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THE EFFECTS OF TRANSIENT STARVATION PERSIST THROUGH DIRECT INTERACTIONS BETWEEN CaMKII AND ETHER-A-GO-GO K+ CHANNELS IN *C. ELEGANS* MALES

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Abstract—Prolonged nutrient limitation has been extensively studied due to its positive effects on life span. However, less is understood of how brief periods of starvation can have lasting consequences. In this study, we used genetics, biochemistry, pharmacology and behavioral analysis to show that after a limited period of starvation, the synthesis of *egl-2*-encoded *ether-a-go-go* (EAG) K+ channels and its C-terminal modifications by *unc-43*-encoded CaMKII have a per-during effect on *C. elegans* male sexual behavior. EGL-2 and UNC-43 interactions, induced after food deprivation, maintain reduced excitability in muscles involved in sex. In young adult males, spastic contractions occur in cholinergic-activated sex muscles that lack functional *unc-103*-encoded ERG-like K+ channels. Promoting EGL-2 and UNC-43 interactions in *unc-103* mutant adult males by starving them for a few hours reduce spastic muscle contractions over multiple days. Although transient starvation during early adulthood has a hormetic effect of suppressing mutation-induced muscle contractions, the treatment reduces the ability of young wild-type (WT) males to compete with well-fed cohorts in siring progeny. Published by Elsevier Ltd on behalf of IBRO.

Key words: mating behavior, *C. elegans*, CaMKII, *ether-a-go-go* K+ channels, starvation, cholinergic signaling.

Organisms commonly encounter varying levels of nutrient availability in their natural environment. Until the development of modern farming techniques, humans also did not have a stable food source and had to withstand periods of famine. Organisms developed mechanisms enabling them to respond to nutrient deprivation via down-regulating circuits not involved in food foraging behaviors while up-regulating those behaviors directly involved in food acquisition. For example, in *C. elegans*, this involves down-regulating male mating behavior, while in *Drosophila*, food acquisition behaviors are increased (Wu et al., 2005; Gruninger et al., 2006). In addition, restricting caloric intake has positive long-term effects and will extend lifespan in organisms from yeast to primates (Jiang et al., 2000; Lin et al., 2000; Colman et al., 2009). The nutrient-limited environment induces changes that increase the probability of survival.

Once the stress of food deprivation is lifted, an organism reverses the suppression of behaviors that would not be beneficial in a starved state. However, transient starvation, especially during development, has lasting consequences, from stunted growth to various brain abnormalities (Hulshoff Pol et al., 2000; Victora et al., 2008). Inadequate nutrient availability positively correlates with the development of schizophrenia, indicating that transient starvation results not only in morphological defects but has a lasting impact on behavior (Susser et al., 1996; Wahlbeck et al., 2001; Victora et al., 2008). Determining the mechanisms via which the lasting consequences of transient starvation are realized provides avenues for alleviation of these effects later in life.

The mechanisms activated by a period of starvation that persist once the organism is returned to food remain largely unknown. In an attempt to address this deficiency, we use the tractable model organism *C. elegans*. As in vertebrates and insects, food deprivation during adulthood extends lifespan regardless of when it is applied, indicating the organism is capable of responding to nutrient deprivation throughout its life (Mair et al., 2003; Dhahbi et al., 2004; Smith et al., 2008). Food deprivation during larval stages is also able to activate pathways that regulate adult lifespan (Lee and Ashrafi, 2008). In addition to extending life span, limited nutrient availability affects behavior, such as inhibiting hermaphrodite egg laying, increasing movement, and reducing male mating ability. This suggests that the excitability of some behavioral circuits is down regulated while others are up regulated in response to starvation (Croll, 1975; Horvitz et al., 1982; Trent et al., 1983; Sawin et al., 2000; Gruninger et al., 2008). Laboratory cultures of *C. elegans* are grown in nutrient-rich environments but *C. elegans* is often found starved in its natural environment (Barriere and Felix, 2005; Mohri et al., 2005). A period of transient starvation has been shown to impact behaviors, such as an increased ability to distinguish between odors and decreased male mate searching (Colbert and Bargmann, 1997; Lipton et al., 2004). The observed behavioral effects combined with the ease of molecular manipulation make *C. elegans* an ideal organism for studying the mechanisms used by transient starvation to influence behavioral response programs.

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Abbreviations: ACh, acetylcholine; ARE, arecoline; CFP, cyan fluorescent protein; EAG, *ether-a-go-go*; EGL, egg-laying defective; ERG, *ether-a-go-go* related gene; fog, feminization of the germ line; gf, gain-of-function; GST, glutathione S-transferase; HA, hemagglutinin; hsp, heat shock protein; MBP, maltose binding protein; NMDAR, N-methyl D-aspartate receptor; Prc, protraction constitutive; UNC, uncoordinated; WT, wild-type; YFP, yellow fluorescent protein.

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We used *C. elegans* male mating behavior to uncover the mechanisms responsible for the perduring effects of transient starvation. Successful mating requires the male to complete a series of stereotyped steps, starting with response to the hermaphrodite, then proceeding to vulva location, insertion of their copulatory spicules, and finally sperm transfer. Spicule insertion includes a series of substeps that result in vulva penetration. Protractor muscles attached to the base of the spicules undergo rapid, rhythmic contractions that result in the spicules prodding at the vulva until it is breached. Once the vulva is breached, the protractor muscles tonically contract, forcing the spicules into the vulva and allowing sperm transfer (Ward and Carrel, 1979; Sulston et al., 1980; Loer and Kenyon, 1993; Liu and Sternberg, 1995; Garcia et al., 2001). The ability to successfully impregnate a mate is reduced when males are food deprived, and spicule muscle response is reduced as well (Gruninger et al., 2008). Previous work established that starvation is able to attenuate spicule muscle spasms induced by the loss of the *ether-a-go-go*-related gene (ERG) K+ channel *unc-103* (Gruninger et al., 2006). In this study we report that increased expression of the *ether-a-go-go* K+ channel *egl-2* in the male sex muscles, in response to a period of transient starvation, produces lasting consequences in the excitability of the male mating circuit. In addition, the activity of EGL-2 is promoted by the calcium/calmodulin-dependent protein kinase II (CaMKII) *unc-43*.

EXPERIMENTAL PROCEDURES

Strains and culture methods

C. elegans strains used to obtain males for this study contained the allele *him-5(e1490)* on LGV (Hodgkin et al., 1979). The *him-5(e1490)* mutation results in a higher incidence of spontaneous males and allows one to obtain sufficient numbers of males in the hermaphroditic species. Additional alleles used were: *unc-103(n1213)* (Park and Horvitz, 1986), *pha-1(e2123)* (Schnabel and Schnabel, 1990), *unc-64(e246)* (Brenner, 1974) on LG III; *unc-43(sy574)* on LG IV (LeBoeuf et al., 2007); and *fog-2(q71)* (Schedl and Kimble, 1988), *egl-2(rg4)* (LeBoeuf et al., 2007), *egl-2(n693)* (Reiner et al., 1995), and *egl-2(n693n904)* on LG V (Weinshenker et al., 1999). The *fog-2(q71)* strain did not contain the *him-5* mutation.

Animals were grown at 20 °C on 5 cm NGM agar plates containing *E. coli* OP50 as the food source (Brenner, 1974). For behavioral assays, virgin males were isolated from non-crowded plates either at the late L4 stage (when cells in the male tail spike have completely migrated anteriorly) or after they newly crawled out of their L4 cuticle. They were kept solitary or in groups of 20 on 1–2 cm diameter lawns of bacteria. For experiments that required monitoring of behavior over multiple days, the males were transferred daily to new OP50-seeded NGM plates.

To starve males transiently, they were serially transferred to 3.5 cm *E. coli*-less NGM agar plates using a mouth pipette and water as a vehicle. To inhibit potential bacterial growth, the plates contained streptomycin at a final concentration of 30 μg/ml. An 8 M glycerol ring was applied to the edge of the agar to discourage males from crawling and desiccating on the sides of the plates. After the starvation period, males were transferred using a worm pick to NGM plates containing *E. coli*.

To assay the effects of translational inhibitors on male growth and behavior, cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) freshly dissolved in water at a concentration of 20 mg/ml was added to the surface of NGM plates with or without *E. coli*. The drug was allowed to soak into the plate overnight. The final concentration of cycloheximide in the plates was 250 μg/ml. This concentration was either lethal to the larva or stalls larval growth. We determined if the concentration of cycloheximide was effective in inhibiting protein translation in adult males by measuring how well the antibiotic interfered with the expression of a heat shock-induced transgene. *syIs38* transgenic males contain a constitutively active allele of the $G\alpha_q$ G-protein controlled by the *hsp-16* heat shock promoter (Bastiani et al., 2003). The behavioral effects of this transgene are rapidly potent, and all males in the presence or absence of food will artificially protrude their spicules in less than an hour after a 30 min heat pulse at 30 °C (n=10 animals for both conditions). When cycloheximide was present, males did not display the effects of the induced transgene well after 2 h post induction (n=10 animals for both conditions). However, ~20 h later, all the antibiotic-treated animals displayed the effects of the transgene; at 250 μg/ml, cycloheximide either breaks down, or small amounts translation can occur over time. To ensure that the effects of cycloheximide were due to its inhibition of protein translation, we also used puromycin (Sigma-Aldrich) freshly dissolved in water at a concentration of 40 mg/ml and added it to plates with or without *E. coli* to obtain a final concentration of 128 μg/ml. Puromycin is a compound that causes premature termination of the elongating polypeptide during protein translation. Like cycloheximide, puromycin prevented larval growth. Males responded similarly to puromycin as they did to cycloheximide. Eight% of wild-type males protracted their spicules when they were fed for 18 h in the presence of puromycin (n=36). Seventy two% of *unc-103(0)* fed 18 h males protracted their spicules on puromycin, indicating protein synthesis was necessary to compensate for the loss of *unc-103* (n=72, P-value =0.0001) compared to *unc-103(0)* fed. Three% of *unc-103(0)* males starved for 18 h on puromycin protracted their spicules, which was similar to the 5% of *unc-103(0)* males starved for 18 h without puromycin (n=35 and 169, respectively). Thirty two% of *unc-103(0)* males that had been starved for 18 h on puromycin and then fed for 7 h on puromycin protract their spicules (n=35), which was significantly higher than the 0% of *unc-103(0)* males that protracted their spicules after having been starved for 18 h and then fed for 7 h (n=20, P-value =0.004, Fisher's exact test).

Statistical tests performed

GraphPad InStat v.3.06 and GraphPad Prism 5 software were used to perform statistics on all data. Fisher's exact test was used when comparing two or more experiments where the outcome was a categorical variable (such as non-protracted spicules vs. protracted spicules (Table 1), expression of the marker gene vs. no expression (Fig. 2A), sensitivity to the acetylcholine (Ach) agonist vs. resistance to the ACh agonist (Table 3) and siring progeny vs. siring no progeny (Fig. 4)). Unpaired t-test was used to compare the means of experimental replicates from two groups (Fig. 3).

Mutation and drug-induced spicule protraction

To score spontaneous spicule protraction, L4 males in groups of 20 or less animals were kept on NGM agar plates containing or lacking food. 18–24 h later, using a dissecting stereomicroscope, we counted the number of males with one or both spicules extending from their cloaca. We disposed of the spicule-protracted males and transferred the non-spicule protracted animals to fresh plates for rescoring 18–24 h later.

To assay agonist-induced spicule protraction, we dissolved the acetylcholine agonist arecoline (Indofine Chemical Company, Hillsborough, NJ, USA) in water to make a stock solution of 1 M. We then serially diluted the stock solution in water as needed. We