, 46] with few adaptations for the *C. elegans* model. Worms treated or not with QUIN were washed three times with M9 buffer and approximately 10.000 worms were transferred for conical tube 1.5 mL and the animals were sonicated on ice in an ultrasonic homogenizer for approximately 15 minutes in an interval of 30 seconds in each session. Both animals, treated or not with 20 mM QUIN .

For the mitochondrial membrane potential, safranin O (2 mM) and the respiratory substrates Glu (1.5 mM) and succinate (150 mM) were incubated with approximately 2 μg protein/mL of *C. elegans* homogenate in the bucket of fluorimeter.

The medium was kept at constant stirring during the assay period. The variation between the absorbance values before the addition of DDNP and 40s after was corrected with an absorbance safranin curve; and converted to percentage of the untreated WT mean control. The test was performed in duplicates and repeated three different times. The fluorescence analysis was performed at 495 nm for excitation and 586 nm for emission, with slit widths of 5 nm [47].

2.6. Mitochondria Isolation

To isolate functional mitochondria, we used a protocol previously described with minor modifications [48]. Approximately 10.000 adult nematodes per group were treated with or without 20 mM QUIN, washed three times, and posteriorly were sonicated using the same protocol performed in mitochondrial membrane potential and posteriorly the homogenate was centrifugated at 800 G for 5 minutes at 4° C. The resultant supernatant from the first centrifugate was transferred to a new conical tube 1.5 mL and centrifugated at 9000 G for 10 minutes at 4° C. Next, the supernatant was discarded and the pellet contained the mitochondria enriched medium was resuspended in 200 μL of mitochondrial respiration medium MirO5 (0.5 mM EGTA, 3 mM MgCl2, 60 mM lactobionic acid, 20 mM taurine, 10

mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/mL fatty acid-free BSA, developed by Oroboros) for high-resolution respiratory experiments [1]. Afterwards, high-resolution respirometry was performed using fresh mitochondria preparations

2.7. High-resolution Respirometry

A High-resolution Respirometry was performed according to the previously described method [48–50] with few adaptations for *C. elegans* model (O2k Oxygraph, Oroboros Instruments, Innsbruck, Austria). For each measurement, an aliquot of 200 µL mitochondria enriched medium in MirO5 was inserted into the electrode chamber. The protocol used is known as SUIT protocol, consisting of a sequential titration of multiple substrates, uncouplers, and inhibitors. The experiments were initiated after signal stabilization and the following were added sequentially: pyruvate (5 mM), malate (2 mM) and Glu (10 mM) (Leak stage); ADP (5 mM) (OXPHOS CI stage); succinate (10 mM) (OXPHOS CI and CII stage); oligomycin (2.5 µM) (LEAK stage - ATP synthase inhibitor); carbonyl cyanide-4-(tri-fluoromethoxy) phenylhydrazone (FCCP - titrations of 0.25 µM until maximal oxygen consumption was reached) (ETS CI and CII - mitochondrial decoupler); rotenone (0.5 µM) (ETS CII - CI inhibitor); malonate (5 mM) (ETS CIII - CII inhibitor) and antimycin (2.5 µM) (ROX - CIII inhibitor). For analysis, the provided DatLab software (Version 7.0.0.2, Oroboros Instruments, Innsbruck, Austria) was used. All data related to the SUIT protocol were normalized with citrate synthase activity in each sample [51, 52]. The assays were independently performed a minimum of five times.

2.8. Citrate-Synthase Activity

For determination of citrate synthase activity, 2 mL of suspension was removed from the Oxygraph-2k chamber at the end of each experiment and stored at −80 °C until further analysis. The citrate synthase activity was assessed spectrophotometrically at 412 nm, recording the linear reduction of 0.1 mM 5,5’dithiobis-2-nitrobenzoic acid (ε 412: 13.6 ml·cm-1·µmol-1) in the presence of 0.10 mM acetyl-CoA, 10 mM oxalacetic acid and 0.1 M Tris/HCl, (pH 8.1) at 37 °C for 6 minutes [49, 53]. The assay was independently performed a minimum of three times.

2.9. Protein determination

The protein content was determined according to Bradford’s test [54] using bovine serum albumin (BSA) as standard performed. After acute exposure to QUIN or control, animals were washed three times using M9 buffer, and sonicated in 1.5 mL conical tubes as performed in the membrane potential determination assay. Then, the samples were centrifuged for 30 min at 15,000 × g, and the supernatants (lysate) collected. The samples are transferred to read plates e was measured using a plate reader in SpectraMax i3 (absorbance 596 nm).

2.10. Lifespan

Lifespan analyses started at the L4 larvae stage in NGM plates seeded with *E. coli* OP50 in the absence or presence of 20 mM QUIN (day 0). Animals were individually transferred each two days to fresh new NGM plates (seeded with *E. coli*), and the worms that did not

respond to a mechanical stimulus of an eyelash was scored as death. Worms that crawled off the plate were censored. Lifespan assays were repeated three times with around 60–120 worms per assay [55, 56].

2.11 Statistical analysis

Statistical analysis was performed using *GraphPad* (Version 6.0, San Diego, CA). Significance was assessed using t-tests or two-way ANOVA, followed by Tukey's Test for *post hoc* comparison. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1. QUIN did not alter body parameters but changed the offset curvature parameters

We demonstrate that 20 mM QUIN failed to alter the body length, width, and area of WT animals and knockouts of NMDA receptors subunits *nmr-1* and *nmr-2* (Table 1). However, 20 mM QUIN increased the wavelength (~26.5%) and mean amplitude (~20.2%) of the body curvature in WT worms, but not in *nmr-1* and *nmr-2* knockout worms (Table 1).

3.2. QUIN increased parameters related to movement in an NMDA receptor-dependent manner

WT animal treated with 20 mM QUIN showed an increase in travel speed (~23.1%); Fig. 1A), track length (~18.8%; Fig. 1B), straight-line distance (~120%; Fig. 1C), distance traveled forward (~25.3%; Fig. 1D), and in reversal (~63.6%; Fig. 1E), but not in the cumulative forward (Fig. 1F) and reversal (Fig. 1G) time.

Both knockout strains for NMDA receptors treated with 20 mM QUIN had no significant difference from their respective control groups in speed travel (Fig. 1A), track length (Fig. 1B), straight-line distance (Fig. 1C), and distance traveled forward (Fig. 1D). In addition, both strains had decreased traveled distance in reversal (Fig. 1E), and the *nmr-2* knockout showed decreased cumulative forward time (Fig. 1G). Moreover, both NMDA receptor knockout strains showed an increase in the cumulative forward time (Fig. 1F) compared to their respective controls. In addition, the 20 mM QUIN failed to alter body bends, the number of reversals, and omega curvature in WT animals, or knockout to NMDA receptors subunits *nmr-1* and *nmr-2* (data not shown).

3.3. QUIN reduced time response to 1-Octanol

WT worms treated with 20 mM QUIN displayed reduced time-response to 1-octanol compared to the control group. This effect was absent in *nmr-1* and *nmr-2* mutant worms after treatment with 20 mM QUIN compared to its respective control groups (Fig. 2).

Despite this, it was also possible to observe that knockout animals for both NMDA receptor subunits in basal situations present a delay in response to 1-octanol compared to WT control animals, which was not worsened by the acute exposure to QUIN.

3.4. The brood size increased with QUIN treatment is *nmr-2* dependent in *C. elegans*.

Treatment acutely with 20 mM QUIN increased the number of eggs produced over the worms' life in WT (~10%) animals and the *nmr-1* (~13.2%) knockout (Fig. 3A). However, in the *nmr-2* knockout animals, 20 mM QUIN did not affect brood size (Fig. 3A).

When we evaluated the number of eggs produced each day, WT worms treated with 20 mM QUIN showed increased egg production only on the second day (~20.5%) compared to the control untreated group (Fig. 3B). In *nmr-1* knockout animals, treatment with 20 mM QUIN increased egg production on the first (~36.2%), third (~25.5%), and fourth day (~400%) (Fig. 3B) in comparison to their respective control groups. However, in *nmr-2* knockout animals, treatment with 20 mM QUIN decreased egg production on the second day (~40.4%) while increasing it on the third day (~31.4%) in comparison to their respective control groups, which recovered on the third day (Fig. 3B).

3.5. QUIN acute treatment failed to alter survival in *C. elegans*

WT and *nmr-1* knockout worms treated with 20 mM QUIN did not display an altered mean and maximal lifespan compared to their respective controls. However, in *nmr-2* worms, 20 mM QUIN decreased the maximal but not the medium lifetime compared to their respective control groups (Fig. 4 and Table 2).

3.6 QUIN affect oxygen consumption and mitochondrial membrane potential in *C. elegans*

Oxygen consumption was reduced in WT (~55.7%) and *nmr-2* animals (~29.8%) treated with 20 mM QUIN compared to their respective control groups (Fig. 5A). Twenty millimolar QUIN did not alter oxygen consumption in *nmr-1* worms (Fig. 5A). A similar effect, as we found in oxygen consumption, was observed in membrane potential in WT animals after treatment with 20 mM, except in *nmr-1* worms where 20 mM QUIN also induced a decrease in membrane potential (Fig. 5B).

The animals remained for the same time in the sonication process, with no changes in the integrity of the cuticle and membrane of the animals treated with QUIN compared to the control.

3.7 QUIN decreased mitochondrial functionality but not viability

Treatment with 20 mM QUIN did not alter citrate synthase activity in WT worms, suggesting that mitochondrial viability was uncompromised (Fig. 6A). Nonetheless, when we evaluated the electron flow in the electron transporter chain, we observed reduced electron flow in oxidative states (OXPHOS) and electron flow independent of ATP production (ETS) in WT worms treated with 20 mM QUIN (Fig. 6B). This result established that QUIN induced a decrease in the electron flow in ETS and OXPHOS states evaluated in the SUIT protocol in *C. elegans* compared to WT control animals (Fig. 6). The electron transporter chain data was converted to percentual of control, being means to all data of each control used to convert the data in each complex. This was necessary because the data found in this analysis of the electron transporter chain are discrete, and this way helps us to visualize the effect.

4. Discussion

In this novel study, we evaluated the QUIN acute exposure inducing toxicity on the glutamatergic system and metabolism of the nematode, *C. elegans*. We showed that QUIN increased reactive oxygen species (ROS) generation and induced neurotoxicity in *C. elegans* affecting analogous pathways inherent to mammals [20]. Our data indicate that acute QUIN failed to affect bodily measures of the worm yet had prominent effects on locomotion and sensory parameters, which were mediated by NMDAR [57, 58]. Moreover, QUIN altered brood size and mitochondrial metabolism, suggesting that QUIN induced toxicity in *C. elegans* by perturbing glutamatergic neurotransmission in an NMDAR-dependent manner [59, 60].

Recent studies have shown that sensory glutamatergic neurons modulate locomotion, as reflected by increased track length, both in zebrafish and mammals [61–63]. Locomotion is primarily modulated by acetylcholine (ACh) and gamma-aminobutyric acid (GABA) [64, 65]. Nonetheless, Glu plays a crucial role in the mechanosensory response mediated by the sensory neuron ASH [32]. Moreover, several locomotion-related behaviors in *C. elegans* are mediated by Glu, such as backward movement and reversal [66, 67]. Here, we showed that body wavelength (Fig. 1A) and mean amplitude (Fig. 1B) were increased in WT worms exposed to QUIN and that a knockout of NMDA subunit receptors led to a decrease in these parameters upon QUIN exposure (Fig. 1A, 1B). QUIN also increased the speed, track length, and straight-line distance in WT worms, and these effects were abolished in *nmr-1* or *nmr-2* knockouts (Fig. 1C). Corroborating these effects, we established that in WT animals, QUIN significantly increased the amplitude and the wavelength of the body curvature, inducing increased speed and track length [68]. Notably, it is well established that for larger wavelengths, it is necessary to use dorsal and ventral muscles, facilitating displacement [69], and that when worms engage in swimming, their wavelength can be twice as long as the body length [70, 71].

Locomotive parameters, such as reversal of locomotion, are also modulated by Glu transporters like GLT-1. The absence of this transporter induces an increase in glutamatergic signaling and consequently increases the number of reversals [72]. Here, we found that QUIN increased the distance traveled in reverse in WT worms (Fig. 1E), absent a significant effect on the number of body bends, reversals, omega (data not shown), and the time traveled in reversal (Fig. 1G). The same effect was observed in distance and time traveled forward (Fig. 1D and 1F). However, when we evaluated these same behaviors in *nmr-1* and *nmr-2* knockouts, the effect was abolished or inverted (Fig. 1). These findings suggest that QUIN increased the displacement in general by increasing the wavelength and mean amplitude of movement without changing the number of body bends.

In addition to mechanosensory behaviors, Glu has an important role in chemosensory behavior [30, 42]. The ASH neurons are a pair of sensory neurons present in the head of the worm, and it performs import synapsis with some interneurons, which express in your membranes the ionotropic Glu receptors, such as NMR-1 and NMR-2 [73]. The 1-octanol response is a chemosensory behavior regulated by the ASH neurons and dopamine [30, 74]. Furthermore, NMR-1 is required for dopamine modulation in the 1-octanol response,

and the absence of NMR-1 increases 1-octanol response time in *C. elegans* [30]. Our results showed that QUIN decreased the response time in WT worms but not in knockout NMDA receptor strains (Fig. 2), establishing a role for the NMDA receptor in regulating glutamatergic neurotransmission and its overactivation by QUIN.

In basal metabolism, it is known that QUIN is produced endogenously, being an intermediate metabolite in NAD^+ *de novo* synthesis. The NAD^+ *de novo* synthesis is conserved in *C. elegans*, with worms expressing all enzymes of the kynurenine pathway, except for quinolinic acid phosphoribosyltransferase (QPRTase), an enzyme that is responsible for the synthesis of NAD^+ from QUIN in mammals [75]. In *C. elegans, de novo* synthesis of NAD^+ depends on the concentrations of intermediates in this pathway, such as QUIN. However, uridine monophosphate phosphatidyl transferase (UMPS), which acts in pyrimidine biosynthesis, a nitrogenous base that makes up the nucleotides, is the one that biosynthesizes NAD^+ instead of the uncoded QPRTase [22]. It is known that UMPS is directly involved in cell division and worm reproduction [22].

We found that QUIN exposure increased the brood size in *C. elegans* in an *nmr-2*-dependent manner, suggesting that this subunit of the NMDA-type receptor is necessary for the hyperstimulation of these receptors by QUIN, as well as the intracellular signaling cascade generated by this neurotoxin. Nonetheless, we did not observe any effect of QUIN on the mean lifetime of worms, suggesting that QUIN could not reduce the total lifespan (Fig. 4). This could be explained by the fact that a single acute QUIN exposure is of insufficient magnitude to reduce lifespan. Notably, another study has shown that acute exposure to toxic compounds, such as 3-nitropropionic acid and QUIN, failed to reduce lifespan despite the toxic effects generated in worms [21]. Still, some pathological events like a convulsive event or a mild trauma not necessarily induced a reduction in lifespan, although it can alter the health span by causing some kind of mental confusion, locomotive disturbance, and oxidative stress [33, 76].

To determine if QUIN exposure affects worm metabolism, we evaluated oxygen consumption, mitochondrial membrane potential, and respiratory chain capacity. We found that QUIN altered both oxygen consumption (Fig. 5A) and membrane potential (Fig. 5B) in WT worms, but this effect was absent in NMDA-receptor knockout animals (Fig. 5A and B). This may reflect the fact that for the oxygen consumption assays, we used intact worms, and for the studies addressing membrane potential, we used a medium enriched with fresh mitochondria obtained by breaking the cuticle of the worms. Similar effects generated by different sample preparations occur with other methods used in *C. elegans*, such as the quantification of ROS with 2.7-dichlorodihydrofluorescein diacetate (H2DCFDA). However, in these methods, the values obtained from fluorescence in intact animals are considerably more discrete, depicting minor differences between the groups than those found in homogenate worms [77].

Hyperstimulation of NMDA receptors by QUIN induces increased intracellular Ca^{2+} levels, triggering mitochondrial dysfunction and loss of mitochondria membrane potential, in turn altering energy production and redox homeostasis and consequently causing cell death [78–80]. Our findings in mitochondrial alterations likely reflect decreased oxygen consumption

and the membrane potential secondary to QUIN treatment, in contrast to other studies utilizing other model neurotoxins, where increased oxygen consumption, and consequently, a reduction in membrane potential has been noted [49, 78]. This suggests that QUIN toxicity is dependent on the NMDA receptor, analogous to mammals [12, 15, 81], and is directly linked to the expression of the *nmr-1* subunit.

To better understand our findings, we assessed whether the QUIN-induced reduction in oxygen consumption and mitochondrial membrane potential was correlated with mitochondrial viability or whether these effects were linked to mitochondrial functionality. The viability of mitochondria can be measured by the activity of citrate synthase, as it is known that upon reduction or loss of this enzyme's activity, a decrease in substrates available for the electron transport chain ensues, and consequently, a decrease in electron flow [82]. Citrate synthase metabolizes oxaloacetate into citrate, thus providing a requisite substrate for the electron transporter chain (ETC) [53, 83]. We found that QUIN did not alter the activity of citrate synthase (Fig. 6A) in WT worms, suggesting that the substrate concentration available to the electron transporter chain was not affected by this neurotoxin. Further, we found that QUIN reduced mitochondrial functionality by decreasing the electron flow in the OXPHOS and ETS stages (Fig. 6B). In OXPHOS, acute QUIN exposure decreased the electron flow in the leak stage and complex I, where pyruvate, malate, and Glu were added (flow independent of the ADP). This could have triggered a reduction in the flow of all electrons considering OXPHOS CI and OXPHOS CII. The addition of oligomycin (OMY, an ATP synthase inhibitor) to the assay induced maximum electron flux in mitochondria in the absence of ATP synthase activity, thus the flux being independent of ATP production, stages are now called ETS. At this stage of the analysis, we observed that QUIN still reduced the electron flow capacity in all ETS stages tested, as well as in ROX, a residual electron flow stage upon the inhibition of all complexes. Different ETS stages can be observed via mitochondria decoupling induced by the carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Fig. 6B). Taken together, our data indicated that QUIN altered basal metabolism in worms, inducing mitochondrial dysfunction.

In addition to aerobic metabolism, it is known that in flatworms and nematodes, anaerobic metabolism may occur and that it is related to a rhodoquinones pathway, which is absent in mammals [84]. Rhodoquinone is used as a mechanism for survival in environments with low oxygen concentrations, and it is activated in the absence of oxygen as the final electron acceptor in the transport chain, where fumarate is reduced to succinate using electrons from the hydroxyrhodoquinone molecule, which is oxidized generating rhodoquinone [85]. Recent studies have shown that the production of rhodoquinone is dependent on metabolites of the kynurenine degradation pathway, such as tryptophan and 3-hydroxykynurenine [86]. Thus, exposure to QUIN might be activating anaerobic metabolism in *C. elegans* and consequently would lead to a reduction in oxygen consumption as well as in the flow of electrons in the ETC. However, these studies also showed that other metabolites produced downstream in this pathway did not influence the production of rhodoquinone [85, 86]. Thus, we speculate that the reduction observed here in ETS and OXPHOS in response to QUIN is not secondary to a shift from aerobic to anaerobic metabolism, as QUINS is not a metabolite that induces the production of rodoquinone, essential for the activity of the anaerobic route in *C. elegans*.

Our novel results established the propensity of QUIN to alter mitochondrial membrane and oxygen consumption, thus compromising the mitochondrial proton pump, and in turn, reducing the flow of electrons along the ETC, and consequently, the need for oxygen consumption. The findings in worms are analogous to those previously reported in rodents, where acute exposure to QUIN reduced the activity of all mitochondrial complexes [18].

5. Conclusion

In this work, we showed that exposure to QUIN induced NMDA-type excitotoxic effects associated with altered motor behavioral and metabolic parameters, reinforcing the utility of the nematode as an invaluable tool for future studies on glutamatergic dysfunction in *C. elegans*.

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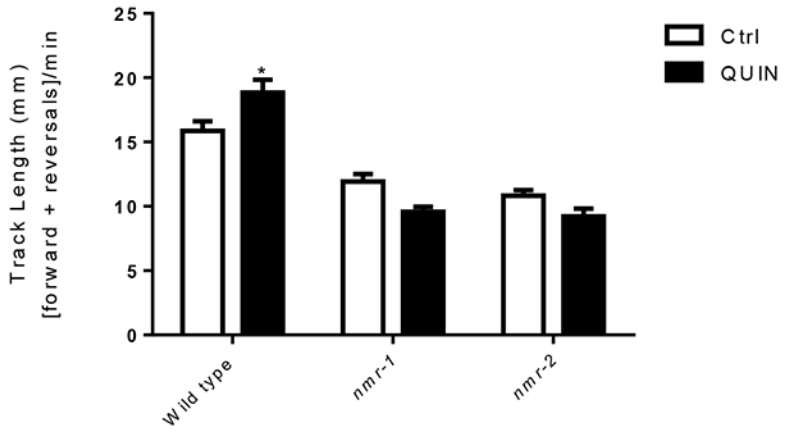
Highlights

- QUIN changes locomotor parameters in *C. elegans*.
- QUIN improve brood size, but not affect the lifespan in *C. elegans*;
- QUIN reduces mitochondrial metabolism in NMDA-dependent *C. elegans*.
- QUIN can be used in *C. elegans* as a model of glutamatergic excitotoxicity.

A)



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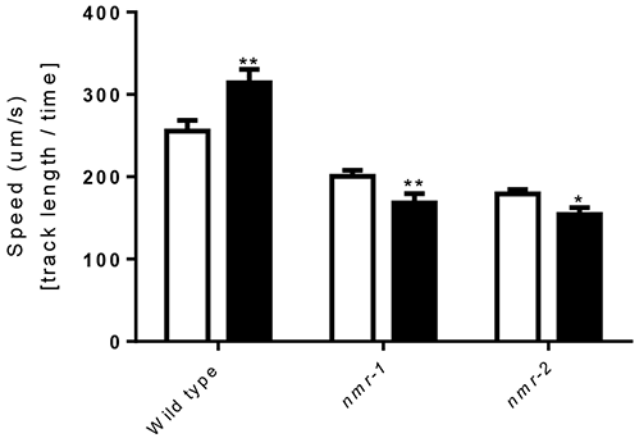

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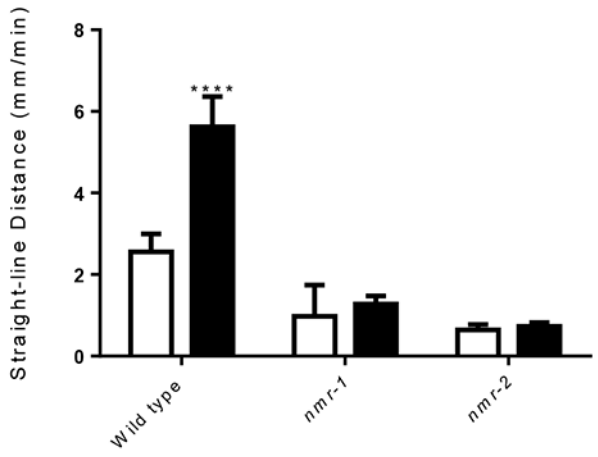

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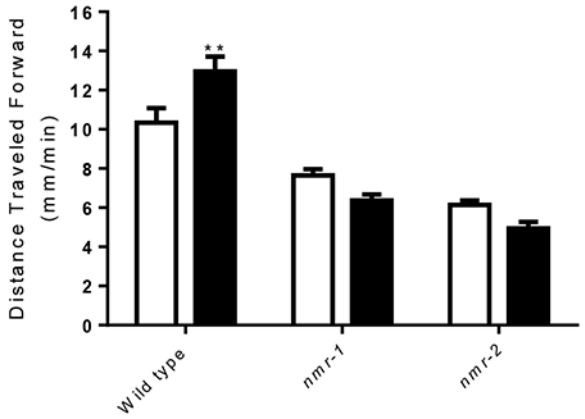

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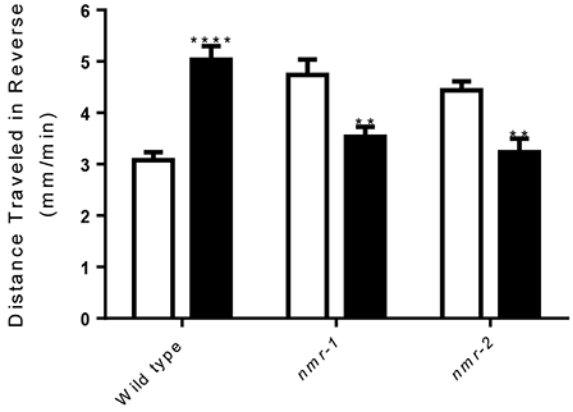

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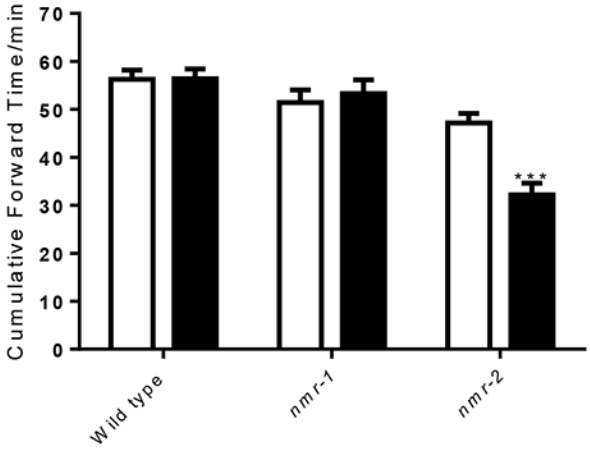

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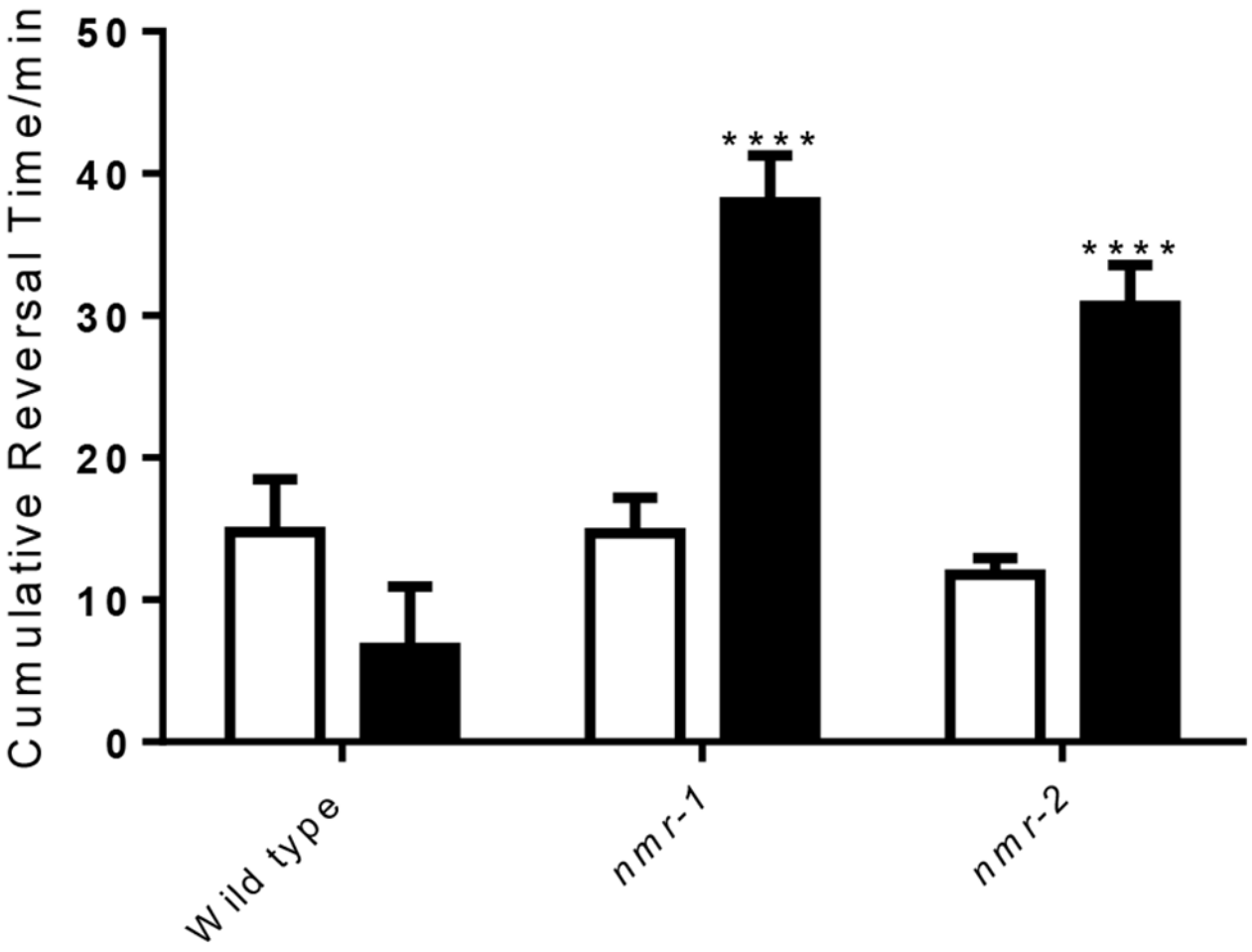

Figure 1. Effect of QUIN in locomotion parameters of the worms.

WT N2, VM487 (*nmr-1)* and VC2326 (*nmr-2*) mutant worms treated or not with 20 mM QUIN in A) track length. B) speed, C) straight-line distance, D) distance traveled forward, E) Distance traveled in reverse, F) cumulative forward time, G) cumulative reversal time. Data are expressed as latency of response to 1-Octanol derived from 3 independent assays (n = 10-23). Error bars are represented as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 compared to each untreated group (Test-t).

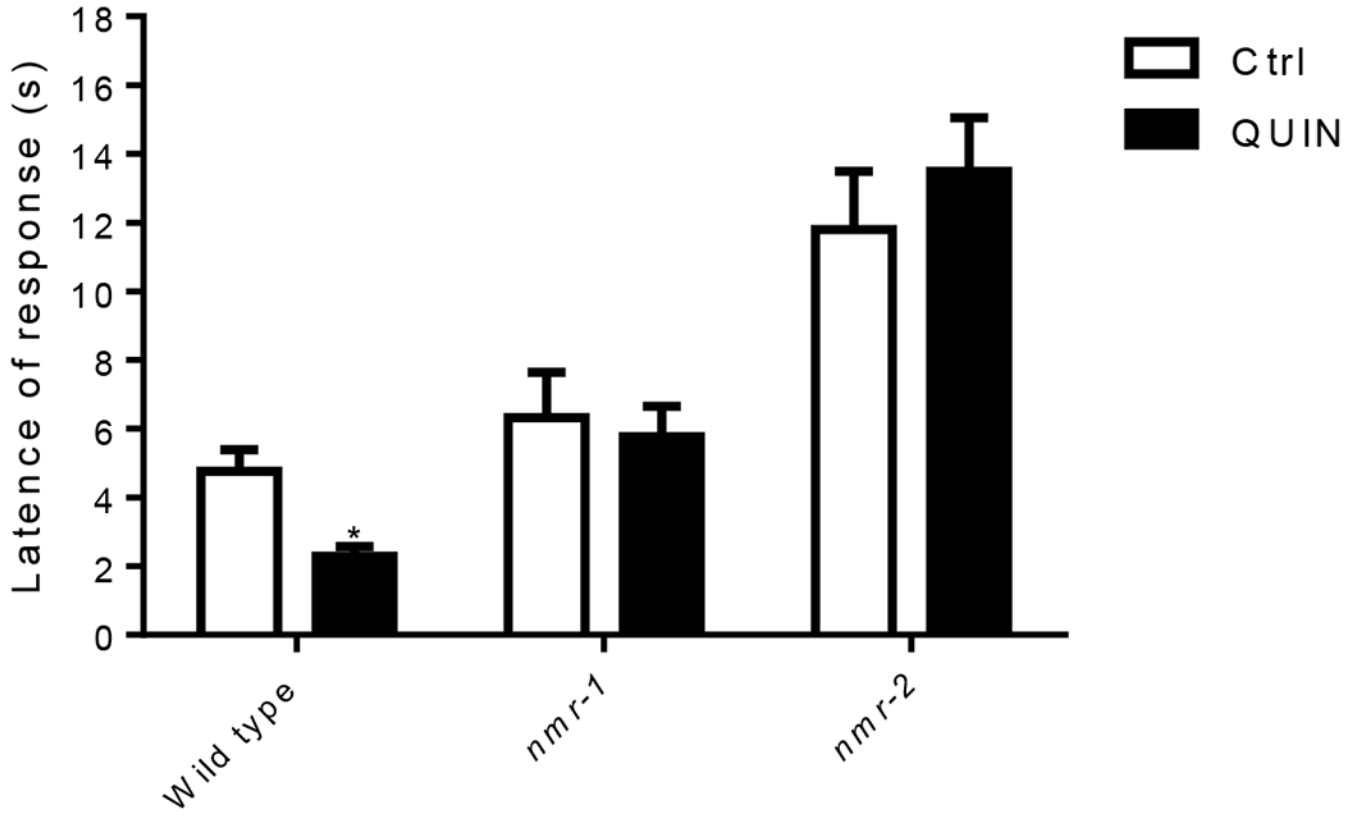

Figure 2. Effect of QUIN to 1-Octanol response of the worms.

WT N2. VM487 (*nmr-1)* and VC2326 (*nmr-2*) mutant worms treated or not with the 20 mM QUIN. Data are expressed as latency of response to 1-Octanol derived from 3 independent assays (n = 10-23). Error bars are represent as mean ± S.E.M. *p < 0.05 compared to each untreated group (Test-t).

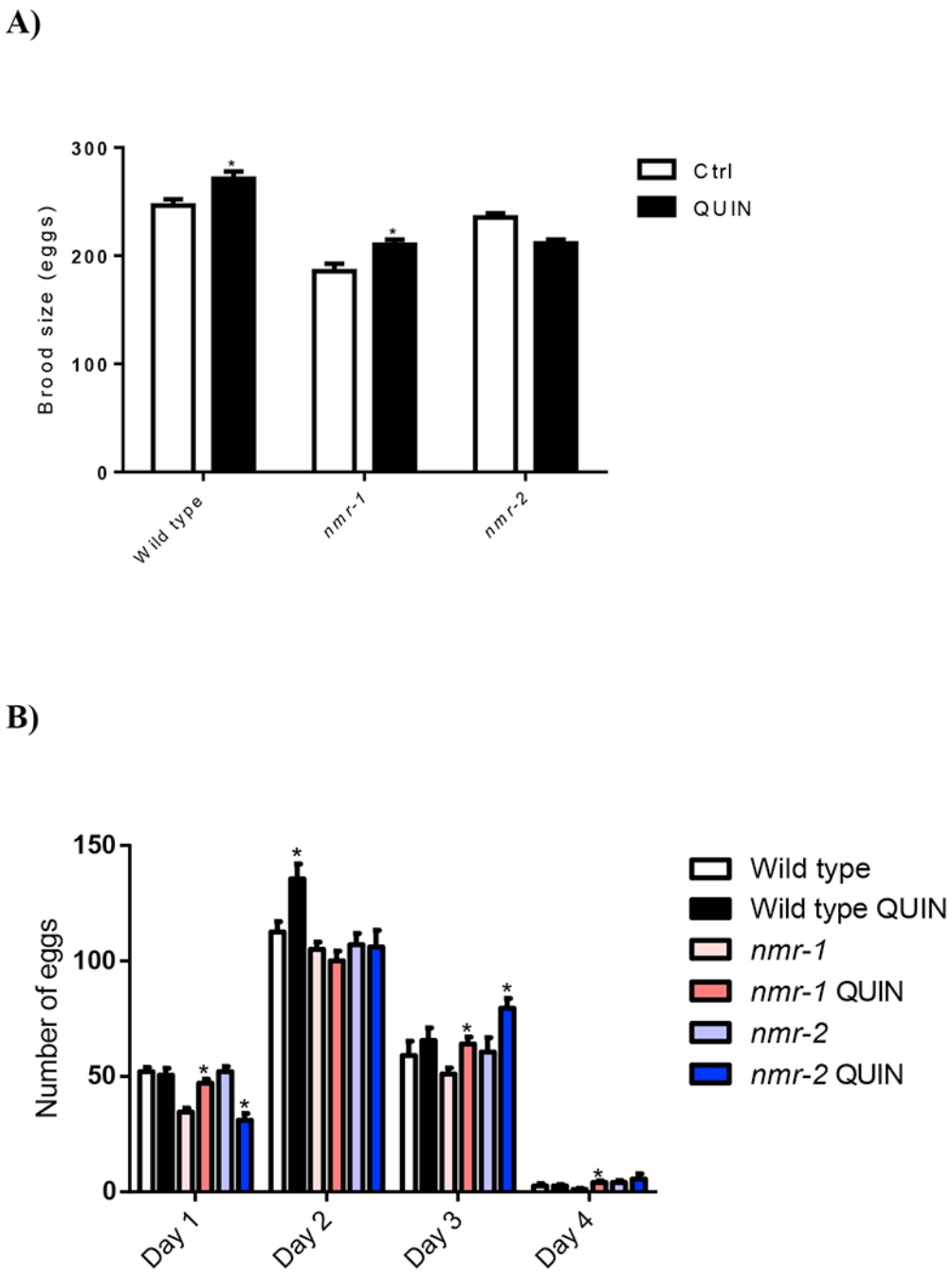

Fig 3. Effect of QUIN in eggs production of worms.

A) Oviposition per day for 5 days and B) brood size in WT N2, VM487 (*nmr-1)* and VC2326 (*nmr-2*) mutants treated or not with the 20 mM QUIN. Data are expressed as number of eggs per worm of each strain derived from 3 independent assays (n = 10-23). Error bars represent as means ± S.E.M. *p < 0.05 compared to each untreated group (Test-t).

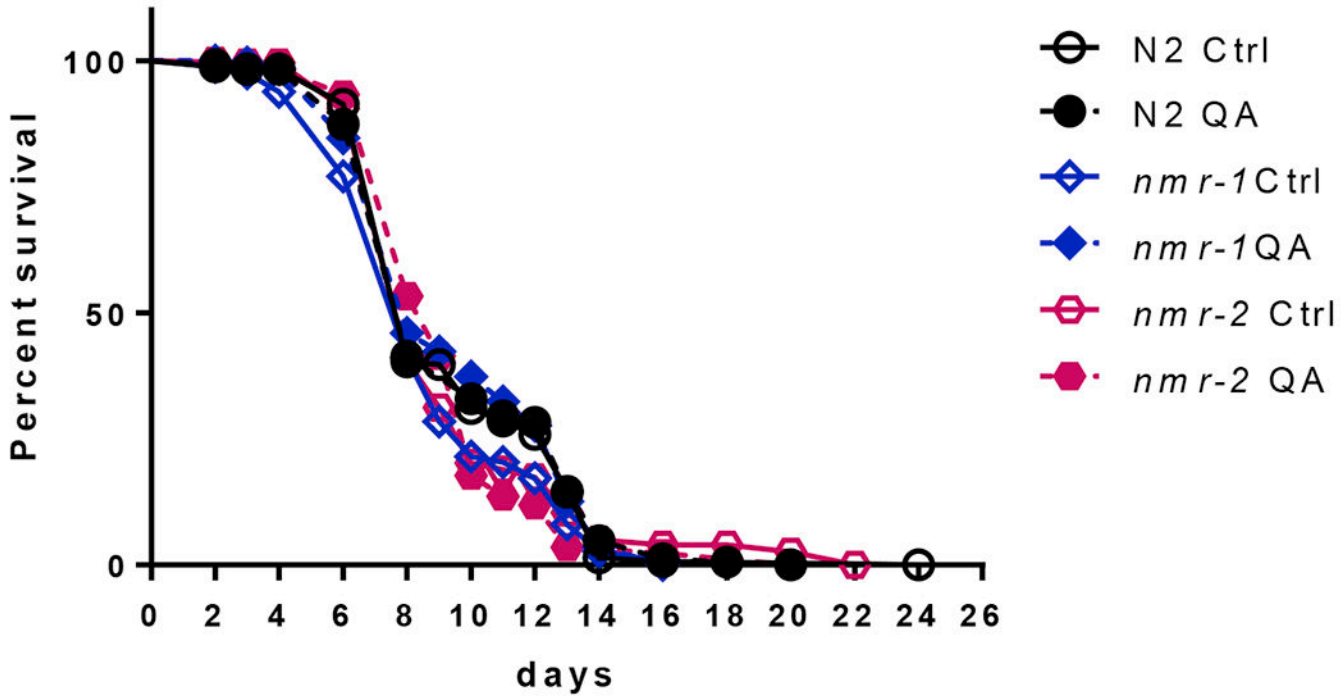

Figure 4. Effect of QUIN maximum lifetime of the worms.

WT N2. VM487 (*nmr-1)* and VC2326 *(nmr-2)* mutant worms treated or not with the 20 mM QUIN in survival curve for maximum lifetime. Data are expressed as time of from 3 independent assays (n = 10-23). Error bars represent as means ± S.E.M (Test-t).

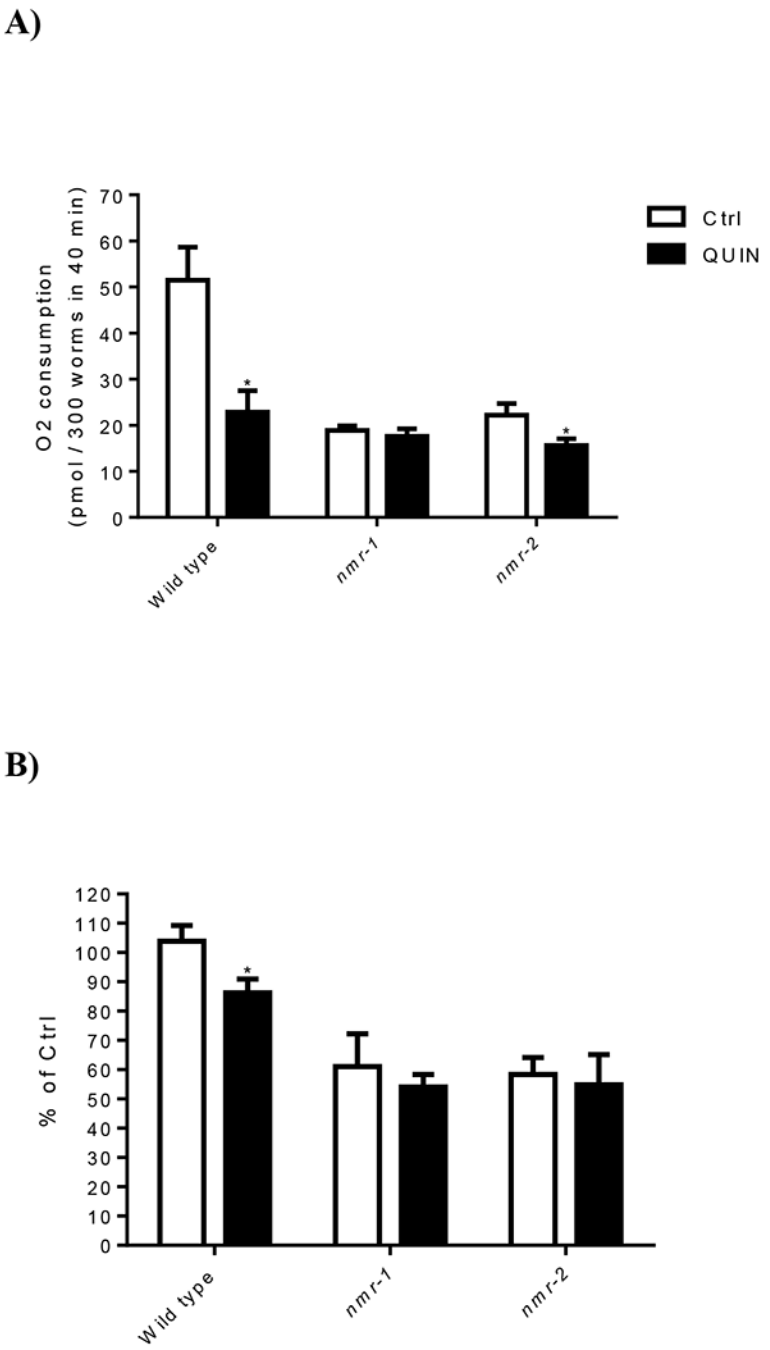

Fig. 5. Effect of QUIN in oxygen consumption (A) and membrane potential (B).

Data are expressed as O2 consumption (pmol / 300 worms) for oxygen consumption (A), and as percentual (%) of control in membrane potential (B) in WT N2. VM487 (*nmr-1*) and VC2623 (*nmr-2*) mutants treated or not with the QUIN 20 mM derived from 8 independent assays (n = 8). Error bars represent as means ± S.E.M. *$p < 0.05$ compared to each untreated group (Test-t).

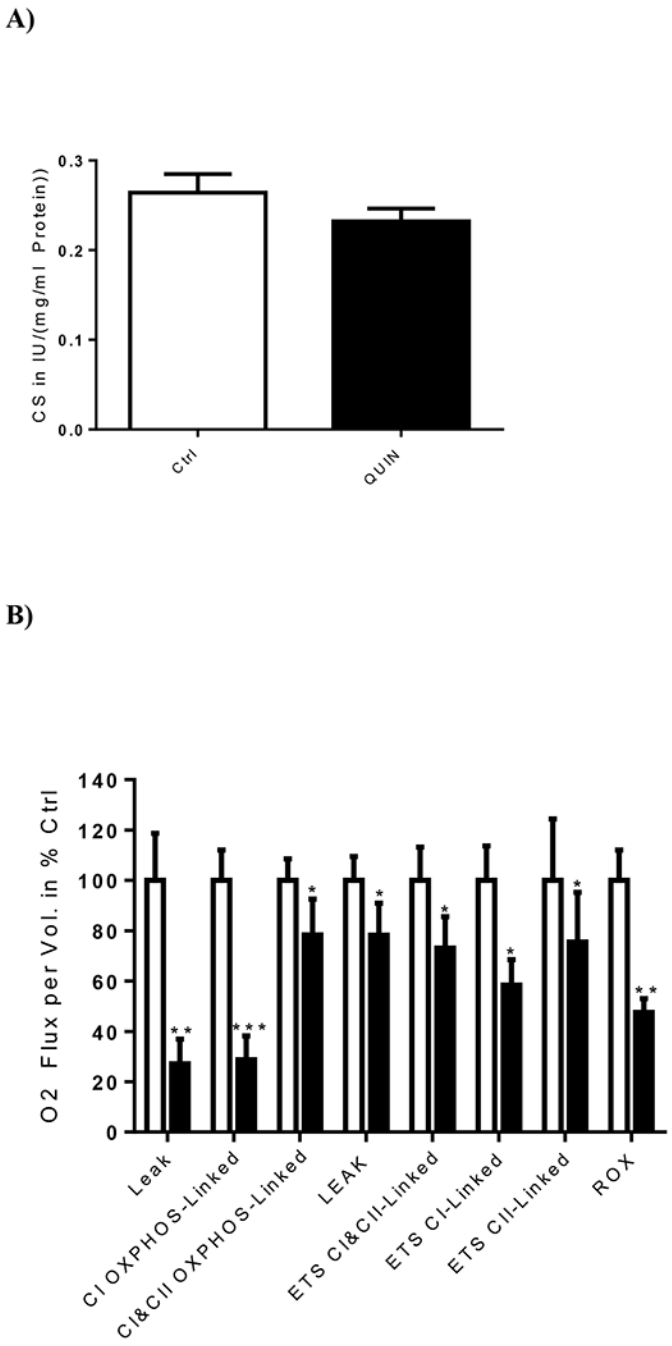

Figure 6. Effect of QUIN in viability and functionality mitochondrial.

WT animal treated or not with 20 mM QUIN in citrate synthase activity (A) and electron flow in electron transporter chain (B). Data are expressed as CS in IU/(mg/ml Protein) in A, and in percentual of control in B. Data are derived of three independent assays (n=4-8). Error bars represent as means ± S.E.M. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to untreated group (Test-t).

Table 1.
Effect of QUIN in body parameters of worms.

Mean Worm Length (mm). Mean Width (mm), Mean Area (mm^2). Wavelength (mm), Mean Amplitude (mm), Max Amplitude (mm) are evaluated using WormLab software. Data are expressed in um of from 5 independent assays (n = 14-36).

Analysis	Genotype	QUIN (mM)	Mean ± SD (Mm)
Mean Worm Length (mm)	**Bristol N2**	0	1.294 ± 0.218
		20	1.267 ± 0.416
	nmr-1	0	1.463 ± 0.197
		20	1.366 ± 0.132
	nmr-2	0	1.406 ± 0.14
		20	1.372 ± 0.228
Mean Width (mm)	**Bristol N2**	0	0.097 ± 0.015
		20	0.096 ± 0.026
	nmr-1	0	0.112 ± 0.011
		20	0.108 ± 0.011
	nmr-2	0	0.114 ± 0.009
		20	0.114 ± 0.010
Mean Area (mm^2)	**Bristol N2**	0	1.410 ± 0.168
		20	1.368 ± 0.549
	nmr-1	0	1.478 ± 0.215
		20	1.428 ± 0.215
	nmr-2	0	1.644 ± 0.258
		20	1.503 ± 0.252
Wavelength (mm)	**Bristol N2**	0	0.743 ± 0.045
		20	0.94 ± 0.169 ****
	nmr-1	0	0.767 ± 0.062
		20	0.652 ± 0.062 ***
	nmr-2	0	0.669 ± 0.096
		20	0.665 ± 0.092
Mean Amplitude (mm)	**Bristol N2**	0	0.94 ± 0.019
		20	0.113 ± 0.019 *
	nmr-1	0	0.116 ± 0.019
		20	0.092 ± 0.018 ***
	nmr-2	0	0.111 ± 0.019
		20	0.108 ± 0.022
Max Amplitude (mm)	**Bristol N2**	0	0.235 ± 0.038
		20	0.25 ± 0.060
	nmr-1	0	0.249 ± 0.040

Analysis	Genotype	QUIN (mM)	Mean ± SD (Mm)
		20	0.214 ± 0.035 *
	nmr-2	0	0.253 ± 0.032
		20	0.238 ± 0.043

Error bars represent as means ± S.E.M.

*
p<0.05,

**
p<0.01,

***
p< 0.001 and

****
p< 0.0001 compared to each untreated group (Test T).

Table 2.

Effect of QUIN on mean lifetime of the worms.

WT N2, VM487 (*nmr-1*) and VC2326 (*nmr-2*) mutant worms treated or not with the 20 mM QUIN in mean lifetime. Data are expressed as mean time the survival of the worms of from 4 independent assays (n= 170-220) (Test-t).

Strain	Mean lifetime (days)
WT Control	8
WT QUIN	8
nmr-1 Control	8
nmr-1 QUIN	8
nmr-2 Control	8
nmr-2 QUIN	9