The transcription factor unc-130/FOXD3/4 contributes to the biphasic calcium response required to optimize avoidance behavior

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The central neural network optimizes avoidance behavior depending on the nociceptive stimulation intensity and is essential for survival. How the property of hub neurons that enables the selection of behaviors is genetically defined is not well understood. We show that the transcription factor unc-130, a human FOXD3/4 ortholog, is required to optimize avoidance behavior depending on stimulus strength in Caenorhabditis elegans. unc-130 is necessary for both ON responses (calcium decreases) and OFF responses (calcium increases) in AIBs, central neurons of avoidance optimization. Ablation of predicted upstream inhibitory neurons reduces the frequency of turn behavior, suggesting that optimization needs both calcium responses. At the molecular level, unc-130 upregulates the expression of at least three genes: nca-2, a homolog of the vertebrate cation leak channel NALCN; glr-1, an AMPA-type glutamate receptor; and eat-4, a hypothetical L-glutamate transmembrane transporter in the central neurons of optimization. unc-130 shows more limited regulation in optimizing behavior than an atonal homolog lin-32, and unc-130 and lin-32 appear to act in parallel molecular pathways. Our findings suggest that unc-130 is required for the establishment of some AIB identities to optimize avoidance behavior.

Behavioral optimization has a vital function shared by many species: it enables the central nervous system to take reasonable action in response to environmental information. Optimization of avoidance responses to harmful stimuli plays a critical role in defense, and failure directly leads to life-threatening situations. Most animals choose various avoidance behaviors, such as reflexes, retreats, and U-turns, depending on the situation. The neural circuit that evaluates risk to decide the appropriate behavior is not fully understood because of the complexity of these responses compared to simple all-or-none responses. Thus, elucidating the neural and molecular basis is a great challenge.

The zebrafish (Danio rerio) and the nematode Caenorhabditis elegans (C. elegans) have been used as model animals to elucidate the mechanism at the synaptic level. In zebrafish, two Mauthner cells in the hindbrain directly control the muscles and determine the escape direction. Mauthner cells can alter glycine receptor expression in a stimulus-dependent manner in addition to following the genetic and developmental programs. The excitation levels of the Mautner cells vary in response to stimulus intensity, but their association with behavioral changes remains unclear.

Previously, we reported that C. elegans primarily uses three types of avoidance behaviors depending on the stimulus intensity: short reversals, long reversals, and omega turns. C. elegans has an advantage as a model animal because all synaptic connections are anatomically described. The following question remains: how is information processed, and how does it drive behavioral output at the synaptic level? We aimed to reveal a simple prototypical neural circuit at the synaptic level that optimizes avoidance behavior in C. elegans by using a complete neural wiring diagram and genes with human orthologs in OrthoList.

As part of a neural circuit, ASH sensory neurons mainly perceive nociceptive stimuli, which is the first step, and excite at least three downstream circuits. In the first circuit, ASHs directly form glutamatergic synapses with AIB interneurons, which increases the probability of omega turns. In the second circuit, ASHs indirectly drive
Mild osmolarity → Reversal

High osmolarity → Omega turn

Wild type

Unc-130

Harsh tap → ?

Harsh tap

ASH::ChR2(H134R)

Omega turn

Behavioral frequency for 4 M sorbitol (%)
**Figure 1.** Unc-130 is necessary for turning behavior during optimization of avoidance behavior. (a) A schematic of optimization of avoidance behavior in *C. elegans*. During exposure to a mild osmotic stimulus, the animal exhibits a reversal behavior, such as body retraction or backward movement. Strong stimulation induces body bending, causing an omega turn so that the animal can return to its original location. (b) In wild-type *C. elegans*, the total frequency of avoidance increased with increasing sorbitol concentration (*P* < 0.001 in 0 M vs. 2–6 M, 1 M vs. 2–4 M, n = 11, 12, 15, 17, 20 (0–6 M, respectively)). In particular, the omega turn frequency was significantly higher under 2–6 M compared to 0 M, under 4 M compared to 1 M, and under 6 M compared to 1 M (*P* < 0.001, *P* < 0.01, *P* < 0.001). (c) Nematodes with a null mutation in the unc-130 had a lower omega turn frequency than wild-type animals (Fig. 1b) (*P* < 0.05 in 1 M and 6 M; n = 12, 14, 20). On the other hand, the total avoidance rate (sum of reversals and omega turns) was similar to that of wild-type animals because of the increase in reversal frequency in all conditions (*P* > 0.05). (d) A schematic of the harsh tap assay. A platinum wire placed in the forward direction is lightly applied to the tip of the nematode’s nose. *e. unc-130* mutants showed the omega turn rate comparable to the wild-type animals. *P* = 0.392 (n = 10, 10, t-test). (f) Schematic of the analysis method using channelrhodopsin 2 (CHR2[H134]). CHR2 (H134) was selectively expressed in primary nociceptive sensory neurons (ASH sensory neurons). Exposure to blue light to selectively activate ASH sensory neurons and their downstream neurons. (g) Adding all-trans retinoic acid (ATRA) and blue light triggered turn behavior. *unc-130* mutants had a significantly lower turn frequency than the wild-type animals. *** indicates *P* < 0.001, * indicates *P* < 0.05 (*n* = 13, 14, 14, 14). (h) Self-promoter-driven UNC-130 expression rescued the decreased turn frequency of *unc-130* mutants. See Fig. S1–l. *** indicates *P* < 0.001, * indicates *P* < 0.05, ns indicates *P* > 0.05 (n = 6, 6, 4). All statistical analyses were performed by Kruskal–Wallis test with Dunn’s multiple comparisons test as a post hoc test. The error bars in the figures represent the ± SEM.

AIB activity through cholinergic AIA inhibitory interneurons, but the neurotransmitter receptor between AIAs and AIBs is unidentified. In the last circuit, ASHs form glutamatergic synapses with AVA interneurons, whose ON responses and OFF responses (calcium increases) induce reversals and omega turns. AIBs can also affect the OFF-calcium response in the AVA neurons via chemical synapses. AIBs and AVs coordinate muscle contractions required for turns via motor/interneurons. We have reported that a lack of AIB cells or gap junction *innexin-1* (inx-1) in AIBs reduces turn frequency but increases reversal frequency. These results suggest that AIBs play a central role in optimization and that electrical synapses are components involved in turning. Calcium imaging using a multilucid system has revealed that AIBs show strong calcium induction after a hyperosmotic stimulus following a decrease during stimulation.

AIB neurons develop in two steps. First, transcription factors encoded by proneural genes determine progenitors’ neuronal fates, and then the progenitors differentiate into mature neurons with unique properties through the combined activity of several transcription factors. Previously, we screened 210 neural transcription factors and found that *abnormal cell Lingeage 32, lin-32*, a nematode homolog of the proneural gene *atonal/ATOH1*, is essential for the optimization of avoidance behaviors via AIB neuron development. lin-32 mutants lack a number of neural function genes in AIBs, and lin-32 may contribute to the initiation of neurogenesis rather than the determination of AIB neuron identity. Therefore, transcription factors that establish the unique features of AIBs have been left unexplored.

Here, we report that *unc-130*, an ortholog of the forkhead transcription factor 3 or 4, *FOXD3*/*FOXD4*, plays an essential role in avoidance optimization, mainly by adjusting turn frequency. *unc-130* null mutants lack both ON responses (calcium decreases) and OFF responses (calcium increases) in AIBs. Inhibitory AIAs affect turn frequency, implying their involvement in AIB ON responses (calcium reductions). OFF responses (calcium increases) in the AVs directly downstream of the AIBs may depend on the AIBs. We report that *unc-130* upregulates three genes: the putative *Nematode Calcium channel 2* (*nca-2*), a homolog of the vertebrate cation leak channel *NALCN*, AMPA-type glutamate receptor 1 (*glt-1*)7,22, and *eat-4*, a hypothetical L-glutamate transmembrane transporter23. However, it does not upregulate *synaptobrevin* (*sub-1*)22,24 or electrical synapse *innexin 1* (*inx-1*)9,10. Our results suggest that *unc-130* partially determines AIB neuron identity to aid in the optimization of avoidance behaviors.

**Results**

A *FOXD3*/*FOXD4* ortholog, *unc-130*, affects the frequency of turn behavior during avoidance optimization. *C. elegans* tends to show reversal behavior in response to mild osmolarity changes but is biased toward omega turns in a manner correlating with stimulus strength (Fig. 1a). In our previous paper, we mentioned that *unc-130* null mutants exhibited reduced avoidance frequency in response to noxious stimuli. However, it remained unclear whether *unc-130* affected the choice of appropriate behavior or simple locomotion. To clarify this issue, we analyzed the avoidance behavior patterns in response to four gradient osmotic concentrations of noxious stimuli. The results clarified that the *unc-130* mutants were less likely to take omega turns upon hyperosmotic stimulation than wild-type animals (Fig. 1b and c; Fig. S1a–h). Instead, the number of reversals increased in the mutants (Fig. 1c). The critical point is that the *unc-130* mutants had no significant changes in the sum of omega turn and reversal frequencies, suggesting that they can avoid stimuli but not optimize how to do so (Fig. 1b and c; Fig. S1a–h).

Meanwhile, such behavioral phenotype left the possibility that the *unc-130* mutant was unable to make the omega turn because of its uncoordinated movements. We hypothesized that the omega turn might occur by a more intense stimulus. If this were the case, we could conclude that the uncoordinated movements were not the reason for the inability to turn. In the drop test, it was difficult to use a sorbitol solution thicker than 6 M due to the viscosity. Therefore, to investigate whether the *unc-130* mutant can mediate the omega turn as well as the wild-type animals, we established and performed an original harsh tap assay with a platinum wire. In this...
Studies, calcium imaging has been performed in AIA neurites. One reason is that AIA neurites, but not the increased calcium concentrations in AIA neurites during osmotic stimulation. In previous stimuli have not yet been reported, we started by observing the detailed patterns in wild-type animals. We found on a central neuron defect rather than "primary hypoalgesia," a simple decrease in perception. We also analyzed that via inhibition of AIBs during the stimulus.

In the drop test, a sorbitol solution accompanying touch or mechanical stimuli might trigger sensory neurons other than ASHs. To confirm whether the optimization defect in unc-130 mutants is dependent on noxious stimuli, we observed the behavior induced by selective excitation of ASH sensory neurons using channelrhodopsin-2 (Fig. 1f). Optogenetic excitation separate from touch or mechanical stimuli reproduced the optimization defect (P < 0.001, n = 13, 14, 14, 14, Kruskal–Wallis test with Dunn's multiple comparisons test as a post hoc test) (Fig. 1g), suggesting that unc-130 optimizes avoidance patterns in response to the strength of the noxious stimuli. UNC-130 driven by its promoter rescued the behavior of the null mutants, indicating that the optimization defect was due to the loss of unc-130 gene function (P < 0.05, n = 6, 6, 4, Kruskal–Wallis test with Dunn's multiple comparisons test as a post hoc test) (Fig. 1h; Figs. S1i–1 and S2a–e).

unc-130 is necessary for biphasic calcium responses of the central neurons for behavioral optimization. Previously, we showed that AIB neurons are the central neurons for turning and the biphasic response involving a calcium reduction during osmotic stimulation and an increase after stimulation. Considering that unc-130 expression is relatively restricted in cell lineages including AIB cells (ABplaapa, ABpraapa), during embryogenesis, we hypothesized that the optimization defect of unc-130 mutants likely resulted from AIB neuronal dysfunction. To test this hypothesis, we attempted to analyze AIB neural responses, including calcium reductions during stimulation, using the calcium indicator inverse-pericam 2.0 (IP2.0), whose fluorescence intensity increases as the calcium concentration decreases. As we expected, the fluorescence intensity of IP2.0 increased during osmotic stimulation and decreased after stimulation in wild-type animals, showing that AIBs received inhibitory input during stimulation and excitatory input after the stimulus was removed (Fig. 2a and Fig. S3a). In contrast, the unc-130 mutants showed no responses (Fig. 2a). Baseline fluorescence values before correction was close to minimum IP2.0 fluorescence values (Fig. S3b and c) (P = 0.077, n = 14, 18, t-test), implying relatively high resting calcium level in both wild-type and unc-130 animals (P > 0.05, n = 14, 18, t-test). These data suggest that reduced responses in AIB neurons cause the optimization defect in unc-130 mutants.

Increases in calcium in AIBs induce turning, but it remains unclear whether the AIB ON response (calcium decrease) contributes to the turning behavior. In C. elegans, ASH sensory neurons indirectly inhibit AIBs via predicted AIA inhibitory interneurons. Therefore, we analyzed whether ablation of AIAs reduces AIB neuronal dysfunction. To test this hypothesis, we attempted to analyze AIB neural responses, including calcium reductions during stimulation, using the calcium indicator 4CaMP6s. There was no significant difference in the peak of ON calcium increase in the wild-type animals compared with unc-130 mutants (P = 0.892, n = 21, 23, t-test). (f) Calcium imaging of AVA neurons, one of the types of downstream AIB neurons. Wild-type animals showed a small ON calcium increase during stimulation and a large increase after stimulation (n = 20). In the unc-130 mutants, OFF calcium responses were abolished (n = 19). The area in orange color is enlarged in the right view. The error bars in these figures represent the ± SEM values.
**Figure 3.** *unc-130* upregulates the expression of putative cation channels in AIB neurons. (a) All-trans retinoic acid and blue light induce turning. The turning frequency was 77.9% in the wild type but only 15.3% in *unc-130* mutants. "" indicates *P < 0.001,* " indicates *P < 0.05* (n = 14, 14, 17, 15, Kruskal–Wallis test with Dunn’s multiple comparisons test as a post hoc test). (b) *nca-2* mutants showed significantly reduced turn frequencies in response to 4 M sorbitol than wild-type animals. "" indicates *P < 0.001* (n = 13, 11, t-test). (c) Expression of *nca-2* promoter-driven GFP in AIB cell bodies (arrowheads). *inx-1* promoter-driven mCherry was coexpressed as a cell identification marker for AIBs. *nca-2* showed weaker AIB expression in a typical *unc-130* animal than in a typical wild-type animal. Scale bar = 5 μm. (d) The ratiometric analysis of the intensity of *nca-2* promoter-driven GFP expression was significantly lower in AIBs of *unc-130* mutants than in those of wild-type animals. "" indicates *P < 0.001* (n = 10, 11, t-test). (e) AIB-selective expression of NCA-2a rescued the decreased turn frequency of *nca-2* mutants. ** indicates *P < 0.01* (n = 12, 12, 12, one-way ANOVA followed by Tukey’s post hoc test). See Fig. S7a–d. The error bars in these figures represent the ± SEM values. (f) Model diagram of the hypothetical function of *nca-2* in AIB. NCA-2 may involve the release of neurotransmitters from synaptic vesicles via cation influx.

response in the AIB. Then, we observed the AIA reaction in the *unc-130* mutants and clarified that there was no statistically significant variation in peak ΔF/F amplitude (*P = 0.892, n = 21, 23, t-test), although the response was slightly dampened in mutants compared within wild-type animals (Fig. 2e).

Finally, to understand the output function of AIBs on the avoidance circuit, we observed the calcium response in AVAs. AVAs receive input from the presynaptic AIB neurons (Fig. 2b). As expected, *unc-130* mutants showed remarkably impaired OFF responses (calcium increases) in AVAs (Fig. 2f). In our previous paper, we discussed how low ON responses (calcium increases) in AVAs during osmotic stimulation might induce reversal behavior independent of AIBs. Since the *unc-130* mutants can perform reversal behavior (Fig. 1c; Fig. S1f and g), we explored whether the AVA ON response (calcium increase) remains in the *unc-130* mutants. The *unc-130* mutants showed an ON response (calcium increase), although it was reduced in size (see the enlarged view of the area in Fig. 2f, inset), consistent with our hypothesis. These results suggest that AIBs are critical neurons for determining appropriate behaviors in response to various stimuli.

The inhibitory synaptic receptors on AIBs have not yet been identified. Since AIAs are cholinergic neurons, we first considered the possibility of the downregulation of inhibitory acetylcholine-gated chloride channels expressed on AIBs in the *unc-130* mutants. We observed the expression patterns of six hypothetical acetylcholine-gated or ligand-gated chloride channels to focus on the inhibitory channels expressed on AIB neurons. In this experiment, we found that all *unc-130* mutant animals expressed *innexin 1* (*inx-1*) on AIBs (n = 25); we used the *inx-1* promoter to mark AIBs and identify AIB-specific gene expression. AIBs expressed *acc-1* (*Acetylcholine-gated Chloride Channel 1*)39, *lgc-46* (*Ligand-Gated Ion Channel 46*, predicted to have chloride channel activity), and *lgc-49* (*Ligand-Gated Ion Channel 49*, predicted to have chloride channel activity)39 promoter-driven GFP (Fig. S4a). However, AIBs did not express *acc-2*, *acc-3*, or *acc-4* (*Acetylcholine-gated Chloride Channel 2, 3, or 4*, respectively)39 (Fig. S4b), nor did they express *lgc-47* or *lgc-48* (*Ligand-Gated Ion Channel 47 or 48*, respectively, predicted to have chloride channel activity)39. We analyzed the turn behavior of deletion mutants of *acc-1*, *lgc-46*, and *lgc-49*, but there were no differences between the single or double mutants and wild-type animals (Fig. S4c and d).

We next assumed a contribution of G protein-coupled acetylcholine receptors. We observed turn behavior in deletion mutants of *goa-1* (*G protein Q, alpha subunit 1*), an ortholog of human GNAO139, which exhibits G protein-coupled acetylcholine receptor activity39. In *C. elegans*, only *goa-1* encodes a member of the mammalian Gi/o class of Gs subunits, and the predicted amino acid sequence of *C. elegans* GOA-1 is over 80% identical to that of mammalian Goα. Hypothetical *goa-1* null mutants showed a lower turn frequency (13.3 ± 4.41%) than wild-type animals (56.3 ± 5.96) (*P < 0.001, n = 9, 8, t-test) (Fig. S5a), but the expression levels of *goa-1* promoter-driven GFP in AIBs were comparable in both strains (Fig. S5b and c) (*P = 0.450, n = 10, 9, t-test). Thus, we concluded that the reduction in turning was independent of the *goa-1* function in AIB neurons. In *C. elegans*, there are three types of muscarinic-type acetylcholine receptors: *gar-1* (*G-protein-linked Acetylcholine Receptor 1*)41, *gar-2*42, and *gar-3*43. Although we analyzed the behavior of triple gene mutants44, turn frequency was not significantly lower in these animals than in wild-type animals (Fig. S5d) (*P = 0.226, n = 9, 8, t-test). Consequently, we could not identify the AIA-AIB inhibitory receptors, but the possibility remains that an unknown G-protein coupled acetylcholine receptor participates.

**unc-130** upregulates the expression of a predicted cation channel. *unc-130* mutants showed no stimulus-dependent OFF responses (calcium increases) (Fig. 2a). Therefore, we tested whether the turn behavior could be recovered by cation flux through ChR2(H134R) selectively expressed in AIBs. Contrary to our expectations, turn behavior induction was minor in *unc-130* mutants (Fig. 3a). In optogenetics, cation flux is reported to be milder than that under natural stimulation45. Therefore, we proposed three hypotheses: 1. that there is dysfunction in the calcium signaling pathway in AIBs, 2. that there are reductions in presynaptic output from AIBs, and 3. AIBs are developmentally altered in *unc-130* mutants.

First, we tested the former. There are eight of nine predicted voltage-gated calcium channel subunits in *C. elegans*46. We excluded three genes from our analysis. One of them, *ccb-1* (*Calcium Channel, Beta subunit 1*), predicted to have high voltage-gated calcium channel activity, is not expressed in AIBs46. We performed a drop test for mutants of the other seven channels and found that only *nca-2* (*putative Nematode Calcium channel 2*, a homolog of the vertebrate cation leak channel NALCN) mutants showed a lower turn frequency in response to a 4 M sorbitol drop than wild-type animals (Fig. 3b; Fig. S6a and b) (*P < 0.001, n = 13, 11, t-test). The mutants for
nca-2 reduced fluorescence intensity compared with wild-type animals (Fig. 3c and d). The neural subsets expressed nca-2 relatively similar to that of wild-type animals (Fig. 3e; Fig. S7). Thus, we conclude that nca-2 the defect in formed a rescue experiment with the AIB-selective expression of the NCA-2a protein. The results revealed that neurons (Fig. 3f).

e dodge expression in the AIBs may upregulate synaptic transmission from AIBs to downstream inter-/motor is necessary for avoidance behavior optimization, suggesting a voltage-gated cation channel or a cation leak receptors in AIB neurons50. GLR-1 is the primary glutamate receptor between ASHs and AIBs. Among the wild-type animals, 65.0% of individuals expressed GFP in two AIB cells, while 35.0% expressed it in one (Fig. 4a and b). Notably, transgenic type animals, 65.0% of individuals expressed GFP in two AIB cells, while 35.0% expressed it in one (Fig. 4a and b); mutants showed a significant difference compared with the wild-type animals. This unc-130 mutants. “ns” indicates no significant difference (P = 0.262, n = 28, 24, t-test). (g) The ratio of total intensity of mCherry expression in AIB presynaptic regions was not different between wild-type and unc-130 mutants. “ns” indicates no significant difference (P = 0.326, n = 20, 20, t-test). The error bars in these figures represent the ± SEM values.

unc-130 upregulates the expression of functional molecules for glutamatergic synapses. Transcription factors frequently determine neural identity by regulating the expression of multiple genes46. To determine whether unc-130 contributes to the excitatory input from upstream neurons to the AIB, we observed glr-1 (Glutamate Receptor family (AMPA)1 promoter-driven GFP expression. glr-1 is one of the primary excitatory receptors in AIB neurons50. GLR-1 is the primary glutamate receptor between ASHs and AIBs. Among the wild-type animals, 65.0% of individuals expressed GFP in two AIB cells, while 35.0% expressed it in one (Fig. 4a and b). Notably, transgenic C. elegans carrying extrachromosomal transgenes frequently display mosaic expression47. Among the unc-130 mutants, only 5.0% of individuals expressed GFP in two AIB cells, while 52.4% expressed it in one (Fig. 4a and b); mutants showed a significant difference compared with the wild-type animals. This result indicates a reduction in excitatory synaptic input from the ASHs. We also tried to use the Pnx-1-driven markers, but the transgenic strains could not be maintained, so we used Podr-2 as the marker promoter in this experiment. AIBs are glutamatergic neurons51. To determine whether typical glutamatergic synaptic vesicles are formed in AIBs, we analyzed the expression level of eat-4 (a predicted L-glutamate transmembrane transporter), which fills vesicles with glutamate5. We detected a significant reduction in the fluorescence intensity of eat-4 promoter-driven mCherry in the AIBs of unc-130 mutants (P < 0.05, n = 20, 20, Mann–Whitney test) (Figs. 4c and d), suggesting that unc-130 is required for the release of proper amounts of glutamate. A decrease in the expression of eat-4 might cause the decrease in turns upon AIB-ChR2 stimulation shown in Fig. 3a.

unc-130 does not alter the expression of electrical synapses in AIBs. Previously, we reported that lin-32 upregulates a broad spectrum of genes in AIB neurons, including the dominant electric synapses inx-1, and that its mutation causes secondary hypoesthesia similar to that caused by unc-130 mutation5. Therefore, we analyzed the property of inx-1 positive cells, expression rate, cell morphology, and expression intensity, as one AIB indicator of functional overlap between unc-130 and lin-32. First, in terms of expression rate, GFP-positive cells were observed in all unc-130 mutants (n = 128) (Fig. S8a and b). 116 (90.6%) animals had normal AIB location and morphology, while 12 animals (9.38%) had an ectopic dendrite extending to the tip of the nose (Fig. S8a and b). However, the occurrence rates were not significantly different (P = 0.0714, n = 40, 128, Fisher’s exact test),
Figure 5. unc-130 does not regulate electrical synapses in the AIBs. (a) Comparison of the expression of inx-1 promoter-driven GFP. unc-130 mutants showed similar expression levels. Scale bar = 5 µm. (b) There were no differences in the intensity of inx-1 promoter-driven GFP expression measured in cell bodies between the wild-type animals and the unc-130 mutants. "ns" indicates no significant difference (P = 0.960, n = 20, 20, Mann–Whitney test). (c) The double mutants of unc-130 and lin-32 almost completely lacked turn behavior (n = 26), whereas unc-130 and lin-32 single mutants exhibited moderately lower turn frequencies (n = 19, 25) than wild-type animals (n = 14). Each mutant had an increased reversal frequency instead of exhibiting turning. (d) unc-130 and lin-32 single mutants (n = 19, 25) showed significantly different turn frequencies than the wild-type animals (n = 14) and the double mutants (n = 26). ***indicates P < 0.001, * indicates P < 0.05 (Kruskal–Wallis test with Dunn’s multiple comparisons test as a post hoc test). The error bars in these figures represent the ± SEM values.
and there was no association between the presence of ectopic neurites and turn/reversal frequency (Fig. S8c) \((P = 0.892, n = 40, 128, \text{Fisher's exact test})\). They suggest that this morphological is not related to the optimization of avoidance behavior. Next, to determine whether \textit{unc-130} might affect the amount of \textit{inx-1} expression, we quantified the intensity of GFP driven by the \textit{inx-1} promoter in the mutants. The \textit{unc-130} mutants expressed the same amount of \textit{inx-1} as wild-type animals (Figs. 5a and b) \((P = 0.326, n = 20, 20, \text{Mann–Whitney test})\), indicating that \textit{unc-130} regulates genes, \textit{inx-1} different from those regulated by \textit{lin-32}. Finally, we performed a drop test on the double mutants to confirm that \textit{unc-130} and \textit{lin-32} act in different molecular pathways. The double mutants exhibited more remarkable optimization defects than the single mutants (Fig. 5c), and the turns almost completely disappeared (Fig. 5d). Meanwhile, the reversal ability remained (Fig. 5b; Fig. S12), suggesting that \textit{unc-130} and \textit{lin-32} regulate parallel molecular pathways for turning. We conclude that \textit{unc-130} impacts behavioral optimization by regulating the characteristic gene expression of calcium dynamics and glutamatergic synapse function, unlike \textit{lin-32}.

\textit{unc-130} starts to be expressed in early embryogenesis but not detected in neurons from the larvae stage onward\cite{53}. In order to clarify whether \textit{unc-130} contributes to AIB development and determination decisions in embryogenesis or gene expression in the adult stage, we examined behavioral rescue in the adult driven \textit{unc-130} by the \textit{inx-1} or \textit{npr-9} promoters, which cause \textit{AIB}-selective expression from larva onward. As a result, all three or four independent transgenic lines driven UNEC-130 from the larva onward could not rescue (Figs. S9 and S10), suggesting that function during embryogenesis is essential. Behavioral optimization defects in \textit{unc-130} adults were not rescued by overexpression of \textit{nca-2} or \textit{eat-4} driven by the \textit{inx-1} promoter (Figs. S10 and S11). Co-transduction with multiple genes in AIB regulated by \textit{unc-130}—at least \textit{nca-2}, \textit{eat-4} and \textit{glr-1}—might be able to rescue behavioral defects.

**Discussion**

\textit{unc-130} regulates the restricted genes for specific neuronal phenotypes to optimize behavior. We have reported that the proneural gene \textit{lin-32} promotes the expression of a wide range of genes in AIBs, including gap junctions\cite{6}. However, \textit{unc-130} seems to play a more limited role in AIB identity. The \textit{unc-130} mutants had reduced expression of \textit{glr-1}, \textit{nca-2}, and \textit{eat-4} in the AIBs but not the electrical synapse component \textit{inx-1} and standard chemical synaptic component \textit{SNB-1} in AIB neurons, suggesting that the \textit{unc-130} mutation causes behavioral optimization defects by disrupting selective glutamatergic synaptic functions (Fig. 6a).

During intense osmotic stimulation, the AIBs receive excitatory inputs through a direct circuit between the ASHs and AIBs via glutamate and GLR-1 receptors as well as inhibitory inputs mediated by AIAs (Fig. 6a).
Considering the AIB suppression that occurs during osmotic stimulation (Fig. 2a), inhibitory input may exceed GLR-1-mediated excitatory input during hyperosmolar stimulation. We could not identify the inhibitory receptors on AIBs in this study (Figs. S4 and S5). In addition to the unanalyzed inhibitory acetylcholine receptors, neuropeptide receptors might be involved in AIA-AIB suppression. The unc-130 mutation may regulate either inhibitory receptors or genes in the downstream calcium signaling cascade.

The molecular basis of regulation after intense osmotic stimulation is also intriguing. After stimulation, OFF responses (calcium increases) in ASHs can induce glutamate release. GLR-1, a receptor for glutamate on AIBs, induces a change in conductance to open NCA-2, a predicted voltage-gated cation channel or a cation leak channel, in order to promote the intracellular influx of cations. This contributes to the increase in the OFF-calcium concentration response in AIBs. We showed that overexpression of NCA-2a in AIBs of unc-130 mutants rescued the optimization defect (Fig. 3c), and unc-130 partially downregulated nca-2 and glr-1 (Figs. 3d and 4b). These results imply that excitatory input is reduced and suggest the amplification mechanism.

The vesicular glutamate transporter EAT-4 transports glutamate into synaptic vesicles. Calcium induction promotes the secretion of glutamate-releasing synaptic vesicles that transmit information to downstream inter-/motor neurons, including neck motor neurons. We have shown that the contraction strength of the neck positively correlates with turn behavior². Thus, the AIBs appear to integrate the two excitatory and inhibitory (E/I) pieces of information from the AIs and ASHs to evaluate the intensity and exposure time of the stimulus and to output avoidance behavior with appropriate intensity and timing.

We demonstrated that unc-130 mutants show reduced expression of glr-1 and nca-2 (Figs. 3c, d, 4a, b), suggesting that insufficient excitatory input from ASHs and voltage-dependent calcium influx occur. Additionally, the reduced expression of EAT-4 may lead to insufficient glutamate loading in the synaptic vesicles, resulting in reduced transmission of information to the downstream neural circuit.

On the other hand, since AIB-specific driven unc-130 has not showed behavioral abnormalities (Figs. S3 and S4), showing the importance of an act of unc-130 in a developmental stage. Often, with the loss of transcription factors during developmental stages, unexpected circuits may form or differentiate into different cells that are similar in lineage. It has also been reported that unc-130 is required to make a difference between AWA and ASG, chemosensory neurons generated from the ABp(l/r)apa lineage from which AIB is derived⁴, unc-130 specifies two glial types that arise from the neighboring lineage (ABp(l/r)apa)⁵. As for the AIBs that we are focusing on in this study, they express at least ins-1, which is selective for AIBs, and show a consistent number, location, and interneuron-like morphology. We consider that they are incomplete differentiated AIBs rather than sister cells of sensory neurons or glial cells.

Now we conclude that unc-130 is involved in both the function and development of AIBs, and comprehensive discussion summarizing the importance of both sides is more appropriate for the role of unc-130. unc-130 is required for the establishment of some AIB identities (e.g., biphasic response, expression of nca-2, glr-1, and eat-4, and role as a behavioral optimization center), and unc-130 contributes to behavioral optimization through its role in regulating the expression of a group of genes that are necessary for AIB identities. unc-130 has the defect in the lineage determination, so the possibility remains that it reflects an unidentified reorganization of neurons in addition to AIB defects. It might explain the synthetic defect in the behavior of unc-130;lin-32 double mutants.

**Biphasic neural response of AIB neurons.** We provide the first evidence for a biphasic neural response of AIBs: calcium decreases during osmotic stimulation followed by a calcium increase after the stimulus (Fig. 2a and Fig. S3a). First, we discuss the neural mechanisms of the calcium decrease in wild-type C. elegans during stimulation. Calcium imaging results showed that ASHs and AIAs were excited and that AIBs were inhibited during the stimulus (Fig. 2a, d, and e; Fig. S1a, e and f). Since ASHs and AIAs form synapses and since AIA-AIB connections are likely to be inhibitory (Fig. 2b)⁶,⁷, we speculate that ASHs may directly excite AIAs during stimulation and that excited AIAs inhibit AIBs (Figs. 2b and 6a), resulting in decreased intracellular calcium concentrations in AIBs (Fig. 6b). It has been implied that excitation of AWC chemosensory neurons might cause calcium decreases in AIBs during stimulus exposure⁶⁵,⁶⁶, but this has not been stringently verified. This study clearly showed such responses with the indicator IP2.0, which monitors the decrease in intracellular calcium concentration associated with osmotic stimulation received by ASHs.

Next, we considered the neural mechanisms of calcium increases in wild-type C. elegans after stimulation. Calcium imaging results showed that ASHs and AIBs, but not AIAs, were excited after the stimulus (Figs. 2a, d, and e; Fig. S3a, e and f). Although removing the CO2 stimulus evokes an AIA OFF response (calcium increase)⁷⁷, it is likely due to neural circuits distinct from those involved in response to noxious osmolality. Anatomical analysis showed a direct synaptic connection between the ASHs and AIBs (Fig. 2b)⁸, but the details of ASH-AIB synapses are not well understood. Since ASHs show the biphasic (ON/OFF-increase) response (Figs. 2d and 6a), we speculate that the ASH OFF response (calcium increase) engages the disinhibition of AIB after stimulation⁹.

Physiologically, such a biphasic interneuron response has also been observed in mammals. In rodents, over 60% of suprachiasmatic nucleus neurons may use such “rebound responses” or “postinhibitory rebounds,” and the probability of the response is positively correlated with the duration of hyperpolarization⁹. One of the next questions will be to determine whether the duration of ON-hyperpolarization of AIBs is correlated with the probability of OFF-calcium responses in C. elegans, so that similarity with a property of suprachiasmatic nucleus neurons can be clarified. Rebound responses are also observed in striatal neurons and imply the existence of an essential mechanism for fear processing and decision-making⁶⁰. C. elegans AIBs share similarities in that they are involved in escape behavior optimization and behavioral choice in response to harmful stimuli. The biphasic neural response with excitatory-inhibitory association seems likely to have been evolutionarily acquired for behavioral diversity. We consider that the biphasic neural response in C. elegans may be one of the neural mechanisms of primitive excitation-inhibition association. In addition, rebound firing is also associated with
Involvement of FOXD3/4 in avoidance behavior optimization. In this study, we demonstrated, for the first time, that a FOXD3/4 ortholog, unc-130, specifies avoidance behavior patterns using C. elegans. FOXD3 is widely conserved from invertebrates to vertebrates, including humans. FOXD3 defines the early pluripotency of neural crest stem cells in vertebrates to differentiate into diverse cells, such as neurons and muscles. Even in C. elegans, unc-130 has a similarity to FOXD3/4 with regard to its expression in both neural progenitors and adult muscle cells. Future unc-130 studies may lead to universal molecular insights into primary neurogenesis. In addition, whole-human genome analysis has implied that FOXD4 is a risk factor for suicide and obsessive-compulsive disorder. Such outcomes may be attributable to vulnerability to stress. unc-130 might be useful for illustrating a prototypical circuit for improved coping behavior under exposure to harmful stimuli.

During development, combinations of transcription factors determine neuron identities. Hobert et al. have comprehensively mapped the combination of transcription factors expressed in all neurons of C. elegans. They speculate that combinations of homeobox (Hox) transcription factors can code for almost all neural identities; these will accelerate the understanding of individual neuronal characters. In addition, basic helix-loop-helix (bHLH) transcription factors, including proneural genes, act in the initial phase and have a crucial role in neurogenesis. However, forkhead box (Fox) transcription factors were grouped relatively recently in 2000. Our findings suggest that a FOXD3/4 ortholog, unc-130 contributes to behavioral optimization mediated by pre- and postsynaptic function to mediate biphasic neural responses. In summary, reductions in the ON and OFF calcium responses required for integrating this information and producing behavioral outputs result in incorrect behavioral choices in response to stimuli of different intensities in unc-130 mutants.

Methods

Nematodes and maintenance. We cultured C. elegans strains using modified standard techniques. NGM agar plates containing 67 mg/ml antibiotic streptomycin and 10 μg/ml mycarn were used. Escherichia coli OP50-1 was seeded as food. unc-130(tm320), acc-1(tm3268), nca-2(tm1305), unc-2(65S), and lin-32(tm2044) mutants were backcrossed twice with N2, lgc-46(ok2949), lgc-49(tm6556), and goa-1(sa734) mutants were backcrossed three times, four, and five times with N2, respectively. cca-1(gk30), nca-1(gk9), unc-36(ec251), unc-36(ok862), and ccb-2(ok862) were not backcrossed because of the pilot screening (Fig. S6b). The deletion and point mutation sites are described in Fig. S13. The strain information is summarized in Table S1.

Plasmid construction. For the own-promoter rescue experiment (Fig. 1h; Figs. S1i–l), pPD_Punc-130::UNC-130 was constructed by subcloning the sequence from 5854 bp upstream of the ATG to the end of the 3' UTR of the unc-130 genomic sequence into the pPD95.75 plasmid instead of gfp. For the AIB-specific promoter rescue experiment (Figs. S10 and S11), promoters of inx-1 or Pnpr-9 were transferred into the pPD_Punc-130::UNC-130 plasmid instead of Punc-130. For calcium imaging, we constructed pPD_Psra-6::GCaMP6s (Fig. 2a; Fig. S3a) or pPD_Psra-6::GCaMP6s (Fig. 2d) by subcloning IP2.0 from pDEST-IP2.0 (codon for C. elegans) or both the 2409 bp sra-6 promoter and GCaMP6s sequences from N2 genomic DNA and pGp-CMV-GCaMP6s into pPD95.75_Pnca-2::gfp or pPD95.75 plasmids instead of gfp, respectively. For expression analysis of inhibitory acetylcholine receptors (Fig. S4a and b), the 5022 bp goa-1 promoter, 5354 bp acc-1 promoter, 5007 bp lgc-46 promoter, 3485 bp lgc-49 promoter, 7838 bp acc-2 promoter, 5777 bp acc-3 promoter, and 2385 bp acc-4 promoter sequences upstream of the ATG from N2 genomic DNA were cloned into pPD95.75_Pnca-2::gfp, respectively. For expression analysis of nca-2 (Figs. 3c and d), pPD_Pnca-2::gfp was constructed by subcloning a total of 9990 bp containing the nca-2 promoter and the first exon and intron of the nca-2a region using the N2 genome as a template into the pPD95.75 vector. For rescue analysis of nca-2 (Fig. 3e; Fig. S7), the 5611 bp nca-2 coding region using the cDNA template into the pPD_Pnca-2::gfp instead of gfp. For rescue analysis of eat-4 (Fig. S13), the 2218 bp eat-4 coding region using the cDNA template into the pPD_Pnca-2::gfp instead of gfp. For observation of synaptic localization (Figs. 4e–g), we constructed pPD_Pnca-2::snb-1::mCherry by subcloning the 327 bp snb-1 coding region using the cDNA template except for the stop codon into the pPD_Pnca-2::mCherry vector.

Transgenic lines and strains. For all rescue experiments, we created three or more independent transgenic lines. For unc-130 rescue experiments, to generate tm320;tmEx5292 (Fig. 1g; Fig. S1i–l), tm320;jskEx0002, tm320;jskEx0003, tm320;jskEx0004, tm320;jskEx0024, tm320;jskEx0028, tm320;jskEx0029, tm320;jskEx0011, tm320;jskEx0013 and tm320;jskEx0014 transgenic animals, pPD_Punc-130::UNC-130-3':3' UTR (2 ng/μl), pPD_Pnca-2::UNC-130-3':3' UTR (2 ng/μl), pPD_Pnca-2::UNC-130-3':3' UTR (2 ng/μl or 0.2 ng/μl) or pPD_Pnpr-9:UNC-130-3':3' UTR (2 ng/μl), pPD95.75_Pnca-2::mCherry plasmids were obtained from Addgene (www.addgene.org).
(20 ng/µl), and pBluescript KS (+)T1 (140 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) as an injection marker into tm320 mutants, respectively. For calcium imaging, to generate tmEx5274 transgenic animals, pPD_Pinx-1::IP2.0 (20 ng/µl) and pBluescript KS (+)T1 (160 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. For AIA ablation experiments, to generate tmEx5494 transgenic animals, pPD_odr-2p:ced-3p(15) (80 ng/µl), pPD_ser-2p:ced-3p(17) (80 ng/µl), and pFD_DrDrosT:gfp::2(aa1 + int.) (20 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. To generate tmEx5137 transgenic animals for ASH calcium imaging, pPD_Pnca-6::GCaMP6s (180 ng/µl) was coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. To generate the tmEx5293 transgenic animals, pFX_Pgyc-28::GCaMP6s (50 ng/µl), pPD_Pins-1(short)::mCherry (30 ng/µl), and pBluescript KS (+)T1 (100 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. For expression analysis of inhibitory acetylcholine receptors, acc-1, acc-2, acc-3, and acc-4, to generate tmEx5408, tmEx5409, tmEx5411, and tmEx5412 transgenic animals, pPD_Pacc-1::gfp (100 ng/µl) or pPD_Pacc-2::gfp (100 ng/µl), pPD_Pacc-3::gfp (100 ng/µl) or pPD_Pacc-4::gfp (100 ng/µl), and pPD_Pinx-1::mCherry (80 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. For lgc-46 and lgc-49, to generate tmEx5454 and tmEx5449 transgenic animals, pPD_Plgc-46::gfp (100 ng/µl) or pPD_Plgc-49::gfp (100 ng/µl), pPD_Pinx-1::mCherry (40 ng/µl), and pBluescript KS (+)T1 (40 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. To generate tmEx5614 transgenic animals, pPD_Pgoa-1::gfp (100 ng/µl), pPD_Pinx-1::mCherry (20 ng/µl) and pBluescript KS (+)T1 (60 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) into N2 animals. For expression analysis of the hypothetical voltage-dependent calcium channels and nca-2, to generate tmEx5615 and transgenic animals, pPD_Pnca-2::gfp (100 ng/µl) and pPD_Pinx-1::mCherry (40 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) and pBluescript KS (+)T1 (40 ng/µl) into N2 animals. For AIB-specific rescue, to generate tm1305, tm1305;jskEx0017 and tm1305;jskEx0018 transgenic animals, pPD_Pinx-1::NCA-2a (20 ng/µl) and pPD_Pinx-1::gfp (20 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) and pBluescript KS (+)T1 (40 ng/µl) into tm1305;jskEx0019, tm1305;jskEx0020 and tm1305;jskEx0021 transgenic animals, pPD_Pinx-1::EAT-4 (20 ng/µl) and pPD_Pinx-1::gfp (20 ng/µl) were coinjected with lin-44p:gfp (20 ng/µl) and PBluescript KS (+)T1 (140 ng/µl) into unc-130(tm320) animals. For precise synaptic localization, to generate tmEx5377 transgenic animals, pPD_Pinx-1::SNB-1::mCherry (80 ng/µl) and pPD_Pinx-1::gfp (20 ng/µl) were coinjected with unc-122p::mCherry (100 ng/µl) into N2 animals. The strains tmIs825[Pin-6::ChR2(H134R):mCherry + Pges-1:EGFP], tmEx4532[Pnpr-4::G-CaMP6s + Pmrn-1::mCherry + Pin-44::gfp], tmEx4456[Pin-1::ChR2(H134R);::mCherry + Pinx-1::gfp + Plin-44::gfp], rhls4[glr-1::GFP + dpy-1(+)]; tmEx3532[DsRedT-odr-2(aa1 + Pbluescript KS + T1] (Fig. 4a and b), otls292[eat-4::mCherry + rol-6]; tmEx3958[Pinx-1::gfp + Plin-44::gfp + pBluescript] (Fig. 4c and d), and tmIs1260[Pinx-1::gfp + Punc-122::mCherry] were generated in our previous study6. tmIs825, tmIs1260, and rhls4 were backcrossed twice and on N2. Supplemental Table 1 summarizes the strain names, genotypes, injected plasmid concentrations, injected recipients, number of outcross with N2, and methods of crossing with mutants of the alleles used in the paper.

Drop test. We performed a drop test as we have previously described9. In this study, we used 1–6 M sorbitol dissolved in S basal10. “n = x” in the figure legends indicates the number of plates analyzed. Responses were classified as omega turns, long reversals, or short reversals, as previously described9. Each score was calculated as the average percentage for 10 ± 3 animals. For a rescue experiment, we selected Pinx-1::gfp marker-positive animals as AIB-rescued animals since extrachromosomal transgenic animals exhibited a mosaic expression pattern. The experimenter was blinded to the nematode strains during the experiment.

Harsh tap assay with a platinum wire. We tapped the nematodes on the tip of their noses with a platinum wire (diameter: 0.23 mm) on NGM plates, respectively. The platinum wire has a smooth polished surface to avoid damaging the nematode. “n = x” in the figure legends indicates the number of plates analyzed. Responses were classified as omega turns and others, as previously described8. Each score was calculated as the average percentage for 10 animals. The experimenter was blinded to the nematode strains during the experiment.

Channelrhodopsin 2-induced avoidance assay. We performed a ChR2(H134R)-induced avoidance assay as previously described7. The animals were individually irradiated with 100% blue light (approximately 2.47 µW/cm²) using a CFP filter (365 nm) at their heads for 2 s. Each score was calculated for 10 ± 3 animals. We performed the experiments on at least three different days and calculated the average percentage. “n = x” in the figure legends indicates the number of plates analyzed. The experimenter was blinded to the nematode strains and to whether all-trans retinoic acid (ATR) was added or not.

Calcium imaging of neurons. As previously described, we performed calcium imaging using an olfactory chip8. We used 2 M sorbitol dissolved in S basal as a stimulus. All optical recordings of neurons were performed on an IX71 microscope with a 40X immersion objective (Olympus Optical) and an ORCA-Flash2.8 CMOS camera (Hamamatsu Photonics) and analyzed with MetaMorph software (Molecular Devices). We captured time stacks of the fluorescence images at one frame per second. The images were analyzed as previously described18. We calculated the percent change in the fluorescence intensity relative to the average intensity during the 5 s before stimulation. In the IP2.0 analysis, the same baseline fluorescence was seen in both wild-type animals and unc-130 mutants, we shifted them to 0 for subtraction and normalization as corrected baselines. In the statistical analysis of AIA, we compared the maximum ΔF/F values from the 5 s before stimulation to 100 s after stimulation. We performed image tracking using a custom ImageJ (NIT, https://imagej.nih.gov/ij/) plugin. We drew a rectangular region of interest (ROI) surrounding the cell body, and the ROI was shifted according to the new position of the center for every frame.
**Microscopy.** Nematodes were immobilized with M9 buffer containing 50 mM sodium azide on a 5% agarose pad containing 10 mM sodium azide. We obtained fluorescence images (Figs. 3c and 4a; Figs. S4a, b, S5b, and S8a) using a BX51 microscope equipped with a DP30BW CCD camera (Olympus Optical). Confocal microscopic images were captured with Zeiss LSM710 confocal microscopes with either 40X oil immersion objectives for single-plane projections (Fig. 4c) or Z-stacks spanning the focal depths (1 μm/step) of the AIB neurons (Figs. 4e and 5a) using ZEN 2011 software (Zeiss, https://www.zeiss.co.jp/microscopy/downloads/zen.html). We drew an ROI surrounding the cell body (Figs. 3d and 4d; Fig. S2c) for neurite (Figs. 4g and 5b) and measured the total fluorescence intensity using a custom ImageJ (NIH, https://imagej.nih.gov/ij/) plugin. We performed ratio-metric analysis for an accurate discussion of expression levels in extrachromosomal arrays, comparing marker fluorescence to nca-2 (GFP) (Fig. 3d), SNB-1 (mCherry) (Fig. 4e), and goa-1 (GFP) (Fig. S5c) fluorescence by drawing the same ROI position. We calculated the synapse dimensions (Fig. 4f) using a custom ImageJ plugin.

**Quantification and statistical analysis.** We performed statistical analyses using GraphPad Prism 6 (GraphPad Software). Pairwise comparisons of omega turn frequencies within two groups were carried out via a post hoc test according to the results of the Shapiro–Wilk normality test for multiple comparisons (GraphPad Software). Pairwise comparisons of omega turn frequencies within two groups were carried out via a post hoc test according to the results of the Shapiro–Wilk normality test for multiple comparisons test as a post hoc test according to the results of the Shapiro–Wilk normality test for multiple groups. We produced bar graphs with the mean ± SEM values from three or more independent experiments. In both cases, “n” is the number of plates (cohorts) of 10 ± 3 animals each. In the bar graphs without error bars (Fig. 4b), “n” is the number of individuals (animals). We have provided the statistical information and the total number of experiments, animals, or cells analyzed per experiment in the figure legends.

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Author contributions
Data curation, formal analysis, funding acquisition, investigation, project administration, validation, writing original draft: S.H. Conceptualization, methodology, and writing review & editing: S.H., S.M. Supervision: S.M. The results and the interpretation were discussed by both authors. Both authors read and approved the final manuscript.

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Competing interests
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