Altered Function of the DnaJ Family Cochaperone DNJ-17 Modulates Locomotor Circuit Activity in a Caenorhabditis elegans Seizure Model

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ABSTRACT The highly conserved cochaperone DnaJ/Hsp40 family proteins are known to interact with molecular chaperone Hsp70, and can regulate many cellular processes including protein folding, translocation, and degradation. In studies of Caenorhabditis elegans locomotion mutants, we identified a gain-of-function (gf) mutation in dnj-17 closely linked to the widely used e156 null allele of C. elegans GAD (glutamic acid decarboxylase) unc-25. dnj-17 encodes a DnaJ protein orthologous to human DNAJA5. In C. elegans DNJ-17 is a cytosolic protein and is broadly expressed in many tissues. dnj-17(gf) causes a single amino acid substitution in a conserved domain, and behaves as a hypermorphic mutation. The effect of this dnj-17(gf) is most prominent in mutants lacking GABA synaptic transmission. In a seizure model caused by a mutation in the ionotropic acetylcholine receptor acr-2(gf), dnj-17(gf) exacerbates the convulsion phenotype in conjunction with absence of GABA. Null mutants of dnj-17 show mild resistance to aldicarb, while dnj-17(gf) is hypersensitive. These results highlight the importance of DnaJ proteins in regulation of C. elegans locomotor circuit, and provide insights into the in vivo roles of DnaJ proteins in humans.

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KEYWORDS GABA glutamic acid decarboxylase acetylcholine receptor excitation inhibition balance
The nicotinic acetylcholine receptor subunit *acr-2* is expressed in the cholinergic motor neurons. A gain-of-function mutation of *acr-2* causes increased cholinergic motor neuron activity accompanied by decreased GABAergic motor neuron activity, generating excitation–inhibition (E/I) imbalance in locomotor circuit. *acr-2(gf)* animals exhibit a characteristic repetitive convulsion behavior, the frequency of which provides a quantitative measure of E/I imbalance (Jospin et al. 2009; Starwicki et al. 2013).

Through studying the effects of defective GABAergic transmission in *acr-2(gf)* animals, we unexpectedly found a gain-of-function mutation in a cochaperone protein *dnj-17* to be present in the widely used strain CB156 *unc-25(e156)*. We show that the DNJ-17 gain-of-function mutation behaves in a hypermorph manner, and exacerbates excitation–inhibition imbalance in *acr-2(gf)*. Null mutations of *dnj-17* exhibit mild resistance to aldicarb, suggesting a role in modulating neurotransmission. Homologs of DNJ-17 include human DNAJ5, which is expressed in the brain and other tissues. Our findings provide insights into the *in vivo* function of these cochaperone proteins.

**MATERIALS AND METHODS**

**Strains**

*C. elegans* strains were kept at 22.5°C according to standard procedures. Supplemental Material, Table S1 lists strain information with alleles and transgenes. Galaxy platform (Giardine et al. 2005) and CloudMap workflows (Minevich et al. 2012) were used to analyze the whole-genome sequence data of MT6648 unc-25(e156) *dnj-17(ju1162)III*; *acr-2(n2420)X* and CZ19995 unc-25(e156) *dnj-17(ju1162)III*; *acr-2(n2420)X*, obtained by Beijing Genomics Institute (Shenzhen, China). Subsequent analyses based on chromosomal linkage and recombination mapping identified the *ju1162* missense mutation in *dnj-17*. We verified the presence of *dnj-17(ju1162)* in CB156, and generated CZ22168 unc-25(e156) that lacks *dnj-17(ju1162)* through multipstep recombination as follows: We verified that the Caenorhabditis Genetics Center (CGC) strain SP1104 unc-25(e156) *bli-5(c518)III* is wild type for *dnj-17*. We outcrossed SP1104 to N2, and isolated recombinant animals that showed unc-25(0) shrinker phenotype without blister phenotype. We performed genotyping on isoegenic strains of the recombinants, and confirmed the presence of unc-25(e156) and the loss of *bli-5(c518)*. In this process, we also found SP1104 has another mutation linked to chromosome III that caused egg-laying defects. Through further outcrossing to N2, we reisolated unc-25(e156) based on behavior and genotyping and established strain CZ22168 which does not exhibit the egg-laying defects. Primers used for PCR and genotyping were as follows: YJ10801 CCGTAGAAAACCTTCACTTGGC and YJ10802 CATGAAATGGCATTACGAGTGTGTC for *dnj-17(ju1162)*, YJ11985 CATTGGCCGACGACTTATTGTC and YJ11986 AATTGCTCAGGAACTACATCT for unc-25(e156), YJ10799 TACTGGTATCCAGCTCTCC and YJ10800 ATTATT TGACAGTATTACGCCACC for *bli-5(c518)*. The information on the alleles and CB156 is deposited in CGC and Wormbase. Several researchers noted that unc-25(e156) appeared to behave differently from other unc-25 alleles or GABA mutants, in a number of behavioral and pharmacological assays (C. Bargmann, E. Jorgensen, S. Chalasani, J. Kaplan, personal communications). For future experiments on unc-25 mutants, we recommend CZ22168, as well as other unc-25 alleles.

**Molecular biology and transgenes**

Molecular biology was performed following standard methods. Gateway recombination technology (Invitrogen, CA) was used for expression vectors. Table S2 describes the details of constructs generated in this study. We amplified 3.5 kb genomic sequences of *dnj-17* with 0.9 kb 5’ upstream sequences to 0.1 kb 3’ downstream region using the following primers: YJ11212 AAAGTCAATCAACTGTTCCTTG and YJ11212 TTGCAATTTATTTCCCGAACAC. To determine the gene structure of *dnj-17*, we isolated mRNAs from mixed-stage animals of N2 wild type and CB156 unc-25(e156) *dnj-17(ju1162)* using Trizol (Thermo-Fisher Scientific). Complementary DNA (cDNA) synthesis was performed using SuperScript III (ThermoFisher Scientific), with random primers according to the manufacturers’ instructions. We performed RT-PCR using SL1 primer GTTAAATTCCAGAATTTTGA and a reverse primer p3 GCGGACAGATTCTTTGCTGTC designed on the exon 3 and exon 4 to determine the first exon of *dnj-17* mRNA, and p2 ATGAAATGGCATTACGAGTGTGTC and p4 ATAGTGTATCACTTATCTCCAC primers designed on the first and sixth exon to verify the coding sequence. Sequences of all clones were verified by Sanger sequencing. Protein domain analysis was performed using NCBI domain database (Marchler-Bauer et al. 2015) and TreeFam (Li et al. 2006).

**Generation of deletion alleles of *dnj-17* by CRISPR-Cas9 editing**

*dnj-17(ju1239)* and *dnj-17(ju1276)* deletion alleles were generated by CRISPR-Cas9 editing in the germline, using modifications of previously described methods (Dickinson et al. 2013) (Z. Wang and Y.J., unpublished data). Briefly, adult animals were injected with the Cas9-sgRNA expression constructs (pCGZ2646) and pCGZ2646, made from pDD162 with sgRNA and Pmyo-2-mCherry as a co-injection marker. F1 animals expressing mCherry in pharynx were isolated, allowed to lay eggs, and then genotyped for *dnj-17* to detect deletions. The F2 progeny of F1 animals with deletions were isolated to establish strains containing *dnj-17* deletion. sgRNA sequences used to target *dnj-17* are the following: AGAGAAACTACGGCTCAA and GAGTTTTGCCGAAAGGATA. *ju1239* was generated following microinjection into N2 animals, *ju1276* was generated following microinjection into CB156 unc-25(e156) animals.

To analyze the temperature effects on *dnj-17(ju1239)*, we examined the growth and locomotion of N2 and CZ21429 *dnj-17(ju1239)* under different temperature conditions. Briefly, 10 gravid adults of each strain were allowed to lay eggs for 6 hr at 22.5°C. Then, adult animals were removed, and the plates with embryos were kept under 15°C, 22.5°C, or 25°C. Hatched progeny were kept under the same temperature, and their growth and general locomotion were visually scored once within 16–24 hr. When the progeny reached L4 stage, animals from each condition were placed onto individual plates. Number of eggs laid by each animal was scored to compare the brood size. The experiment was repeated twice.

**Generation of single-copy inserted strains**

Single-copy insertion transgenes of *Pdnj-17-dnj-17* (+) and *Pdnj-17-dnj-17* (ju1162) were generated at Chromosome II site (tTi5605 using modified vectors (Z. Wang and Y.J., unpublished data)). Briefly, N2 young adult animals were injected with the following constructs: a construct (pCGZ3031 or pCGZ3032) containing *dnj-17* sequence with tTi5605 homology arms and a copy of hygromycin resistance gene, and *dnj-17* (ju1162) was generated following microinjection into N2 animals, *ju1276* was generated following microinjection into CB156 unc-25(e156) animals.
Each insertion line was outcrossed twice before being used in experiments.

Quantification of convulsion behavior

Scoring of convulsions was performed as previously described (Stawicki et al. 2013). Briefly, L4 larvae were transferred to nematode growth medium (NGM) plates seeded with Escherichia coli OP50. On the following day, young adults were transferred to fresh plates with OP50 and visually scored for convulsion behavior under a dissecting microscope. The observer was blinded to the genotype of the animals tested. A convulsion event was defined as a shortening of the animal’s body length. The assay was repeated at least twice per genotype in two different generations. Two independent transgenic lines were used for each construct.

Aldicarb assay

One day before the experiment, L4 animals were transferred to fresh plates seeded with OP50. On the next day, 10 animals were transferred to an NGM plate with 500 μM aldicarb. Animal behavior was scored every 30 min. Animals were scored paralyzed when they did not move for more than 5 sec in response to touch stimulus.

Confocal microscopy

L4 animals were imaged using a Zeiss LSM 710 confocal microscope (63× objective). Animals were immobilized by 1 mM levamisole and placed on 4% agar pads. Images are maximum-intensity projections of z stacks obtained at 1 μm intervals. ImageJ was used to process the images obtained.
RESULTS AND DISCUSSION

Identification of a missense mutation in dnj-17 in unc-25(e156) strains

acr-2(n2420gf) animals show spontaneous convulsion behavior, due to increased cholinergic excitation and reduced GABAergic inhibition (Jospin et al. 2009). We wanted to further examine the effects of GABAergic transmission on the convulsive behavior of acr-2(gf) animals. We generated double mutants of acr-2(gf) with genes essential for GABA signaling, using canonical or null alleles of unc-25/GAD, unc-47/VGAT, unc-49/GABAR. We used three null mutations of unc-25: e156, n2324, and n2328, which cause amber stop codons at Trp383, Trp291, and Glu486, respectively, and which are all predicted to encode truncated proteins that lack the cofactor binding site and enzymatic activity site at the C-terminus. All double mutants showed increased convulsion frequency compared to acr-2(gf) single mutants (Figure 1A). While unc-25(n2324) and unc-25(n2328) enhanced acr-2(gf) behavioral defects to similar degrees as unc-47(gk192) and unc-49(e382), unc-25(e156) increased the convulsion frequency significantly more than these four mutations. Further outcrossing of unc-25(e156);acr-2(gf) (MT6648) did not eliminate this enhancement. We thus hypothesized that the ancestral CB156 strain may contain additional modifier mutations (link) to unc-25(e156).

We performed whole-genome sequencing analysis of MT6648 and of an outcrossed strain CZ19995 unc-25(e156);acr-2(gf). Following chromosomal linkage mapping, we identified a single nucleotide version from thymine to adenine in the coding sequence of the gene dnj-17, approximately 0.5 map units right of unc-25 on chromosome III (Figure 1B), and hereafter referred to as dnj-17(ju1162). The ju1162 mutation was present in the CGC strain CB156 unc-25(e156), but not in SP1104 unc-25(e156) bli-5(e518) III, which was generated in about 1987 through recombination from trans-heterozygous animals of unc-25(e156) with a chromosome containing bli-5(e518) (R. Herman, personal communication). dnj-17(ju1162) was also not present in MT5957 unc-25(n2324) III and MT5969 unc-25(n2328) III. Therefore, dnj-17(ju1162) did not arise as a spontaneous mutation in strain passage in our laboratory, but was inherited from the original CB156 stock.

dnj-17 encodes a homolog of human DNAJA5
Gene structure (Wormbase WS251) showed that dnj-17 contains seven exons, generating a mature mRNA predicted to encode a protein of 510 amino acids. To verify the dnj-17 gene structure we performed cDNA analyses using mRNA isolated from N2 and CB156 unc-25(e156) dnj-17(ju1162). RT-PCR analyses using SL1 and gene-specific primers revealed that the 5′ end of dnj-17 mRNA contained an SL1 leader, but predicted exon 1 was not present in the mature mRNA. We obtained full-length dnj-17 cDNA and found that DNJ-17 protein consists of 485 amino acids (Figure 1, B and C). From NCBI protein domain analysis, the N-terminus of DNJ-17 has a highly conserved Dnaj domain, known to interact with Hsp70 family proteins, and the C-terminal half contains two C2H2-type zinc finger motifs that have been implicated to be important for polypeptide binding (Banecki et al. 1996; Lu and Cyr 1998; Szabo et al. 1996). Relatives of DNJ-17 are found widely in eukaryotes, with orthologs named as JJJ1 in yeast, DNAJA5/DNAJC21 in human, DNAJC21 in mouse, and CG2790 in Drosophila (Figure 1D). The J domain of DNJ-17 also has a highly conserved HPD motif that is crucial for interaction with Hsp70 proteins (Tsi and Douglas 1996). Yeast JJJ1 activates ATPase activity of Hsp70, and lack of JJJ1 results in cold sensitivity (Meyer et al. 2007). On the other hand, functions of the DNJ-17 family proteins in animals remain mostly unknown, though human DNAJA5 is expressed in several tissues including the brain (Chen et al. 2004). We confirmed that cDNAs from

Data availability
Strains and constructs are described in Table S1 and Table S2 respectively, and are available upon request.

Figure 2. dnj-17(ju1162) acts as a gain-of-function mutation. (A,C,D) Quantification of convulsion frequency. (A) Loss-of-function deletion alleles of dnj-17 do not affect acr-2(gf) convulsion frequency. (C) Removal of dnj-17(ju1162gf) reduces the convulsion frequency. (D) dnj-17(ju1162gf) shows semidominant effects on convulsion frequency. Statistics, one way ANOVA followed by Bonferroni’s post hoc test. * P < 0.05. Numbers in the column indicate sample sizes. (B) Brood size is not affected by deletion of dnj-17. n = 6 for each condition. Error bars indicate SEM.
CB156 unc-25(e156) dnj-17(ju1162) contained a single nucleotide change, which causes Asp77 to Lys amino acid substitution (N77K) in the region immediately adjacent to the DnaJ domain (Figure 1, B and D).

**dnj-17(ju1162) is a gain-of-function mutation**

Several lines of evidence support that ju1162 is a gain-of-function mutation of dnj-17. First, we examined the effect of a dnj-17 deletion allele dnj-17(tm570), which removes the C-terminal half of the protein (Figure 1B). dnj-17(tm570) homozygous animals showed normal growth rate, wild-type locomotion, and did not affect convulsion frequency ofacr-2(gf) (Figure 2A). We also generated unc-47(gk192) dnj-17(tm570);acr-2(n2420) triple mutants and found that they resembled unc-47(gk192);acr-2(n2420) double mutants in their convulsion frequency. As dnj-17(tm570) mutants potentially produce mRNAs encoding a truncated protein with intact DnaJ domain, we next generated a deletion allele targeting the DnaJ domain using CRISPR-Cas9-mediated genome editing technology (Dickinson et al. 2013; Friedland et al. 2013) (Z. Wang and Y.J., unpublished results). dnj-17(ju1239) removes a large portion of the DnaJ domain and is predicted to cause a frameshift and premature stop after amino acid 34 (Figure 1B). Since the null mutation of a yeast protein with J domain, Jjj1, was previously reported to cause cold sensitivity in yeast (Meyer et al. 2007), we examined the viability and locomotion ofdnj-17(ju1239) mutants. The mutant animals had similar brood size as wild type under three temperature conditions (Figure 2B). Their growth rate, body shape, and movement were also indistinguishable from wild type. Finally, dnj-17(ju1239) did not affect the convulsion frequency ofacr-2(n2420) (Figure 2A). These observations show that dnj-17 is a nonessential gene for C. elegans development and behavior, and that dnj-17 loss-of-function does not affect convulsion ofacr-2(gf) by itself or when GABA transmission is eliminated inunc-47(null) animals.

We further examined if removing dnj-17(ju1162) from unc-25(e156) background would eliminate the increased convulsion frequency phenotype ofacr-2(gf). As dnj-17 is located 0.5 map unit apart from unc-25, it is challenging to separate unc-25(e156) and dnj-17(ju1239) by genetic recombination. We therefore generated another deletion allele in the unc-25(e156) dnj-17(ju1162) background using CRISPR editing (Figure 1B). dnj-17(ju1276) removed the DnaJ domain and the region including ju1162(N77K), and eliminated the increased convulsion (Figure 2C). Furthermore, through isolation ofunc-25(e156) recombinants after outcrossing SP1104 unc-25(e156) dnj-17(+) bli-5(e118), we obtained CZ22169 unc-25(e156) dnj-17(+)acr-2(n2420). Animals of genotype unc-25(e156) dnj-17(ju1276);acr-2(gf) and unc-25(e156) dnj-17(+);acr-2(gf) showed convulsion frequencies lower than unc-25(e156) dnj-17(ju1162);acr-2(gf), and instead resembledacr-2(gf)double mutants withunc-25(n2324) or with other GABA mutants (Figure 2C). Finally, we also observed thatdnj-17(ju1162) showed semidominant effects on convulsion frequency in theacr-2(gf);unc-25(0) background (Figure 2D). Thus, we conclude thatdnj-17(ju1162) is a semidominant gain-of-function mutation, designated asdnj-17(ju1162gf).

**DNJ-17 is a cytosolic protein expressed in multiple tissues**

We next analyzed the expression pattern ofdnj-17. We first generated an extrachromosomal transcriptional green fluorescent protein (GFP) reporter using 0.9 kb promoter region ofdnj-17. GFP was seen throughout the body with enrichment in the intestine and several cells around the pharynx, and the expression pattern was similar in both wild type andacr-2(gf) background (Figure 3A). We then made GFP-fused translational DNJ-17 reporters. GFP signals fromPdnj-17-dnj-17::gfp localized to the cytosol of head neurons, and in other unidentified cells at lower levels throughout the body (Figure 3B). A weaker but similar pattern was observed in an integrated fosmid expression line which expressesDNJ-17 tagged with C-terminal TY1::EGFP:3xFLAG (not shown) (Zhong et al. 2010). Moreover, DNJ-17(N77K)::GFP showed similarly diffused expression. Both DNJ-17(+)::GFP and DNJ-17(N77K) expression patterns were similar in wild type and inacr-2(gf) background, suggesting that the presence ofacr-2(gf) does not largely affect the localization ofDNJ-17.

**DNJ-17(N77K) behaves as a hypermorph**

We next examined the nature ofDNJ-17(N77K) using transgenic overexpression. Overexpression of wild-type dnj-17 by genomic sequence ofdnj-17 including 0.9 kb upstream promoter region caused increase ofacr-2(gf) convulsion frequency (Figure 4A). This transgene also enhanced convulsion frequency inunc-47(0) mutant background, suggesting that the increase in convulsion by overexpression ofdnj-17 is independent of the effect caused by defects in GABAergic transmission. Interestingly, this enhanced effect was also observed by overexpression ofdnj-17(ju1162gf). ACR-2 is expressed specifically in neurons (Jospin et al. 2009). However, overexpression ofdnj-17 wild type or ju1162(gf) using thePrgef-1 pan-neuronal promoter did not affect convulsion frequency ofacr-2(gf) (Figure 4B). Also, overexpression in muscles usingPmyo-3 promoter did not affectacr-2(gf) convulsion frequency (Figure 4B). These results suggest that the effect ofdnj-17 on convulsion frequency likely requires its expression in multiple tissues.

To precisely compare the effect ofdnj-17(ju1162gf) to wild-typednj-17, we generated a single-copy insertion transgene expressing full-length genomicdnj-17(ju1162gf) ordnj-17(+) on chromosome II. Animals withPdnj-17-dnj-17(ju1162gf) expressed from a single-copy transgene showed...
overall normal locomotion, growth speed, and brood size. We found that *Pdnj-17-dnj-17(ju1162gf);acr-2(gf)* increased convulsion compared to *acr-2(gf)* single mutants, whereas *Pdnj-17-dnj-17(+)* single-copy expression did not (Figure 4C), consistent with *dnj-17(ju1162gf)* acting semi-dominantly in *unc-25(e156)* background (Figure 2D). These results suggest that the DNJ-17(N77K) mutation has higher activity than wild-type DNJ-17, implying that the increase in convulsion by overexpression of wild-type *dnj-17* is caused by excess levels of the protein.

**dnj-17 activity affects the response to aldicarb**

To further assess the effect of *dnj-17* mutations on neurotransmission at the neuromuscular junction, we examined the sensitivity of the mutant animals to an acetylcholine esterase inhibitor aldicarb. *dnj-17(ju1239)* null animals showed mild resistance to aldicarb, which was rescued by single-copy insertion of *dnj-17(+)*, implying that *dnj-17* affects cholinergic transmission at the neuromuscular junction (Figure 4D). Expression of *dnj-17(ju1162gf)* caused mildly increased sensitivity to aldicarb, reaching statistical significance at one time point, consistent with this allele being a hypermorph mutation. These results raise a possibility that the function of *dnj-17* is required for folding and/or function of proteins in multiple tissues that are involved in cholinergic transmission. Overexpression of wild-type DNJ-17 may also lead to a high level of cholinergic transmission by contributing to folding of the proteins in the pathway.

**Perspectives**

Other mechanisms might account for the effects of *dnj-17(ju1162)*. The N77K mutation may make DNJ-17 prone to form aggregates. DnaJ/Hsp40 proteins bind to misfolded proteins and bring them to Hsp70 (Cheetham and Caplan 1998). The N77K mutation could alter the kinetics for DNJ-17 to detach from the protein(s) it binds to, and prevent the misfolded protein from being degraded, resulting in accumulation of misfolded proteins that cause cellular stress. Such cellular stress could alter neuronal and muscular functions. Another possibility is that the mutation disrupts certain cellular functions. Recently it was reported that an Asn to Ser mutation in the DnaJ domain of human DNAJC13 was found in a family with Parkinson disease, where the disease was transmitted in an autosomal-dominant manner (Vilariño-Güell et al. 2014). The mutant protein exhibited a toxic gain-of-function activity affecting endosomal transport. The N77K mutation in *C. elegans* DNJ-17 may affect similar cellular functions such as endocytosis and...
subcellular trafficking, thus disrupting the coordination of the motor neuron circuit.

E. coli has only one gene coding DnaJ/Hsp40, whereas animals typically express multiple DnaJ family members. The DnaJ protein in E. coli has been well characterized, but functions of individual DnaJ/Hsp40 family proteins in animals remain largely unknown. DNAJAS, the closest human homolog of DNJ-17, shows enhanced expression in the brain (Chen et al. 2004) which suggests neuron-specific roles, but its substrates and functions are yet to be characterized. Studies of DnaJ/Hsp40 in animals may lead to further understanding of the physiological mechanisms of protein homeostasis in neurodegenerative diseases.

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