Putative transmembrane transporter modulates higher-level aggression in *Drosophila*

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By selection of winners of dyadic fights for 35 generations, we have generated a hyperaggressive Bully line of flies that almost always win fights against the parental wild-type Canton-S stock. Maintenance of the Bully phenotype is temperature dependent during development, with the phenotype lost when flies are reared at 19 °C. No similar effect is seen with the parent line. This difference allowed us to carry out RNA-seq experiments and identify a limited number of genes that are differentially expressed by twofold or greater in the Bullies; one of these was a putative transmembrane transporter, *CG13646*, which showed consistent and reproducible twofold down-regulation in Bullies. We examined the causal effect of this gene on the phenotype with a mutant line for *CG13646*, and with an RNAi approach. In all cases, reduction in expression of *CG13646* by approximately half led to a hyperaggressive phenotype partially resembling that seen in the Bully flies. This gene is a member of a very interesting family of solute carrier proteins (SLCs), some of which have been suggested as being involved in glutamine/glutamate and GABA cycles of metabolism in excitatory and inhibitory nerve terminals in mammalian systems.

aggression | *Drosophila* | transmembrane neutral amino acid transporter

Aggression is an innate, complex social behavior, whose manifestation is continually modifiable by social context. Great heterogeneity exists in the display of aggressiveness among individuals in competition for desired resources, even within inbred populations of organisms. The root causes of this heterogeneity are relatively unknown, with interactions among genes, development, and environment commonly offered as underlying explanations. Moreover, how organisms transition from safer, lower levels of aggression that initiate conflicts, to potentially damaging, higher levels of aggression that end conflicts and establish rank orders of organisms, also is poorly understood. In this article, we begin to address several of these issues by comparing the genetic makeup of a highly aggressive line of flies called Bullies (selected by inbreeding) to a parental wild-type stock of Canton-S flies (1), and identifying candidate nervous system genes important in establishing higher levels of aggression.

In both vertebrate and invertebrate nervous systems, numerous genes of widely varying functions have been identified as important contributors to aggression (2–8), including small molecules, biogenic amines, and peptide transmitter-related genes, their receptors, and their second messenger targets (9–15); steroid hormones and their membrane and nuclear receptor targets (16, 17); transcription regulators (18); and key metabolic regulators (16, 19, 20). Many of these genes were identified using transgenic mutants directed at specific genes. Recent studies have taken a more unbiased approach and identified multiple new genes by comparing differential mRNA transcript abundance between selected lines of highly aggressive and less-aggressive animals (21).

In recent years, *Drosophila* has been established as a strong model system for the study of aggression displaying characteristic fighting patterns that can be examined by forward and reverse genetic approaches, and by a powerful genetic toolkit, including binary systems that allow gene expression to single-cell levels (22–24). After originally being described briefly in 1915 (25), aggression in *Drosophila* was further described in more detail by

Jacobs (26) and Dow and von Schilcher (27), with the latter paper being the first to describe an escalated high-intensity boxing behavior. In 2002, Chen et al. (28) reported a dyadic fight setup that has since become a standardized aggression paradigm in *Drosophila*. As in vertebrate models, a majority of the genes identified through a candidate gene approach in studies of *Drosophila* aggression belong to the biogenic amine and peptide families. Thus, the amines serotonin (29, 30), dopamine (31), and octopamine (32) have been shown to modulate levels of aggression, along with the peptides tachykinin (33) and NPF (29). In addition, candidate gene approaches also have identified tailless, a fly ortholog of mouse *nr2e1*, and its corepressor, atrophin, as influencing aggressive behaviors (34). Unbiased screens have also been used to identify behavioral candidate loci in *Drosophila* by choosing extreme phenotypes in selection experiments (35, 36). In relation to aggression, similar approaches have been taken in recent years by several laboratories via selection of hyperaggressive flies (1, 37, 38). Transcriptome analysis of aggressive compared with less-aggressive lines generated lists of many genetic differences with little agreement between the results of different laboratories. One gene that was followed up on by several laboratories was a member of the cytochrome P450 gene family that is closely associated with sensory signal detection (37, 39). Even after such initial forward and reverse genetic screens, the genetic basis of aggression in *Drosophila* remains poorly understood.

Significance

How high levels of aggression are generated in any organism is poorly understood, especially the genetic basis. Analyses of a hyperaggressive line of fruit flies (Bullies) generated by a genetic selection approach revealed a loss of the aggressive phenotype when animals were reared at a lower temperature. This effect offered an opportunity to perform RNA-seq analyses searching for genetic differences specifically related to hyperaggression. The results showed a small number of gene differences of twofold or more in the Bullies; one is a member of a neutral amino acid family of transporters suggested to be important in glutamate and GABA neurotransmitter regulation. Lowering levels of this gene in *Drosophila melanogaster* partially duplicates the high-aggression phenotype.

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Fig. 2. Hyperaggression is influenced by a temperature shift during a short preeclusion window. Several aspects of the Bully phenotype were examined when flies were grown at 19 °C or 25 °C throughout development or were shifted from 25 °C to 19 °C at different points during pupal life (shifts were made at APF⁰ or APF⁴⁸). All flies were shifted to 25 °C after eclosion and until used in experiments. (A) The latency to the first lunge was longer when Bullies were reared at 19 °C (Bully¹⁹) and in the APF⁰ and APF⁴⁸ groups compared with Bullies reared at 25 °C (Bully²⁵) (APF⁰ Wilcoxon test, $P = 0.08$; APF⁴⁸ Wilcoxon test, $P = 0.067$; Bully²⁵ Wilcoxon test, $P = 0.0004$). (B) Bully²⁵ flies showed higher numbers of lunges than those displayed by Bully¹⁹ flies (Wilcoxon $P = 0.02$) and by the APF⁰ and APF⁴⁸ groups of flies. (C) Bully²⁵ flies spent a greater amount of time boxing than all other groups (Wilcoxon test, $P = 0.02$), and (D) engaged in a higher number of boxing events than the Bully¹⁹ and the other two groups of flies (Wilcoxon test, $P = 0.03$).

these groups and searched for differentially expressed genes (DEG) between the groups that were selectively related to the Bully phenotype.

A comparison of RNA profiles of heads from Bully²⁵ and Canton-S²⁵ flies revealed 336 genes (Dataset S1) that were differentially expressed at a threshold of twofold or greater differences in gene expression levels. Next we attempted to separate gene differences that were selectively related to maintenance of the hyperaggression phenotype and were not related to the differences in genetic background. For this purpose, we examined gene expression differences in Bully²⁵ and Bully¹⁹ fly heads and compared these to the differences seen in the initial group. This examination reduced the list of candidate genes from 336 to 11. Finally, to control for any additional genes that changed due to temperature alone, the Canton-S²⁵ and Canton-S¹⁹ parent groups that showed no hyperaggression phenotype at either temperature were included as well, leaving only nine candidate genes (Fig. 3A). The genes involved were *CG13646*, *rha*, *CG10445*, *fit*, *CG33459*, *CG32199*, *CG13059*, *osi2*, and *drc*.

We attempted to verify the expression levels of the nine candidate genes using qRT-PCR in an additional three independent samples per group. Six of the nine genes consistently showed expression level differences that were in the same direction and of similar magnitude to the values found in the RNA-seq analysis (underlined in Fig. 3B and Dataset S2). To further support the notion that the expression differences of the group of six were selectively related to the Bully phenotype, we again used qRT-PCR to measure levels of gene expression in RNA samples from heads of flies reared under the APF⁰, APF⁴⁸, and Bully²⁵ experimental protocols (Fig. 3C and Fig. S1). In this experiment, we anticipated that the largest gene differences would be seen in the

Bully²⁵ group. Among the six candidate genes, the most consistent results with the least variation were seen with two genes: *CG13646* and *osi2* (Fig. 3C and Fig. S1). Focusing on *CG13646* (see below for selection of this gene), we asked whether several indicators of high-level aggression might correlate with the relative expression level of this gene. To explore this further, we constructed regression plots of transcript abundance in the RNA samples from the APF⁰, APF⁴⁸, and Bully²⁵ groups of flies vs. the median latency to the first lunge and the median numbers of lunges seen during 10 min after the first lunge (graph not shown). A strong positive correlation ($R^2 = 0.86$; $P < 0.0007$) was shown only between the median lunge latency and the expression level of *CG13646* (Fig. 3D).

***CG13646* Is a Putative Neutral Amino Acid Transporter Gene That Modulates Higher-Level Aggression.** To begin the analysis of possible functional roles of genes important in maintaining the Bully phenotype, we selected *CG13646* for further examination. This gene is a postulated member of a large family of closely related amino acid transporter genes found in many species, including humans and *Drosophila* (41). Of particular interest in this transporter group is that family members have been suggested to serve important roles in neurons and glia relating to GABA, glutamate, and glutamine metabolism in nerve terminals (42, 43). Moreover, as noted above, reduced expression of *CG13646* is well correlated

Fig. 3. Identification of candidate genes correlated with hyperaggression using differential expression profiles. (A) Relative transcript abundance in fly heads was measured using RNA-seq. The genotypes compared for differential expression were as follows: Bully²⁵ and Canton-S²⁵; Bully²⁵ and Bully¹⁹; and Canton-S²⁵ and Canton-S¹⁹. Using FDR < 0.05 (adjusted P values) and minimum FC > 2, nine genes were identified that show consistent differential expression that was correlated with hyperaggression. (B) Relative expression of the nine candidate genes in the Bully²⁵ groups were compared with Canton-S²⁵. qRT-PCR confirmed six (underlined) of the nine genes as candidates for the higher-level aggression phenotype (three biological replicates per group). (C) The expression of *CG13646* in Bully²⁵ flies was significantly down-regulated compared with both Bully APF⁰ (Student's t test $P = 0.003$), and Bully APF⁴⁸ flies (Student's t test $P = 0.01$). Log fold changes were calculated using *CG13646* expression in Bully¹⁹ as reference. (D) Lunge latency was strongly related to *CG13646* relative expression (linear regression analysis, lunge latency, $R^2 = 0.86$, $P = 0.0007$).

lines of flies. This gene is a member of the evolutionarily conserved solute-linked carrier superfamily of proteins (SLCs) (41, 45). The SLCs are the second largest collection of distinct membrane proteins in the human genome consisting of close to 400 proteins, distributed in 46 families in humans: 42 closely related families are found in the *Drosophila* genome (41). *CG13646* belongs to the SLC38 family of the SLC superfamily (46). In the human genome and in other mammalian species there are 11 members of this family, and they all are Na⁺-dependent amino acid transporters that are widely distributed in many tissues of the body, including the nervous system (45, 47). A recent publication has characterized the *SLC38A8* member of that family in mouse brain and has demonstrated that this transporter is found in both inhibitory and excitatory neurons and that it has a preference for transporting glutamine, alanine, arginine, histidine, and aspartate into neurons via a Na⁺-dependent mechanism (48). Of particular interest is that this transporter is hypothesized to be important in a glutamine/glutamate (GABA) cycle in the brain (49), in which glutamine, the amidated form of glutamic acid, serves to regulate stores of glutamate and GABA in nerve terminals. Another recent publication suggests that the A6 member of the SLC38 family of proteins is selectively localized at synaptic membranes of glutamatergic excitatory neurons (47). Different isoforms of the *SLC38* gene are highly expressed in different regions of the mammalian brain, where they likely serve different functions. For example, the A1 isoform is highly expressed in the thalamus, arcuate nucleus, and amygdala where it is postulated to be involved in aggression (45), whereas the A6 isoform is concentrated in the lateral hypothalamus where it is speculated to be involved in motivation and reward (42).

In the present studies we showed a consistent twofold decrease of *CG13646* expression in the Bully line reared at 25 °C in comparison with Bullies reared at 19 °C and to the control Canton-S parent line reared at either temperature. At least five other genes also showed elevations or reductions of expression in the heads of Bully²⁵ flies compared with all controls, and an even larger number showed smaller and more variable-level changes (Dataset S1). We selected *CG13646* for further study due to the consistent down-regulation of its mRNA levels associated with higher-level aggression, and because it showed a strong and highly significant coefficient of determination with elevated aggression. Because members of SLC family are commonly found in synaptic regions where they have been postulated to serve in glutamine/glutamate (GABA) and glutamine/glutamate cycles of transmitter regulation in nerve terminals (50), we hypothesized that genes like *CG13646* might be functioning at important nodes in pathways of behavioral regulation. We were delighted, therefore, to find that lowering levels of *CG13646* to similar levels of expression as those seen in Bully²⁵ fly heads, partially duplicated the aggression phenotype displayed by Bullies. Heterozygous mutants and RNAi knockdown of *CG13646* both yielded progeny that showed higher levels of aggression measured by lunge latency and total numbers of lunges displayed during fights; these flies also were significantly more likely to win fights against their control counterparts. Down-regulation of this single gene, however, did not result in elevated levels of boxing, the highest level of aggression seen during fights in *Drosophila*. This result may not be surprising given the complex architecture of aggressive behavior, which involves internal arousal, sensory experience, decision-making, and a persistent elevated aggressive state (30, 32, 51, 52).

In summary, in the present studies, beginning with the Bully line of hyperaggressive flies previously generated in our laboratory, we have identified a member of a new and interesting category of genes that is tied selectively to the Bully phenotype. The gene *CG13646* is a member of the SLC38 category of neutral amino acid transporters that have been proposed to be important in mammalian systems in glutamate and GABA neurotransmitter regulation. In the next stages of these studies, we will (i) examine the substrate specificity of the protein product of *CG13646* to ask if it indeed is a glutamine-selective transporter and (ii) develop

reagents that will allow us to localize the protein product in the *Drosophila* central nervous system.

Methods

Stocks and Fly Husbandry. Flies were maintained on standard cornmeal medium on a 12-h light/dark cycle at 25 °C and ambient humidity. The following strains were used for this study: Canton-S, Canton-S^{Bully} (1), *elav¹⁵⁵-Gal4;UAS-dcr2*, transgenic RNAi lines targeting the following genes: *CG13646* (no. 53701), Chorion protein 36 (no. 57838), and the negative control without any RNAi transgene (no. 36304). For mutant studies, PBac(RB)*CG13646⁶⁰³⁵⁸⁷* (Exelixis Harvard Stock Center) and the corresponding background strain used for the insertion, *w¹¹¹⁸* (no. 6326), were crossed to Canton-S females to generate the heterozygous and control flies, respectively. For RNAi knockdown studies, *elav¹⁵⁵-Gal4;UAS-dcr2* females were crossed to *UAS-CG13646^{RNAi}*, *UAS-chorion protein^{RNAi}*, and the negative control (*attP40* docking site only) to generate the corresponding lines for aggression assays.

For experiments involving different developmental temperatures, control flies were raised at 19 °C or 25 °C throughout development until eclosion (i.e., Bully¹⁹ or Bully²⁵). A temperature shift from 25 °C to 19 °C was performed at two different points during pupal development (APF⁰ and APF⁴⁸). After eclosion, flies were kept at 25 °C for 5–7 d before performing the aggression assays.

Aggression Assays. Socially naive males were isolated at late pupal stage and housed individually in single glass vials (16 × 100 mm) containing ~1.5 mL standard fly food. For flies reared at 19 °C, isolated pupae were kept at 19 °C until eclosion, and then transferred to 25 °C on the day after eclosion. Flies were aged for 5–7 d at 25 °C and ambient humidity before carrying out aggression assays. Aggression assays were always performed between pairs of males of the same genotype and experimental treatment unless otherwise specified. For winner–loser experiments, pairs of flies were briefly anesthetized with CO₂ at least 24 h before the behavioral assays. Different colors of water-based acrylic paint were applied to the dorsal thorax of both flies to facilitate visual tracking of individual flies.

For the experiments involving different developmental temperatures, assays were carried out in 12-well polystyrene plates as previously described (53). For all other experiments, aggression assays were performed in a newly developed behavioral chamber that involves minimal handling of flies (44). Food cups with standard fly food and with a drop of yeast paste on top were used as an attractive resource in all fights. Lunge and boxing behavior was scored as previously described (28): latency to lunge was the time between the first encounter on the food cup (social interaction lasting at least 3 s) and the occurrence of first lunge on the food cup. Winner–loser outcomes were defined as previously described (1). All aggression assays were carried out with age- and size-matched males during the first 3 h of the daily light cycle. Each fight was videotaped for at least 40 min, and videos were analyzed manually.

RNA-Seq and Differential Gene Expression Analysis. Two biological replicates of four groups of flies (CS¹⁹, CS²⁵, Bully¹⁹, and Bully²⁵) were used to carry out an RNA-seq analysis. For each group, 6-d-old socially isolated male flies were frozen in liquid nitrogen. Next, 90–100 frozen heads were collected per group and the samples were separated into two biological replicates. Total RNA extraction and the following RNA-seq analysis were performed at the Harvard Medical School Biopolymers Facility. Sequencing was performed on an Illumina HiSeq instrument, resulting in ~30 million of 50-bp reads per sample. Sequencing reads were mapped to the *Drosophila* transcriptome (dm3 RefSeq annotation) using TopHat (54). Gene expression counts were calculated using GenomicFeatures (55). Calculation of expression values and differential expression analysis were performed using the edgeR package (56). The genes with false discovery rate (FDR) < 0.05 (adjusted *P* values) and fold change (FC) > 2 were considered to indicate significant up-regulation; FDR < 0.05 and FC < 0.5 were considered as significant down-regulation; FC between 0.5 and 2 was considered not significant. For the identification of aggression-related genes, DEGs that showed different expression directions in CS²⁵ and Bully²⁵ flies were considered aggression related, such as genes up-regulated in CS²⁵ but suppressed in Bully²⁵, or down-regulated in CS²⁵ but up-regulated in Bully²⁵. Similarly, DEG of different expression directions in Bully¹⁹ and Bully²⁵ flies were considered aggression related, whereas DEGs between CS²⁵ and CS¹⁹ were considered not aggression related.

Quantitative PCR. Independent samples of fly heads (6-d-old socially naive males) were used to verify the RNA-seq results. Three samples of fly heads, each containing a pool of at least 30 individual heads, were collected from different groups and frozen in liquid nitrogen. Frozen heads were homogenized and total RNA was extracted using TRIzol (Life Technologies) following the protocol described by the manufacturer. cDNA from each sample was generated using the QuantiTect Reverse Transcription Kit (QIAGEN). Quantitative PCR were

performed using the iQ SYBR Green Supermix and CRX96 System (Bio-Rad). The list of primers used in the qRT-PCR analysis is provided in Table S1. Three replicates were included for each sample and the results were normalized against the mRNA level of actin to determine the ΔC_t (cycle threshold) values.

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