Identification of a Novel Gene, Anorexia, Regulating Feeding Activity via Insulin Signaling in Drosophila melanogaster

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Feeding activities of animals, including insects, are influenced by various signals from the external environment, internal energy status, and physiological conditions. Full understanding of how such signals are integrated to regulate feeding activities has, however, been hampered by a lack of knowledge about the genes involved. Here, we identified an anorexic Drosophila melanogaster mutant (GS1189) in which the expression of a newly identified gene, Anorexia (Anox), is mutated. In Drosophila larvae, Anox encodes an acyl-CoA binding protein with an ankyrin repeat domain that is expressed in the cephalic chemosensory organs and various neurons in the central nervous system (CNS). Loss of its expression or disturbance of neural transmission in Anox-expressing cells decreased feeding activity. Conversely, overexpression of Anox in the CNS increased food intake. We further found that Anox regulates expression of the insulin receptor gene (dInR); overexpression and knockdown of Anox in the CNS, respectively, elevated and repressed dInR expression, which altered larval feeding activity in parallel with Anox expression levels. Anox mutant adults also showed significant repression of sugar-induced nerve responses and feeding potencies. Although Anox expression levels did not depend on the fasting and feeding states cycle, stressors such as high temperature and desiccation significantly repressed its expression levels. These results strongly suggest that Anox is essential for gustatory sensation and food intake of Drosophila through regulation of the insulin signaling activity that is directly regulated by internal nutrition status. Therefore, the mutant strain lacking Anox expression cannot enhance feeding potencies even under starvation.

Conclusion: We identified a new gene, Anorexia (Anox), which encodes an acyl-CoA binding protein with an ankyrin repeat domain.

Significance: Identification of Anox can develop a better knowledge of mechanisms underlying animal feeding behavior.
work indicated that certain uncharacterized genes regulate or influence the feeding potencies of Drosophila larvae. The results also implied the validity of the Drosophila GS lines for studying the molecular mechanism of feeding responses in this animal.

In the present study, we focused on the feeding potencies of Drosophila larvae to identify genes that contribute to the integration of external and internal signals into feeding behaviors. To identify mutant Drosophila larvae with feeding activities significantly different from those of control larvae such as y w and wild-type larvae, we screened the GS strains and found that the same GS strain, GS1189, which lacked a significant taste preference, exhibited a significant decreased feeding potency. Therefore, we focused on this strain to investigate the molecular mechanisms that cause the down-regulation of feeding in this strain. Anatomical analysis using a green fluorescent protein (GFP) reporter showed the specific expression of CG33071 in the cephalic chemosensory organs and various neurons in the CNS, including the subesophageal ganglion and antennal lobe region, that have been reported to contribute to the detection of chemosensory information and integration of these gustatory signals. A further finding that CG33071 regulates expression of the insulin-like receptor gene (dInR) indicates the possibility that CG33071 expression in the cephalic sensory organs and CNS contributes essentially to gustatory sensation and mediates the feeding activity of Drosophila through regulation of the insulin-signaling activity. Based on these results, we propose that CG33071 be henceforth referred to as Anorexia (Anox).

EXPERIMENTAL PROCEDURES

Drosophila Culture and Stocks—Drosophila stocks were reared on standard cornmeal-glucose-yeast medium at 23 °C (12). GS strains were supplied by the Drosophila Genetic Resource Centre, Kyoto Institute of Technology. UAS-Anox and Anox-Gal4 transgenic flies were generated using a standard injection protocol with Δ2–3 (y[1] w[67c23]; ry[506] Sb[1] P(Δ2–3)99B/TM6, Ubx) strain (14, 15). The Anox-Gal4 fly contains 0.6-kb Anox upstream regulatory sequences. UAS-TNT, UAS-TNTE, and UAS-IMP TNT strains were supplied by C. O’Kane (University of Cambridge, Cambridge, UK). UAS-dInR strain was supplied VDRC (Vienna Drosophila RNAi Center). UAS-dsCG33071 (Anox) was supplied by NIG-FLY (National Institute of Genetics).

Behavioral Assays—The feeding activities of Drosophila larvae were assayed by measuring the exact volume of diet absorbed by test larvae (15, 16). The volume of the consumed diet was quantified by measuring the absorbance of the gut extract at 630 nm after feeding them test diet containing blue dye for 10 min. To prepare the gut extract, the whole body of larvae were homogenized in 300 μl of 50% chilled ethanol, and the homogenate was left on ice for 10 min. The homogenate was then centrifuged at 20,000 × g for 20 min at 4 °C, and the supernatant was used as the gut extract sample.

Larval mouth hook movement was counted for 30 s. Water was used for confirmation of locomotive aberration. Agar containing 2% yeast and the standard diet were used for quantification of intake rate, as indicated by the frequency of mouthhook contractions.

Capillary Feeder assay previously described (17) was slightly modified. Briefly, flies were starved for 20 h but supplied with water-soaked Kimwipe paper in vials. Twenty flies were introduced into a plastic vial that has a diameter of 2.3 cm and length of 4 cm with the bottom comprising a water-soaked Kimwipe.

Northern Blotting and Quantitative RT-PCR—Total RNAs were prepared from the CNS of test Drosophila larvae. Northern blotting and real-time quantitative RT-PCR was performed as described previously (18).

Production of Polyclonal Antibodies and Immunoblotting—The cDNA fragments containing the ORFs of ACBP-ANK (CG33713) and RRM (CG33714) were respectively cloned into pGEX (Novagen) and expressed as recombinant proteins in Escherichia coli BL21(DE3) as described previously (19). Production of each protein containing six histidine tag residues was induced by 0.4 mM isopropylthio-β-D-galactoside for 3 h at 37 °C. The recombinant proteins were purified by a Chelating Sepharose Fast Flow column (GE Healthcare) charged with nickel. Each purified protein was emulsified by Titer Max Gold (CytRx Co.) and injected into a rabbit to generate a specific polyclonal antibody. The antibodies were precipitated by adding ammonium sulfate to 40% saturation and further purified by an affinity column of protein G-Sepharose (GE Healthcare) (20). Proteins of Drosophila larval brains were separated by SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane filter. After blocking, proteins on the membrane were probed with each antibody as described previously (18). All positive bands were quantified using ImageJ (National Institutes of Health). Activation of Akt was measured by immunoblotting using anti-phosphorylated Drosophila Akt (Ser-505) and anti-Akt antibodies (Cell Signaling Technology, Danvers, MA).

Electrophysiology—Extracellular single unit recordings were carried out as described earlier (21, 22). Briefly, flies 3–6 days old were electrically grounded by using a glass capillary tube filled with Drosophila ringer’s solution inserted into the abdomen. Action potentials were recorded from a labellar l-type sensillum using the tip recording method.

RESULTS

GS1189 Strain Larvae Significantly Lack Their Appetite—We first screened 480 GS strain larvae to identify strains whose appetite was significantly different from those of the control (yw, Canton-S (CS), and Oregon-R (OR)) types. Two strains (GS51 and GS1189) showed decreased feeding activities, whereas two other strains (GS1008 and GS1203) showed increased feeding responses compared with the control larvae (yw, CS, and OR) (Fig. 1A). Of these four mutants, the greatest difference in feeding activities between control and mutant larvae was observed in GS1189, which was previously identified by a two-way choice assay as a strain with an altered food preference during the larval stage (12). We therefore focused on this anorexic strain to precisely analyze the functional role of the target gene into which P[GS] was inserted, as well as the phenotypes of this mutant.
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Detailed analysis of growth rates of GS1189 larvae showed a significant reduction in weight gain and consequent delay of larval growth. An ~70% decrease in weight gain and consequent delay of the mutant larvae by the 5th day of the last instar caused a 2- to 3-day delay in pupation on average (Fig. 1B). This growth retardation was not due to a physical defect in mouth-hook contractions: as shown in Fig. 1C, the mouth-hook contraction rates of the control and GS1189 larvae were no different when they were put into water, but the contraction rates of the mutant larvae were significantly lower than those of the controls when they were put into agar containing yeast or standard diet components, indicating the possibility that GS1189 larvae possess a defect in the ability to sense tastants.

Characterization of Mutated CG33071 (Anox) of GS1189—The GS element P[GS] insertion site in GS1189 was found in the ORF of CG33071 when we first checked the Flybase database in 2007; therefore, we named this gene Anorexia (Anox) based on the phenotype of GS1189 larvae. The current annotation in Flybase describes Anox as a putative dicistronic gene containing a 5′ ORF encoding an RNA recognition motif (RRM) (CG33714) and a 3′ ORF encoding an acyl-CoA binding protein with an ankyrin repeat domain (ACBP-ANK) (CG33713) (Fig. 2A). To test for the transcription of these genes in the CNSs of Drosophila y w and GS1189 larvae, we performed Northern blotting and detected the transcript in y w but not in GS1189 larvae (Fig. 2B). We next examined the protein expression in the larval CNS using antibodies for both proteins (supplemental Fig. S1). Immunoblotting showed a clear band cross-reacted with anti-ACBP-ANK protein IgG but did not show the positive band cross-reacted with anti-RRM protein antibody (Fig. 2C), indicating that at least the ACBP-ANK protein expressed in normal fly larvae could act as an Anox gene product. However, although we did not detect the RRM protein band, we could not rule out the possibility that the expression level was too low to detect it in the larval CNS.

Distribution of Anox Expression—The 588-bp 5′ flanking region of Anox (putative promoter region) drives GFP reporter expression of Drosophila larva mainly in the external mouth region in addition to the CNS of Drosophila larva (Fig. 3, A and B). Strong expression in the CNS was observed mainly in the brain and the subesophageal, thoracic, and abdominal ganglia (Fig. 3, C and E). The dorsal view showed expression in the neurosecretory cells in the pars intercerebralis region of the protocerebrum that is close to the cells reported to express insulin-like peptide genes (Fig. 3, D). The ventral view showed GFP expression in cell clusters of the antennal lobe and subesophageal ganglion (Fig. 3F). The cells that expressed GFP in the thoracic abdominal ganglia were slightly more abundant in the ventral region than in the dorsal region (Fig. 3, C and E). The GFP signal was also observed in external and internal gustatory organs. Several sensory neurons close to the terminal organ (TO) and dorsal organ (DO) showed strong expression, but no positive neuron was feeding behavior, whereas agar containing yeast or standard diet components was used for testing an active feeding potency. Each value represents the mean ± S.D. for 20–30 independent determinations. Significant differences are indicated by Tukey’s HSD (*, p < 0.05; **, p < 0.01).

![A Drosophila Gene Regulating Feeding Activity via Insulin Signaling](image-url)
observed beside the ventral organ (VO) (Fig. 3, G and H). Dorsal and ventral views of the pharynx region displayed clear signals in the dorsal pharyngeal sense organ (DPS) and ventral pharyngeal sense organ (VPS), respectively (Fig. 3, I–L). In adults, the GFP signal was also clearly observed in external sensory organs such as the sensilla of the labella, maxillary palpi, and tarsi (supplemental Fig. S2).

**Contribution of Anox to Control of Feeding Potency**—To reveal effects of Anox on the regulation of feeding potencies, we examined whether physiological and environmental conditions alter CNS Anox expression levels in *Drosophila*. Although no hunger-driven increase in Anox expression was observed, heat or desiccation stress conditions significantly repressed its expression in the CNS of test larvae (Fig. 4, A and B). The stress-induced repression of the CNS Anox expression was also observed in the adults. These results indicated that a constant level of Anox expression is required irrespective of nutritional states in normal physiological conditions, although we could not exclude the possibility that the hunger-driven increase in Anox expression occurred in too small a subset of cells and neurons to be detected by our present analyses.

To evaluate the effects of Anox expression on feeding potencies of larvae, Anox expression was either up-regulated by expressing a functional Anox cDNA (UAS-Anox) or down-regulated by expressing Anox dsRNA (UAS-dsAnox) under the direction of an Anox promoter (Anox-Gal4) (supplemental Fig. S3), and the feeding activities of resulting transgenic larvae were assayed. Overexpression of Anox enhanced feeding activities relative to controls, whereas its down-regulation significantly repressed the activities (Fig. 5A). The repressed feeding activities of Anox-knockdown larvae were not elevated even by starvation (Fig. 5B).

To gain further information on the function of Anox-expressing neurons, we then blocked synaptic transmission in these neurons by expressing UAS-TNT under the direction of Anox-Gal4. The feeding activities of these larvae were significantly restricted by the neurotoxin TNT (Fig. 5C), whereas the feeding potentials of control larvae that expressed weaker TNT (TNTE) or imperfect TNT (IMP TNT) were not altered. These data thus indicate that Anox contributes to the regulation of feeding activities of *Drosophila*.

**Signaling Pathway of Anox**—We previously carried out a large-scale oligonucleotide-DNA microarray screening to reveal the genetic mechanism underlying the change of feeding behaviors of GS1189 larvae (13). Although we identified several interesting genes whose expression levels were significantly dif-
different between yw and GS1189 larvae, the experimental information was not sufficient to explain the mechanism. To further characterize the components of an Anox signaling pathway, we here performed differential display RT-PCR on total RNAs prepared from yw and GS1189 strain larval CNS using 60 different primer sets (Annealing Control Primer 1–60, Seegene, Inc.). Of these, one primer (Annealing Control Primer 12) revealed a significant reduction in transcription of one DNA band encoding the Drosophila insulin receptor gene (dInR) in the CNS of GS1189 larvae compared with that in the control. To confirm this finding, we performed quantitative RT-PCR to estimate expression levels of dInR together with insulin-like peptide genes in the control (yw) and GS1189 larval CNS (Fig. 6A). The results showed that expression of most dilps as well as dInR was reduced, further, of all dilp expression levels, the difference in dilp3 expression between yw and GS1189 larvae was the biggest. Further, we confirmed that the repressed dInR transcription levels in GS1189 larvae were not altered by changes in physiological conditions, fed or starved (supplemental Fig. S4).

To assess whether neural dInR expression is regulated by Anox, dInR expression levels were analyzed in larval CNS whose transcription levels of Anox were genetically up- or down-regulated. The results demonstrated that dInR expression was positively regulated by Anox; dInR expression in larvae was elevated by Anox up-regulation, whereas it was clearly reduced by Anox down-regulation (Fig. 6B). We then investigated the effects of neural dInR down-regulation on feeding activities of larvae whose dInR expression was repressed broadly or specifically in Anox neurons (supplemental Fig. S5).

**FIGURE 3. GFP reporter expression in Drosophila Anox-Gal4; UAS-GFP larvae.** A and B, dorsal and ventral view of whole larval body expressing GFP. GFP fluorescence was localized in the CNS and external sensory organs, terminal organ (TO) and dorsal organ (DO). C and D, merged picture of dorsal vision of the CNS. GFP signals were widely distributed in the CNS but strongly expressed in the ventral nerve cord region and the pars intercerebralis region (indicated by arrows) of the protocerebrum. D, enlargement as shown in the insert. E and F, merged picture of ventral vision of the CNS. In the brain region, the antennal lobe (indicated by arrows) and the subesophageal ganglion region (enclosed by dotted line) strongly showed fluorescence. F, enlargement of the central region between brain hemispheres. G and H, ventral view of the larval external sensory organ region. Several neurons projected from TO and DO, but no positive neuron was observed from the ventral organ (VO). I–L, GFP expression in the larval internal sensory organs. Ventral view (I and J) and dorsal view (K and L) showed the GFP signals at ventral pharyngeal sense organ (VPS) and dorsal pharyngeal sense organ (DPS), respectively, but did not show any signal at posterior pharyngeal sense organ (PPS). Ventral view also showed a strong signal at terminal organ ganglion (TOG). Scale bar: 100 μm for C and E; 50 μm for D; 25 μm for F–L. GFP fluorescence images were observed by CellMap confocal imaging system (Carl Zeiss) equipped with a 405/488 nm laser.
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Both types of dInR knockdowns had significantly decreased feeding activities relative to control larvae (Fig. 6C). These results together with the clear repression of dInR expression in larvae with down-regulated Anox expression indicate that the decrease in feeding activities of GS1189 larvae was at least partly due to the repression of dInR expression in the CNS.

To analyze the mechanism of repression of dInR expression in GS1189, we focused on Akt activity, which negatively regulates dInR expression through phosphorylation of the transcription factor dFOXO for dInR: phosphorylated dFOXO cannot enter the nucleus to promote dInR expression (25, 26). Interestingly, active (phosphorylated) Akt levels in GS1189 were constantly maintained above a certain level irrespective of larval physiologies, fed or starved, although active Akt was detected only very low level in starved control GS1189rev larvae (Fig. 6D). Therefore, it is reasonable to conclude that the dInR expression level was kept constantly low irrespective of the physiological conditions in GS1189.

Anox Function in Drosophila Adults—The effects of Anox mutation on adult feeding activity and life span were investigated. Capillary feeder assays showed that Anox-deficient (GS1189), Anox RNAi, and InR RNAi adults had remarkably reduced feeding responses relative to control adults (Fig. 7A). As observed in Drosophila transgenic larvae (Anox-Gal4;UAS-TNT), the adult feeding responses were also significantly restricted by the neurotoxin TNT. In contrast, overexpression of Anox significantly enhanced feeding activities relative to controls. Further, to address whether the electrophysiological response to sugars was defective in GS1189 flies, we recorded tastant-induced action potentials in taste sensilla of GS1189 adults. In control CS flies, sugar-induced action potentials were detected in the labellum gustatory sensilla using glucose and sucrose at concentrations from 10 to 1000 mM, whereas they were significantly decreased in GS1189 flies (Fig. 7B). These results together with the observation that GS1189 rev flies had recovered the nerve responses to sugar reception (supplemental Fig. S6) indicate that Anox is needed for normal sugar perception.

Measurement of longevities of control and mutant flies showed that the average life span was significantly shortened in GS1189 relative to control flies; in particular, earlier mortality was more obvious in males than in females (supplemental Fig. S7). The reduction in the longevity may be explained by a shortage of nutrition due to the marked drop in adult feeding activity.

DISCUSSION

This study demonstrated that GS1189 mutant larvae that lacked normal Anox expression had suppressed appetites in which dInR expression levels were significantly down-regulated. This finding is consistent with our general knowledge that insulin signaling is rapidly modulated in response to changes in nutrition in animals, including Drosophila (26, 27). The fact that the feeding activities of GS1189 larvae continuously remained very low suggested that regulation of feeding potencies by insulin signaling was somehow disordered in the mutant animals. This speculation was at least partially confirmed by the result that dInR expression levels were consistently down-regulated irrespective of nutrient availability. Despite the transcriptional down-regulation of dilps as well as dInR in GS1189 larvae, insulin signaling was continuously activated at a low level via the continuously but only partially activated Akt (Fig. 6D). It is also worth emphasizing that the bodies of full-grown GS1189 larvae reached almost the same size as those of controls when the feeding period was extended; therefore, the adults appeared to be normal size. GS1189 adults nevertheless exhibited greatly diminished feeding activities and shortened life span compared with control flies (Fig. 7A and supplemental Fig. S4). We interpreted these phenotypes of GS1189 in light of the disordered regulation of insulin signaling as follows. In normal animals, inactivation of insulin signaling due to limited nutrition elevates feeding activity, and this allows the animal to achieve a satiated condition, which reactivates insulin signaling; thus, as long as insulin signaling works normally, feeding activity of Drosophila is maintained at a certain level. Further, it is well known that longevity is extended if dietary restriction is applied to adult Drosophila (28). However, GS1189 adults with the decreased appetite die younger than...
normal flies because their feeding activities are continuously too low to ingest enough nutrition to survive as long as or longer than normal flies. This may be mainly due to a defect of the insulin signaling system (transcriptional control of dInR and dips) that regulates the feeding activity in response to the internal nutritional status. Decreases in the life span due to a deficit in nutrition have been reported in several animals, including Drosophila (29, 30).

Anox, which encodes acyl-CoA binding protein with a short ankyrin repeat domain, is mainly expressed in the CNS and cephalic chemosensory organs where the integration of gustatory signals as well as the detection of chemosensory cues proceeds (8, 10, 31). Because changes in dInR expression parallel changes in Anox expression in the CNS, we concluded that the neural dInR expression is regulated by the upstream regulatory gene Anox in the CNS. This conclusion was partially confirmed by the observation that dInR RNAi in Anox-expressing cells reproduced the phenotype of GS1189 or Anox RNAi larvae, i.e. decreased feeding activities and consequent retardation of larval growth. Although we do not have enough data to explain in detail the role of the Anox product in regulating dInR expression at present, there is some evidence to predict its possible

FIGURE 5. Gustatory functions of Anox in the CNS of Drosophila larvae. A, feeding activities of Anox overexpression and knockdown transgenic line larvae. Each value represents the mean ± S.D. for 3–8 independent determinations. Significant differences are indicated by Tukey's HSD (*, p < 0.01; **, p < 0.001). B, starvation effects on feeding activity of Anox transgenic larvae. Note that feeding activities of GS1189 and Anox transgenic (overexpression and knockdown) line larvae were not changed by starvation. Each value represents the mean ± S.D. for nine independent determinations. Significant differences are indicated by t test (*, p < 0.01; **, p < 0.001). C, feeding activities of transgenic larvae in which neurotoxin TNT gene is overexpressed under the control of Anox promoter activity. Each value represents the mean ± S.D. for 8–31 independent determinations. Significant differences are indicated by Tukey's HSD (*, p < 0.001).
role as follows. 1) The result that dInR expression continuously remains very low in GS1189 larvae and adults irrespective of their nutritional status suggests that the Anox product is essential to its transcriptional elevation under the control of dFOXO, a transcription factor for dInR. 2) The result that neural Anox expression in control y w larvae and adults is continuous irrespective of the nutritional status indicates that nutrition-dependent periodic changes in dInR expression are not regulated by Anox expression levels but mainly by Akt-dFOXO. 3) The above results, together with the observation that dInR expression levels are greatly diminished in GS1189 and Anox RNAi larvae, enable us to speculate that Akt-dFOXO-dependent regulation of dInR expression occurs only in the presence of the Anox product; therefore, the Anox product contributes to establishment of the basal condition essential in regulating dInR expression through the Akt-dFOXO system depending on the nutritional conditions.

Anox encodes acyl-CoA binding protein with an ankyrin repeat domain. The Drosophila genome has a total of nine genes encoding putative acyl-CoA binding proteins: Diaze-
pam-binding inhibitor (CG8627), CG8498, CG8814, CG8629, CG15829, CG5804, Anox (CG33713), CG14232, and CG8628. Expression of the first seven genes was observed in the CNS of y w larvae. Of these seven expressed genes, only Anox
contains an ankyrin repeat domain. Ankyrin repeats are 33 amino acids long and involved in protein-protein interactions (32). Indeed, yeast two-hybrid screening using certain proteins with ankyrin repeats as bait has discovered several target proteins with an affinity for the ankyrin repeat domain (33). Based on these molecular characteristics, it is generally thought that many proteins with ankyrin repeats may play roles in integrating signals from multiple protein-protein interactions. Various kinds of ankyrin repeat-containing proteins have been identified as nuclear receptor cofactors that interact with nuclear receptors to enhance or suppress transcription of target genes (34). These reports enabled us to speculate that the Anox product may facilitate initiation of dInR transcription through interaction with dFOXO. However, it is also possible that the acyl-CoA binding protein portion encoded by Anox contributes to the regulation of dInR expression and feeding activity via its effect on lipid metabolism. This possibility does not contradict the fact that the Zellveger syndrome mouse, which lacks normal expression of acyl-CoA binding protein, shows a phenotype similar to the GS1189 fly: the Zellveger syndrome mouse fails to feed normally, which leads to an early death, usually in the first year of life (35). Further analysis of the function of Anox in the feeding activity of Drosophila is certainly required to substantiate these speculations.

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