A genetic screen for aldicarb resistance of Caenorhabditis elegans dauer larvae uncovers 2 alleles of dach-1, a cytochrome P450 gene

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Abstract

Animals exhibit phenotypic plasticity through the interaction of genes with the environment, and little is known about the genetic factors that change synaptic function at different developmental stages. Here, we investigated the genetic determinants of how animal’s sensitivity to drugs that alter synaptic activity is regulated at specific developmental stages using the free-living nematode Caenorhabditis elegans. C. elegans enters the stress-resistant dauer larval stage under harsh conditions. Although dauer is known to have reduced permeability and increased resistance to most exogenous chemicals, we discovered that dauer is hypersensitive to a cholinesterase inhibitor, aldicarb. To investigate genes regulating dauer-specific acetylcholine transduction, we first screened for acetylcholinesterase inhibitor called aldicarb (Blazie and Jin 2018). A working concentration of aldicarb increases the amount of acetylcholine at the neuromuscular junction (NMJ), causing paralysis of the animals within a few hours (Mahoney et al. 2006; Oh and Kim 2017). In this condition, mutations affecting synaptic transmission change the time course of aldicarb-induced paralysis. Such Ric (Resistance to an inhibitor of cholinesterase) phenotype causing slow aldicarb-induced paralysis is associated with reduced acetylcholine syntheses, vesicle loading, vesicle release, or reduced acetylcholine receptor (Nguyen et al. 1995; Rand 2007). Although the Ric phenotype was due to a reduction of acetylcholine, the revealed functions of most Ric genes are not limited to acetylcholine transmission, but rather are involved in all neurotransmitters. This is because vesicle loading or release mechanisms are usually not specific to a particular neurotransmitter. For this reason, the Ric phenotype is an efficient method to find novel genes important for the overall function of synaptic transmission. Although Ric genes were found by many studies, all studies were focused on the phenotype in adults, and no studies were conducted in the different developmental stages.

The onset of neuronal dysfunction often depends on certain conditions such as the developmental stage (Zhang et al. 2017). It is because mechanisms that regulate synaptic activity can be modulated by multiple developmental stages. For example, in the human adolescent brain, the activity of the brain region related to reward is relatively elevated, whereas the region related to the aversive stimulus is relatively weakened (Spear 2013). In the case of C. elegans, new postembryonic motor neurons are differentiated during the transition from L1, the first larva period, to the next developmental stage, and the position of the synapse of the preexisting motor neurons is remodeled (Raptis 2020). Structural changes in neurons according to developmental stages suggest that synaptic function may also vary in specific
developmental stages. If the cholinergetic transmission in NMJ is increased in a certain stage, aldicarb-induced paralysis might be observed even at the subthreshold level of the adult stage. In this study, we utilized the subthreshold level of aldicarb to test whether other developmental stages elicit the aldicarb hypersensitivity. Then, we focused on the aldicarb hypersensitivity in the developmental stage called dauer. Based on the dauerspecific aldicarb hypersensitivity, we conducted a forward genetic screening to discover the mutations that induce dauer-specific aldicarb resistance. From the screening, we found that 2 alleles of dach-1 or cyp-34A4, caused a dauer-specific Ric phenotype. dach-1 has not been isolated from conventional Ric screening using adults and appears to be a mechanism to regulate synaptic transmission through paracrine factors from nonneuronal tissues. Furthermore, we discovered that dach-1 is important for other types of phenotypic plasticity in dauer.

Materials and methods

Maintenance and strains

Most worms were maintained at 20°C as previously described (Brenner 1974), and daf-2(e1370) and other daf-2(e1370) background mutants were maintained at 15°C. The following strains were used: N2, daf-2(e1370), daf-2(e1370);dach-1(ys51), daf-2(e1370);dach-1(ys52), dach-1(ys51), dach-1(ys52), rrf-3(pk1426);daf-2(e1370), rrf-3(pk1426);daf-2(e1370);dach-1(ys51), N2, Ex[Pdach-1::GFP, rol-6(su1006)], dach-1(ys51), Ex[Pdach-1::dach-1::SL2::GFP, Pmyo-2::mCherry], dach-1(ys51), Ex[Pdpy-7::dach-1::SL2::GFP, Pmyo-2::mCherry], dach-1(ys51), Ex[Pmyo-3::dach-1::SL2::GFP, Pmyo-2::mCherry], dach-1(ys51), Ex[Peqg-3::dach-1::SL2::GFP, Pmyo-2::mCherry], ace-3(dC2), and ace-3(3c2);dach-1(ys51).

Molecular biology

Each promoter was subcloned by restriction enzymes. Unspliced genomic sequence of dach-1 containing 3’ UTR was inserted into pPD114.108(SL2::GFP) vector using BSSHII and NotI as the restriction sites. Pdach-1, Pdpy-7, Pmyo-3, and Pegl-3 were inserted into the dach-1::SL2::GFP vector using Sall and BSSHII as the restriction site. All the promoter information was gained from the Promoterome database (Dupuy et al. 2004).

Generation of transgenic lines

Introducing DNA into the gonads of young adult hermaphrodites was carried out as previously described (Mello et al. 1991). After microinjection, worms were quickly recovered with M9 buffer. For the reporter transgenic lines, rol-6(su1006) was used as the injection marker with 50 ng/μl of concentration. For the rescue experiments, Pmyo-2::mCherry was used as the marker with 3 ng/μl concentration, along with 50 ng/μl of rescue construct and 47 ng/μl of empty vector (pPD95.77).

Ethanol and stress sensitivity assay

7% ethanol sensitivity assay was conducted as previously described (Choi et al. 2016). First, 50 worms were moved to an empty, unseeded NGM plate. Dauer larvae were transferred via mouth pipette with M9 buffer, and L3 or nondauer larvae were done via platinum pick. After 10 min, they were harvested with 1 ml of solution containing 7% ethanol and immersed as a droplet in an empty 55-mm Petri dish. The lid was closed and swimming worms were counted. For heat stress, nonseeded NGM plates containing about 20 dauers were incubated at 37°C for 4 h and then transferred to 20°C, and the number of live and dead animals was counted the next day. At a heat stress of 30°C, most of the dauers did not die, so 37°C was used as the heat stress condition (data not shown). To test the heat resistance of L3, worms were incubated at 37°C for 2 h because L3 did not survive after 4 h at 37°C. For high osmotic stress, about 20 worms were transferred to a solution containing 1,500 mM NaCl for dauers and 250 mM NaCl for L3 with a mouth pipette and incubated at 25°C for 20 h. After washing and harvesting the worms, they were transferred to an NGM plate and the number of moving and dead worms was counted the next day.

Dauer formation and selection

Dauer formation was induced with the cocktail of asc#1, #2, and #3 in NGM plate. For 250 ml of pheromone plates, 0.5 g NaCl, 0.75 g KH2PO4, 0.125 g K2HPO4, and 5 g agar were dissolved in 248 ml of distilled water and autoclaved. Before pouring 0.5 ml cholesteral, 0.5 ml Daumone 1 (asc#1 “C7”), 0.5 ml Daumone 2 (asc#2 “C6”), and Daumone 3 (asc#3 “C9”) were added (Ludewig et al. 2013). Bactotryptone was not included in the pheromone plates to limit the growth of bacteria. After 1 day, 100 μl of saturated OP50 culture was seeded and incubated in room temperature for another day and stored at 4°C for up to 1 month. For inducing dauer formation, 7–10 young adults were placed on a pheromone plate and incubated at 25°C for 4 days. For inducing dauer formation in the daf-2(e1370) background, worms were not placed in pheromone plates but NGM plates instead. Several L4 worms were moved to a new NGM plate and incubated at 15°C for 2 days. After L4 worms were grown into adults and laid enough numbers of eggs, the NGM plate was incubated at 25°C for 4 days. Wild-type and mutant dauers were selected based on their radially constricted morphology and darkened appearance. To demonstrate that selected animals were dauers, survival in 1% SDS was tested by incubation for 1 h.

Quantification of nictation behavior

For quantifying the nictation behavior, the protocol from Lee et al. (2011) was followed. Fifty dauer larvae were harvested with M9 buffer and transferred to the microdirt chip. After 30 min, the nictation ratio of each worm was tested for 1 min, and the tested worm was eliminated from the microdirt chip. For each set of experiments, 15 worms were tested and this procedure was repeated 2–3 times.

Quantification of dauer quiescence

On the dauer-induced pheromone plate, the total number of dauers and the number of moving dauers were counted. After 3 measurements on 1 plate, the average value was recorded as a representative value. Independent experiments were repeated 3 times.

Feeding RNA interference method

Clones of C. elegans RNA interference library were from Ahringer or Vidal Library. All the RNAi clones were carried by HT115 bacterial cell line. Each RNAi cell was streaked and cultured on LB containing ampicillin, and transcriptional activation was induced by 1 mM IPTG. rrf-3(pk1426);daf-2(e1370) double mutant was used to conduct RNAi screening in the dauer stage. Worms were placed on RNAi plates at the L4 stage and incubated at 15°C. After 1 generation, L4 stage worms were transferred to new RNAi plates. After 2 days, young adults with eggs were moved to 25°C, and dauer formation was induced. After 4 days, dauer larvae outside the Escherichia coli lawn were harvested and placed on aldicarb plates for the assay.
Aldicarb assay
100 mM aldicarb stock was dissolved in 70% ethanol. For 250 ml of aldicarb plates, 0.5 g NaCl, 0.75 g KH₂PO₄, 0.125 g K₂HPO₄, and 5 g agar were dissolved in 250 ml of DW and autoclaved. Before pouring, add 250 µl of 100 mM aldicarb stock (for a final concentration of 0.1 mM aldicarb). One day after pouring, 0.1 mM aldicarb plates were stored at 4°C for up to 1 month. For the comparison of aldicarb sensitivity of dauer larvae, 10 worms were harvested with M9 buffer and placed on the 0.1 mM aldicarb plate. After 70 or 80 min, moving worms on the plate were counted. Every experiment was repeated 3 times.

EMS mutagenesis
To isolate aldicarb-resistant dauer mutants, random mutagenesis was conducted using EMS. daf-2(e1370) animals were mutagenized. Young adult worms of daf-2(e1370) were treated with a final concentration of 50 mM EMS for 4 h and washed with M9 buffer. After EMS treatment, samples were treated with an inactivation solution containing sodium bisulfate (806 mM) and NaOH (100 mM). Mutagenized P₀ worms were placed on half-seeded NGM 100 mm plates. The total number of F₁ progeny was about 37,800. When F₁ worms lay eggs, they were moved to 25°C. After 4 days, dauer-induced F₂ offspring worms were harvested with M9 buffer and placed on 0.1 mM aldicarb-containing 100-mm plates. After 80 min, each moving worm was selected and placed on NGM plates, respectively, and was incubated at 15°C for dauer recovery.

Whole genome sequencing
To identify the causal genes of daf-2(e1370);ys51 and daf-2(e1370);ys52, whole genome sequencing was conducted. daf-2(e1370);ys51 and daf-2(e1370);ys52 were outcrossed with daf-2(e1370) 2 times independently. F₂ homozygote mutants were screened through the aldicarb-resistant phenotype as described above. At the second outcross, more than 10 independent homozygote mutants were isolated for each allele. Genomic DNA was extracted for each mutant, and the same amounts were mixed. Finally, sequenced samples were daf-2(e1370);ys51 and daf-2(e1370);ys52. The Illumina sequencing condition was HiSeq4000/100PE/5Gb. For the process of raw data, MAQGene software and Wormbase WS195 reference genome were utilized (Bigelow et al. 2009). The final “grouped” information was exported to an excel file, and all the mutation information was grouped into chromosomes. For each allele, all the mutations shared with any other alleles were excluded, and allele-specific mutations were sorted. For each allele-specific mutation, the % homozygosity was obtained by dividing the number of variants read into the number of wild-type reads. Mutations with % homozygosity over 95 were sorted. Then, C→T or G→A substitutions, which are usually induced by EMS mutagenesis, and missense or premature stop classes were finally sorted.

Results
Dauers are more sensitive to cholinesterase inhibitors, but not to an acetylcholine agonist
In adult worms, paralysis has been observed in 0.5–1.5 mM concentration of aldicarb (Mahoney et al. 2006; Oh and Kim 2017). However, 0.1 mM aldicarb induces uncoordinated movement but does not induce paralysis. We tested whether a subthreshold, 0.1 mM aldicarb induced paralysis in other developmental stages including L3, L4, and young adult. And, we discovered that 0.1 mM aldicarb-induced paralysis was induced only in dauer, a hibernation stage after harsh conditions (Fig. 1, a and b, and Supplementary Fig. 1a). The dauer-specific aldicarb hypersensitivity was a surprising result because dauer is known to be impervious to many external chemicals. Indeed, we confirmed that dauer had more resistance than L3 to 7% ethanol, which induces paralysis by changing neuronal activity (Choi et al. 2016) (Fig. 1c). We compared the responses of dauer larvae to another kind of cholinesterase inhibitor, trichlorfon. It is an organophosphate cholinesterase inhibitor with a different chemical structure from that of aldicarb (Nguyen et al. 1995). We confirmed that dauer larvae were sensitive to trichlorfon as well (Fig. 1d). It shows that dauer is sensitive to both classes of cholinesterase inhibitors, carbamate and organophosphate. Overall, it suggests that the mechanism related to acetylcholine transmission of dauer is distinct from that of nondauer. Paralysis due to the excess accumulation of acetylcholine occurs primarily through levamisole-sensitive nicotinic Ach receptors expressed in muscle (Rand 2007; Treinin and Jin 2021). In contrast to dauer-specific aldicarb hypersensitivity, dauer did not show significant hypersensitivity upon levamisole compared to L3, and some dauers were still moving even when L3 showed complete paralysis (Fig. 1e). It suggests that dauer-specific aldicarb hypersensitivity is not due to the regulation of postsynaptic levamisole-sensitive AchRs. Next, we investigated whether changes in synaptic transmission in dauer were maintained during subsequent developmental stages. When a dauer encounters a favorable condition, it develops into an adult after passing through postdauer-L₄ (PD-L₄). Unlike dauer, PD-L₄ had aldicarb resistance similar to that of normally developed L₄ (Fig. 1f). It demonstrates that the increased synaptic transmission in dauer is not imprinted from the experience of early development but is rapidly changed by adapting to a new environment.

dach-1, a cytochrome P450 gene, regulates the dauer-specific aldicarb sensitivity
To elucidate the mechanism of developmental stage-specific alteration in acetylcholine transmission, we performed a forward genetic screening for dauer-specific aldicarb-resistant mutants (Fig. 2a). To induce dauer formation on large scale without dauer-inducing pheromone, a dauer-constitutive mutant, daf-2(e1370) was used for mutagenesis (Riddle et al. 1981). daf-2 is the C. elegans insulin/IGF receptor homolog and the mutation induces dauer at the restrictive temperature (Kimura et al. 1997; Riddle et al. 1981). Similar to wild-type dauer, daf-2(e1370) dauer was sensitive to aldicarb compared to L₃ (Supplementary Fig. 1b). We conducted EMS mutagenesis following the standard protocol (Brenner 1974). The number of F₁ progeny from 420 P₀ worms was 37,800 when laying enough F₂ eggs at permissive temperature. Then, we induced dauer by transferring the F₂ eggs at the restrictive temperature and then screened aldicarb-resistant F₂ dauer (Fig. 2a). After discarding the aldicarb-resistant mutants that show phenotype both in dauer and adult, 2 independent mutants that showed phenotype only in dauer were isolated—ys51 and ys52.

Microscopy
Fluorescence images were taken through a confocal microscope (ZEISS LSM700; Carl Zeiss, Inc). Worms were harvested with 2.5 mM levamisole (dissolved in M9 buffer) and placed on an agarose pad.
Each mutant was outcrossed with daf-2(e1370), and whole genome sequencing was conducted to identify the causal variant for each mutant. Genomic DNA from pooled recombinants was sequenced, and homozygous mutations were sorted and analyzed (Doitsidou et al. 2010) (Supplementary Fig. 2). As the result, we have discovered that ys51 and ys52 shared distinct nonsense mutations in T09H2.1, a cytochrome P450 gene cyp-34A4 (Fig. 2c). ys51 and ys52 failed to complement each other, confirming that they are 2 different alleles of the T09H2.1 mutant (Supplementary Fig. 3). Also, we found that knockdown of T09H2.1 with feeding RNAi showed the aldicarb resistance in the rnf-3(pk1426);daf-2(e1370) background (Fig. 2d). In addition to aldicarb, we found that the dach-1 mutant dauer is resistant to trichlorfon (Supplementary Fig. 4). On the other hand, dach-1 dauer was similar to N2 dauer for ethanol-induced paralysis (Supplementary Fig. 5). Taking these results together, we concluded that cyp-34A4 regulates dauer-specific alteration in acetylcholine transmission and named the gene dach-1 (dauer-specific Ach transmission defect-1).

**dach-1 is expressed in epidermis from dauer and can exhibit cell nonautonomous effects**

To elucidate the role of dach-1, we analyzed its spatiotemporal expression. The transcriptional reporter of the 1.2-kb sized dach-1

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**Fig. 1.** Altered acetylcholine transmission in dauer stage of *C. elegans*. a) Dauer animals (n = 61) are sensitive to aldicarb compared to L3 stage animals (n = 60). b) Dauer animals (n = 21) are sensitive to aldicarb compared to L4 stage animals (n = 19). c) Dauer animals (n = 147) are resistant to 7% ethanol compared to L3 animals (n = 130). d) Dauer animals (n = 59) are sensitive to trichlorfon compared to L3 animals (n = 55). e) Dauer animals (n = 55) are not sensitive to levamisole compared to L3 (n = 61). f) Postdauer-L4 animals (n = 30) are not sensitive compared to dauer (n = 31). Error bar indicates SE. Statistical analysis is performed by log-rank (Mantel-Cox) test.
promoter with GFP is only expressed in the intestine from the nondauer, which is consistent with the previously reported expression pattern of the 2.0-kb sized dach-1 promoter (Joshi et al. 2021). During dauer, the transcription of dach-1 showed a robust increase in nonintestinal tissues (Fig. 3b). We confirmed that the epidermis is the tissue in which dach-1 expression is specifically upregulated in dauers by colocalization with Pdpy-7, an epidermis-specific promoter (Fig. 3c). The aldicarb-resistant phenotype of the dach-1 mutant was rescued when the wild-type genomic regions of dach-1 were expressed in the mutant using the same promoter used in transcriptional reporter (Fig. 3d). Consistently, we confirmed that rescue was successfully achieved when the wild-type dach-1 genomic region was expressed using an epidermis-specific promoter (Pdpy-7). Furthermore, rescue experiments were performed by ectopic expression of the wild-type DACH-1 in body wall muscle (Pmyo-3) or the nervous system (Pegl-3). Although the dach-1 transcriptional reporter was not expressed in neurons or muscles, the ectopic expression of dach-1 genomic regions in muscles or neurons sufficiently rescued dach-1 mutant. Next, we tested whether the role of dach-1 in aldicarb treatment is truly mediated by the level of endogenous acetylcholine, which is released onto NMJ. Released acetylcholine is normally degraded by cholinesterase. If cholinesterase is mutated, the level of extrasynaptic acetylcholine is increased. We confirmed that ace-3, one of the cholinesterase mutants, suppresses the aldicarb-resistant phenotype of dach-1 (Rand 2007; Treinin and Jin 2021) (Fig. 3c). Overall, the results indicate that although dach-1 is not expressed in neurons or muscles, its cell nonautonomous effect can influence the aldicarb sensitivity related to cholinergic transmission in NMJ.

Fig. 2. dach-1 is the cytochrome P450 regulating acetylcholine transmission in the dauer stage. a) Experimental scheme of forward genetics for dauer-specific aldicarb-resistance. b) Compared to daf-2(e1370), ys51 and ys52 mutants show aldicarb-resistant phenotype in the dauer stage. The percentage of animals not paralyzed after 80 min of aldicarb treatment was compared. n = 4 for each experiment. Error bar indicates SE. ****P < 0.0001 (1-way ANOVA, Dunnett’s posttest). c) ys51 and ys52 harbor distinct nonsense mutations in T09H2.1. d) Feeding RNAi of T09H2.1 in a RNAi-sensitive rrf-3;daf-2 phenocopied aldicarb-resistant phenotype in the dauer stage. The percentage of animals not paralyzed after 80 min of aldicarb treatment was compared. n = 6 for both experiments. Error bar indicates SE. ***P < 0.001 (unpaired t-test).
encodes a cytochrome P450 enzyme; therefore, its enzyme products are expected to play a role in the paracrine effect from the epidermis. The rescue effect of dach-1 by ectopic expression in the neuron and muscle suggests that the substrate of dach-1 may exist in other tissues as well as in the epidermis. Another C. elegans cytochrome P450 gene, daf-9, synthesizes a steroid hormone called dafachronic acid from cholesterol, which inhibits dauer entry (Gerisch et al. 2001). Accordingly, when cholesterol depletion occurs, dauers remain in quiescence because DAF-9 cannot synthesize dafachronic acid. We investigated whether dach-1, similar to daf-9, is involved in the synthesis of cholesterol-derived steroid hormone and modulates aldicarb sensitivity. We found that the aldicarb sensitivity of dauer did not change under the condition of cholesterol depletion (Supplementary Fig. 6). Thus, dach-1 appears to regulate the aldicarb sensitivity through mechanisms other than steroid hormone metabolism.

Fig. 3. dach-1 is increased in epidermis in the dauer stage and acts in cell nonautonomous manner. a) The 864-bp upstream from start codon of dach-1 was fused to a GFP minigene. Expression of dach-1 was shown in intestine at the L4 stage. Bar, 20 μm. b) dach-1 promoter showed increased expression at the dauer stage. Bar, 20 μm. c) dach-1 promoter was coexpressed with epidermis marker Pdpy-7::mCherry. Their expressions overlapped in epidermis. Bar, 20 μm. d) Tissue-specific expression of dach-1::SL2::GFP construct in dach-1 (n = 5), dpy-7 (n = 3), myo-3 (n = 6), and egl-3 (n = 6) promoter all rescued aldicarb-resistant phenotype of dach-1 mutants (n = 4) compared to wild-type N2 (n = 3). e) ace-3 fully suppresses the aldicarb resistance of dach-1. n = 3 for each experiment. d, e) The percentage of animals not paralyzed after 80 min of aldicarb treatment was compared. Error bar indicates SE. ****P < 0.0001 (1-way ANOVA, Dunnett’s posttest).

Pleiotropic effects of dach-1 mutation on dauer-specific phenotypic plasticity

We then investigated whether the dach-1-dependent altered neurotransmission is important for phenotypic plasticity of dauer in the physiological conditions. Dauer is the hibernation-like stage of C. elegans with quiescent movement (Cassada and Russell 1975; Gaglia and Kenyon 2009). Most dauers remain in quiescence, but they can react quickly and move normally after receiving a physical stimulus. Although there was no significant difference in movement and harsh touch sensitivity of dach-1 dauers compared to wild-type dauers, we found that dach-1(ys51) dauers showed relatively decreased quiescence (Fig. 4a). When dauers move around 3-dimensional obstacles, they can raise their heads and wave body, which is called nictation (Lee et al. 2011; Yang et al. 2020). Acetylcholine transmission from IL2 is important for nictation (Lee et al. 2011). Also, acetylcholine plays a
role in dauer entry (Lee et al. 2014). However, neither nictation nor dauer entry was affected by dach-1 (Supplementary Fig. 7). It shows that dach-1-induced synaptic transmission changes do not significantly affect the movement of dauer in laboratory culture conditions.

Another important characteristic of dauer is stress resistance to harsh environments (Golden and Riddle 1984; Narbonne and Roy 2009). Previous studies in C. elegans have shown that stress resistance can be regulated by neuronal signaling (Kim and Jin 2015). Interestingly, dach-1(ys51) dauers were highly susceptible to heat shock and high osmotic stress (Fig. 4, b and c and Supplementary Fig. 8). It suggests that the dauer-specific induction of dach-1 is crucial for the stress response of dauer in harsh conditions. In previous C. elegans studies, little was known about the importance of acetylcholine in stress resistance. However, like other aldicarb-resistant mutants, it is possible that dach-1 may be involved in overall synaptic transmission. Therefore, dauer-specific synaptic plasticity may have modulated stress resistance. However, since acetylcholine-depleted mutants such as unc-17 and cha-1 are more lethargic, the high rate of spontaneous movement in dach-1 is probably not due to cholinergic transmission. Peptidergic signaling in RIS neurons induces dauer sleep-like behavior, similar to sleep-like behavior in other developmental stages (Wu et al. 2018). Therefore, the decreased lethargus of dach-1 is likely due to the decreased neurotransmission of RIS or other sleep-inducing neurons.

We found that dach-1 not only regulates dauer-specific aldicarb hypersensitivity but also plays a pleiotropic effect in stress resistance of dauer. Dauer must survive long without eating. The increased neurotransmission in the dauer analyzed from cholinesterase inhibitors seems paradoxical because dauer must use its energy resources efficiently. The stress hypersensitivity of the dach-1 mutant raises the possibility that the use of energy resources at the synapse was compensated for the survival in stressful environments. In the future, the detailed mechanism underlying the pleiotropic effect may be further explored by examining the direct relationship between the regulation of synaptic activity and stress resistance.

In this study, we found that dauers were hypersensitive to cholinesterase inhibitors. The proposed function of dach-1 described in this study would explain the mechanism how dauers become more sensitive to these inhibitors to some extent, but it is not clear what specific changes occur in acetylcholine signaling in the NMJ of dauers. Considering that dauer is not hypersensitive to levamisole, 1 possibility is that the presynaptic activity of motor neurons is changed. Alternatively, the relative levels of several acetylcholine receptors expressed in muscle may have

**Discussion**

In this study, we reported a previously unidentified aldicarb resistance gene specific for dauer. Previously identified aldicarb resistance genes in the adult stage are in most cases genes expressed in neurons, whereas dach-1 is a gene expressed in the epidermis. It suggests that dauer-specific aldicarb resistance is regulated by a mechanism different from that in adults. Consistently, most aldicarb-resistant mutants at the adult stage had a mild or severe locomotion phenotype, whereas in the case of dach-1, no significant difference was found in general locomotion. We discovered that the dach-1 dauers exhibited less quiescent behavior than wild-type dauers. Since acetylcholine-depleted mutants such as unc-17 and cha-1 are more lethargic, the high rate of spontaneous movement in dach-1 is probably not due to cholinergic transmission. Peptidergic signaling in RIS neurons induces dauer sleep-like behavior, similar to sleep-like behavior in other developmental stages (Wu et al. 2018). Therefore, the decreased lethargus of dach-1 is likely due to the decreased neurotransmission of RIS or other sleep-inducing neurons.

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changed. Another possibility is that the aldicarb sensitivity may differ because the dauer has a completely different connectome compared to the nondauer stages.

In this study, we conducted forward genetics and discovered that cyp-34A4 mutant dauer was resistant to aldicarb. It is well established that cytochrome P450 is involved in xenobiotic detoxification (Anzenbacher and Anzenbacherova 2001). In this case, one possible function of dach-1 is detoxification of aldicarb. However, if that were the case, dach-1 mutants would be more sensitive to aldicarb than resistant. On the other hand, it is possible that the cytochrome P450 enzyme activity of dach-1 modifies aldicarb to increase its function as a cholinesterase inhibitor. However, we showed that dach-1 mutant dauer is resistant not only to aldicarb but also to trichlorfon, another class of cholinesterase inhibitor. It raised the possibility that dach-1 may regulate the acetylcholine transmission process itself rather than being involved in the biochemical reaction of exogenous cholinesterase inhibitors. However, since synaptic transmission was not directly investigated in dach-1 mutants in this study, how a single cytochrome P450 enzyme modulates aldicarb sensitivity is an interesting question to be answered in future studies.

Another role of cytochrome P450 is to participate in the metabolism of cholesterol or polyunsaturated fatty acids to produce intercellular signaling molecules including steroid hormones or eicosanoid molecules (Anzenbacher et al. 2001; Konkel and Schunck 2011; Spector and Kim 2015). Since cholesterol seems to be not involved in dauer-specific aldicarb hypersensitivity, dach-1-dependent metabolites from polyunsaturated fatty acids may be involved in acetylcholine transmission. Molecules such as EETs generated through cytochrome P450 metabolism can convey autocrine or paracrine signaling through TRPV channels or ion channels in surrounding cells (Spector and Kim 2015). Hence, the epidermis induction of dach-1 at the dauer stage identified in our study is likely to play a role in paracrine signaling. Based on the anatomy of C. elegans, molecules secreted from the epidermis can be easily transmitted to other neurons and muscles via the pseudocoelom. Also, in C. elegans studies, it has been well established that signals from the epidermis regulate glia growth, which in turn regulates the synaptic position of neurons (Shao et al. 2013). Therefore, we speculate that the role of the epidermis regulated through dach-1 may be similar to that of glia or supporting cells in the mammalian brain. Several cytochrome P450s are also expressed in glial cells and are known to be involved in brain inflammation and brain tumors. It would be interesting if the mammalian cytochrome P450 in glia also plays a role in synaptic transmission.

A recent study using C. elegans discovered that the transcription of intestinal dach-1 is regulated by serotonin or dopamine, and the putative signaling molecules generated by cyp-34A4 regulate the unfolded protein response in the epidermis (Joshi et al. 2021). The role of dach-1 in the epidermis regulating aldicarb sensitivity suggests that a feedback loop exists between the function of nervous system and the nonneuronal expression of dach-1. Another possibility is that dach-1 may play different roles in distinct tissues at different stages of development. The study of the neuron-to-nonneuronal signaling system involving cytochrome P450 is expected to be of interest in the future. Genes regulating the formation of dauer have been extensively studied through random mutagenesis screening (Fielenbach and Antebi 2008), but studies on genes that change physiology after becoming dauer have been rarely performed. Through random genetic screening, our study found a single gene that regulates developmental plasticity that occurs only at specific stages of development. Overall, our study suggests a new function of cytochrome P450 in the nervous system during development.

Data availability
Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. The sequencing results generated for this study were deposited in Korean Nucleotide Archive (KoNA, https://kobic.re.kr/kona) with the accession ID of PRJKA220467. Supplemental material is available at G3 online.

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Author contributions
SS, M-KC, and JL established the concept of the study. SS and M-KC performed the random mutagenesis and genetic analysis. SS performed the genetic mapping, the molecular biology experiments, the nictation, and lethargus assay. M-KC performed the stress resistance assay. DSL drew the schematic images. JS analyzed the whole genome sequencing data. SS, M-KC, and JL analyzed the data and wrote the manuscript. SS and M-KC contributed equally to this work.

Conflicts of interest
None declared.

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