Reduced Function of the Glutathione S-Transferase S1 Suppresses Behavioral Hyperexcitability in Drosophila Expressing Mutant Voltage-Gated Sodium Channels

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ABSTRACT Voltage-gated sodium (Nav) channels play a central role in the generation and propagation of action potentials in excitatory cells such as neurons and muscles. To determine how the phenotypes of Nav-channel mutants are affected by other genes, we performed a forward genetic screen for dominant modifiers of the seizure-prone, gain-of-function Drosophila melanogaster Na+,channel mutant, paraS hu. Our analyses using chromosome deficiencies, gene-specific RNA interference, and single-gene mutants revealed that a null allele of glutathione S-transferase S1 (GstS1) dominantly suppresses paraS hu phenotypes. Reduced GstS1 function also suppressed phenotypes of other seizure-prone Na+,channel mutants, paraSEFS+ and paraBsm. Notably, paraS hu mutants expressed 50% less GstS1 than wild-type flies, further supporting the notion that paraS hu and GstS1 interact functionally. Introduction of a loss-of-function GstS1 mutation into a paraS hu background led to up- and down-regulation of various genes, with those encoding cytochrome P450 (CYP) enzymes most significantly over-represented in this group. Because GstS1 is a fly ortholog of mammalian hematopoietic prostaglandin D synthase, and in mammals CYPs are involved in the oxygenation of polyunsaturated fatty acids including prostaglandins, our results raise the intriguing possibility that bioactive lipids play a role in GstS1-mediated suppression of paraS hu phenotypes.

KEYWORDS Forward genetic screen genetic modifiers epilepsy RNA-sequencing analysis

Defects in ion-channel genes lead to a variety of human disorders that are collectively referred to as channelopathies. These include cardiac arrhythmias, myotonia, forms of diabetes and an array of neurological diseases such as epilepsy, familial hyperekplexia, and chronic pain syndromes (Rajakulendran et al. 2012; Venetucci et al. 2012; Waxman and Zamponi 2014; Dib-Hajj et al. 2015; Jen et al. 2016). The advent of genome-wide association studies and next-generation sequencing technology has made the identification of channelopathy mutations easier than ever before. However, the expressivity and disease severity are profoundly affected by interactions between the disease-causing genes and gene variants at other genetic loci. The significance of gene-gene interactions in channelopathies was demonstrated by Klassen et al. (2011), who performed extensive parallel exome sequencing of 237 human ion-channel genes and compared variation in the profiles between patients with the sporadic idiopathic epilepsy and unaffected individuals. The combined sequence data revealed that rare missense variants of known channelopathy genes were prevalent in both unaffected and disease groups at similar complexity. Thus, the effects of even deleterious ion-channel mutations could be compensated for by variant forms of other genes (Klassen et al. 2011).

Drosophila offers many advantages as an experimental system to elucidate the mechanisms by which genetic modifiers influence the severity of channelopathies because of the: wealth of available genomic information, advanced state of the available genetic tools, short life cycle, high fecundity, and evolutionary conservation of biological pathways (Hales et al. 2015; Ugur et al. 2016). In the current study, we focused on genes that modify phenotypes of a voltage-gated sodium (Na+) channel mutant in Drosophila. Na+ channels play a central role in the generation and propagation of action potentials in excitatory cells such as neurons and muscles (Hodgkin and Huxley 1952; Catterall 2012). In mammals, the Na+ channel gene family comprises nine paralogs.
These genes encode large (~260 kD) pore-forming Na₉-channel α-subunits, Na₉.1- Na₉.1.9, all of which have distinct channel properties and unique patterns of expression involving both subsets of neurons and other cell types. The Drosophila genome contains a single Na₉-channel gene, para (para), on the X chromosome. It encodes Na₉-channel protein isoforms that share high amino-acid sequence identity/similarity with mammalian counterparts (e.g., 45%/62% with the human Na₉, 1.1). High functional diversity of para Na₉ channels is achieved through extensive alternative splicing that produces a large number (~60) of unique transcripts (Kroll et al. 2013).

A number of para mutant alleles have been identified in Drosophila. They display a variety of physiological and behavioral phenotypes: lethality, ocellary defects, spontaneous tremors, neuronal hyperexcitability, resistance to insecticides, and paralysis or seizure in response to heat, cold, or mechanical shock (Suzuki et al. 1971; Ganetzky and Wu 1982; Lilly et al. 1994; Martin et al. 2000; Lindsay et al. 2008; Parker et al. 2011; Sun et al. 2012; Schutte et al. 2014; Kaas et al. 2016). One of these more recently characterized Na₉-channel gene mutants, paraShu, is a dominant gain-of-function allele formerly referred to as Shudderer due to the “shuddering” or spontaneous tremors it causes (Williamson 1971; Williamson 1982). This allele contains a missense mutation that results in the replacement of an evolutionarily conserved methionine (Williamson 1982; Lilly 1982; Sun et al. 2014; Kaas et al. 2016). Adult paraShu mutants exhibit various dominant phenotypes in addition to shuddering, such as defective climbing behavior, increased susceptibility to electroconvulsive and heat-induced seizures, and short lifespan. They also have an abnormal down-turned wing posture and an indented thorax, both of which are thought to be caused by neuronal hyperexcitability (Williamson 1982; Kaas et al. 2016; Kasuya et al. 2019). In the current study, we carried out a forward genetic screen for dominant modifiers of paraShu and found that the phenotypes are significantly suppressed by loss-of-function mutations in the glutathione S-transferase S1 (GstS1) gene. To obtain insights into the mechanisms underlying this GstS1-mediated suppression of paraShu phenotypes, we also performed RNA-sequencing analysis. This revealed changes in gene expression that are caused by reduced GstS1 function in the paraShu background.

MATERIALS AND METHODS

Fly stocks and culture methods

Flies were reared at 25°C, 65% humidity in a 12 hr light/dark cycle on a cornmeal/glucose/yeast/agar medium supplemented with the mold inhibitor methyl 4-hydroxybenzoate (0.05%). The exact composition of the fly food used in this study was described in Kasuya et al. (2019). The Canton-S strain was used as the wild-type control, paraStu, which was originally referred to as Shudderer (Shu) (Williamson 1982) and was obtained from Dr. Rodney Williamson (Beckman Research Institute of the Hope, CA). Drosophila lines carrying deficiencies of interest and gene-specific UAS-RNAi transgenes (CG3930, GD36069; CG6967; GD27769; CG30466; GD40624; CG8946, KK10572; CG6984, GD21650; Gs1, GD1635) were obtained from the Bloomington Stock Center (Indiana University, IN) and the Vienna Drosophila Resource Center (Vienna, Austria), respectively. GstS1M26 was obtained from Dr. Tina Tootle (University of Iowa, IA). Genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome (DS) flies (paraDS and paraDS+) (Sun et al. 2012; Schutte et al. 2014) were obtained from Dr. Diane O’Dowd (University of California, Irvine, CA), and bangsenselss (paraStu) files were obtained from Dr. Chun-Fang Wu (University of Iowa, IA).

Behavioral assays

Reactive climbing: The reactive climbing assay was performed as previously described (Kaas et al. 2016), using a countercurrent apparatus originally invented by Seymour Benzer (Benzer 1967). Five to seven-day-old females (~20) were placed into one tube (tube #0), tapped to the bottom, and allowed 15 sec to climb, at which point those that had climbed were transferred to the next tube. This process was repeated a total of five times. After the fifth trial, the flies in each tube (#0 ~ #5) were counted. The climbing index (CI) was calculated using the following formula: CI = 2(Ni x i)/(5 x ΣNi), where i and Ni represent the tube number (0-5) and the number of flies in the corresponding tube, respectively. For each genotype, at least three groups were tested.

Video-tracking locomotion analysis: Five-day-old flies were individually transferred into a plastic well (15 mm diameter × 3 mm depth) and their locomotion was recorded at 30 frames per second (fps) using a web camera at a resolution of 320 × 240 pixels for 10 min. The last 5 min of the movies were analyzed using pySolo, a multi-platform software for the analysis of sleep and locomotion in Drosophila, to compute the x and y coordinates of individual flies during every frame (Gilestro and Cirelli 2009). When wild-type flies are placed in a circular chamber, they spend most of their time walking along the periphery of the chamber (Besson and Martin 2005), resulting in circular tracking patterns. In contrast, the uncoordinated movements caused by spontaneous tremor or jerking of paraStu mutants lead to their increased presence in the center part of the chambers. The tremor frequency was therefore indirectly assessed by determining the percentage of time that fly stayed inside a circle whose radius is 74.3% of that of the entire chamber. The distance between the fly’s position and the center of the chamber was calculated using the formula (X₂-X₁)²+(Y₂-Y₁)² < 13² where X₁ and Y₁ are the coordinates of the fly, and X₂ and Y₂ are the coordinates of the chamber center (13 mm is 74.3% of the chamber radius).

Heat-induced seizures: Newly eclosed flies were collected in groups of 20 and aged for three to five days, after which the heat-induced seizure assay was performed as previously described (Sun et al. 2012). Briefly, a single fly was put into a 15 × 45 mm glass vial at room temperature (Thermo Fisher Scientific, MA) and allowed to acclimate for two to 10 min. The glass vial was then submerged in a water bath at the specified temperature for two minutes, during which the fly was video-taped and assessed for seizure behavior every five seconds.
Seizure behavior was defined as loss of standing posture followed by leg shaking.

**Bang-sensitive assay:** The bang-sensitive assay was carried out following a previously described protocol (Zhang et al. 2002). Briefly, 10 flies were raised on conventional food for two to three days post-eclosion. Prior to testing, individual flies were transferred to a clean vial and acclimated for 30 min. Next, the vials were vortexed at maximum speed for 10 sec, and the time to recovery was measured. Recovery was defined as the ability of flies to stand upright following paralysis. At least five independent bang-sensitive assays were carried out for each genotype.

**Male mating assay:** Newly eclosed paraShu males with or without one or two copies of GstS1M26 (i.e., paraShu/Y; +/+; paraShu/Y; GstS1M26/+; and paraShu/Y; GstS1M26/GstS1M26) were collected. Each was placed, along with three to five day-old wild-type (Canton-S) virgin females, into a plastic tube (75 x 12 mm) containing approximately 1 ml of fly food. Tubes were kept at room temperature (~22°C) for two weeks, at which point they were examined for the presence of progeny.

**Gene expression analysis**

RNA was purified from one-day-old female flies using Trizol solution (Ambion, Carlsbad, CA) and an RNasy column (Qiagen, Valencia, CA). Flies of four genotypes were used: (1) +/+; +/+, (2) paraShu/+; +/+, (3) +/+; GstS1M26/+; , and (4) paraShu/+; GstS1M26/+; . For each genotype, RNA-sequencing (RNA-seq) analysis was performed (four biological replicates) by the Iowa Institute of Human Genetics (IIHG) Genomics Division (University of Iowa, Iowa). DNase I-treated total RNA (500 ng) samples were enriched for PolyA-containing transcripts by treatment with oligo(dT) primer-coated beads. The enriched RNA pool was then fragmented, converted to cDNA, and ligated to index-containing sequence adaptors using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Cat. #RS-122-2101, Illumina, Inc., San Diego, CA). The molar concentrations of the indexed libraries were measured using the 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and combined equally into pools for sequencing. The concentrations of the pools were measured using the Illumina Library Quantification Kit (KAPA Biosystems, Wilmington, MA) and the samples were sequenced on the Illumina HiSeq 4000 genome sequencer using 150 bp paired-end SBS chemistry. Sequences in FASTQ format were analyzed using the Galaxy platform (https://usegalaxy.org/). The FASTQ files were first evaluated using a quality-control tool, FastQC. The sequenced reads were filtered for those that met two conditions: minimum length >20 and quality cutoff ≥20. After the quality control assessments were made, the reads were mapped to Release 6 of the *Drosophila melanogaster* reference genome assembly (dm6) using the STAR tool. The number of reads per annotated gene was determined by running the featureCounts tool. The differential gene expression analyses were performed using the DESeq2 tool (Love et al. 2014), which uses the median of ratios method to normalize counts. The P-value was adjusted (Padj) for multiple testing using the Benjamini-Hochberg procedure, which controls for the false discovery rate (FDR). For functional enrichment analysis of differentially expressed genes (DEGs), we generated a list of those for which Padj < 0.05 and applied it to the GOseq tool for gene ontology analysis (Young et al. 2010).

**Statistical analysis**

Statistical tests were performed using Sigma Plot (Systat Software, San Jose, CA). For multiple groups that exhibit non-normal distributions, the Kruskal-Wallis one-way ANOVA on ranks test was performed using Dunn’s method *post hoc*. Data that did not conform to a normal distribution are presented as box-and-whisker plots (boxplots). Values of the first, second, and third quartiles (box) are shown, as are the 10th and 90th percentiles (whisker), unless otherwise stated. Two-way repeated measures ANOVA and Holm-Sidak multiple comparisons were used to analyze temperature-induced behavioral phenotypes. Fisher’s exact test was used to analyze the wing and thorax phenotypes of paraShu mutants. For multiple comparison, the P-values were compared to the Bonferroni adjusted type I error rate for significance. Statistical analyses for RNAseq experiments are described in the previous section “Gene expression analysis by RNA-sequencing”.

**Data availability**

Fly strains are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present.
RESULTS

The chromosomal region 53F4-53F8 contains a dominant modifier(s) of para\textsuperscript{Shu}

To identify genes that interact with para\textsuperscript{Shu} and influence the severity of the phenotype, we performed a forward genetic screen for dominant modifiers of para\textsuperscript{Shu} using the Bloomington Deficiency Kit (Cook et al. 2012; Roote and Russell 2012). Females heterozygous for para\textsuperscript{Shu} (para\textsuperscript{Shu}/EM7) were crossed to males carrying a deficiency on the second or third chromosome (+/Y; Df(2)/balancer or +/Y; Df(3)/balancer). The effects of the deficiency on para\textsuperscript{Shu} were evaluated by examining the F1 female progeny trans-heterozygous for para\textsuperscript{Shu} and the deficiency (e.g., para\textsuperscript{Shu}/+; Df/+ for their reactive climbing behavior (see Materials and Methods). As reported previously, para\textsuperscript{Shu} heterozygous females have a severe defect in climbing behavior due to spontaneous tremors and uncoordinated movements (Kaas et al. 2016). Our initial screen identified several chromosomal deficiencies that significantly improved the climbing behavior of para\textsuperscript{Shu} females (Supplemental Table 1; deficiencies that resulted in CI > 0.4 are shaded). The current study focuses on one of these deficiencies, Df(2R)P803-\Delta 15.

The Df(2R)P803-\Delta 15 deficiency spans chromosomal region 53E-53F11 on the right arm of the second chromosome, but a lack of nucleotide level information regarding its break points makes identifying the genomic region responsible for suppression of the para\textsuperscript{Shu} phenotypes challenging. Therefore, we used three additional deficiencies which overlap Df(2R)P803-\Delta 15 and also have molecularly defined break points (Figure 1A). Phenotypic analysis of para\textsuperscript{Shu} females crossed to these deficiencies revealed that Df(2R)Exel6065 and Df(2R)BSC433, but not Df(2R)Exel6066, had a robust suppressing effect similar to that of Df(2R)P803-\Delta 15 (Figure 1B). Of the two suppressing alleles, Df(2R)BSC433 carries the smaller deficiency; it spans genomic region 53F4 to 53F8 (Figure 1A).

The suppressive effect of Df(2R)BSC433 was confirmed by analyzing other para\textsuperscript{Shu} phenotypes. The introduction of Df(2R)BSC433 to the para\textsuperscript{Shu} background (para\textsuperscript{Shu}/+; Df(2R)BSC433/+ significantly reduced the severity of the abnormal wing posture, indented thorax (Figure 2A), spontaneous tremors (Figure 2B), and heat-induced seizures (Figure 2C). Two deficiency lines, Df(2R)BSC273 (49F4-50A13) and Df(2R)BSC330 (51D3-51F9), carry a genetic background comparable to that of Df(2R)BSC433. Unlike Df(2R)BSC433, these deficiencies did not lead to suppression of para\textsuperscript{Shu} phenotypes (Figure 2A-C), showing that the effect of Df(2R)BSC433 is not due to its genetic background. Taken together, these results clearly demonstrate that removal of one copy of the genomic region 53F4-53F8 reduces the severity of multiple para\textsuperscript{Shu} phenotypes, and that a dominant para\textsuperscript{Shu} modifier is present in this chromosomal segment.

GstS1 loss of function suppresses para\textsuperscript{Shu} phenotypes

Based on the molecularly defined breakpoints of Df(2R)BSC433 (2R:17,062,915 and 2R:17,097,315), it disrupts six genes that are localized in the 53F4-53F8 region: CG6950, CG6967, CG30460, CG8946 (Sphingosine-1-phosphate lyase; Sply), CG8984, and CG8938 (Glutathione S-transferase S1; GstS1) (Figure 3A). To identify the gene(s) whose functional loss contributes to the marked suppression of para\textsuperscript{Shu} phenotypes by Df(2R)BSC433, we knocked down each gene separately using gene-specific RNAi and examined the effects on para\textsuperscript{Shu} phenotypes. Expression of each RNAi transgene of interest was driven by the ubiquitous Gal4 driver, da-Gal4. RNAi-mediated knockdown of CG6967 or Sply resulted in developmental lethality, whereas knockdown of CG6950, CG30460, CG6984 or GstS1 did not. Among the viable adult progeny with gene-specific knockdown, those...
in which Gst1S1 was knocked down showed the greatest improvement in wing and thorax phenotypes (Figure 3B). Since the effectiveness of the RNAi transgenes for CG8950, CG30460, and CG6984 was not strictly evaluated, we could not completely rule out the possible involvement of these genes in the observed phenotypic suppression. Nevertheless, reduced GstS1 function most likely contributes to the suppression of paraShu phenotypes by Df(2R)BSC433.

GstS1M26 is a null allele of GstS1 in which the entire coding region is deleted (Whitworth et al. 2005) and homozygotes are viable as adults. We used GstS1M26 to determine how reduced GstS1 function affects paraShu phenotypes. In paraShu/+; GstS1M26/+ flies, both the morphological (downturned wing and indented thorax) and behavioral (spontaneous tremors and heat-induced seizure) phenotypes were considerably milder than in their paraShu/+ counterparts (Figure 4A-C). paraShu phenotypes were not further improved in

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**Figure 3** Glutathione S-transferase S1 (GstS1) as a robust genetic modifier of paraShu. (A) Depiction of six genes that are localized within chromosomal region 53F4-53F8 and disrupted by the chromosomal deficiency Df(2R)BSC433. Arrows indicate the direction of gene transcription. (B) The frequency of paraShu morphological phenotypes following RNAi-mediated knockdown of each gene. Gene-specific RNAi was ubiquitously expressed using da-GAL4 in paraShu heterozygous females (e.g., paraShu/++; da-GAL4/UAS-RNAi). The downturned wing (Wings) and indented thorax (Thorax) phenotypes were scored. Numbers in the bar graph indicate how many flies were scored. Fisher’s exact test with Bonferroni correction was used to analyze the data.

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**Figure 4** GstS1M26 as a dominant suppressor of paraShu phenotypes. The effects of the GstS1 null allele, GstS1M26, on paraShu phenotypes were examined in flies of three genotypes: (1) paraShu/+; +/+, (2) paraShu/+; GstS1M26/+, and (3) paraShu/+; GstS1M26/GstS1M26. (A) Frequencies of down-turned wings (Wings) and indented thorax (Thorax). Numbers in the bar graph indicate how many flies were scored. (B) Severity of spontaneous tremors. 8–10-day-old paraShu/+ females were used. Numbers in the boxplot indicate how many flies were scored. (C) Frequencies of heat-induced seizures. Three groups of 30-50 flies at 4-5 days after eclosion were used per genotype. Averages are shown with SEM. Fisher’s exact test with Bonferroni correction (A), the Kruskal-Wallis one-way ANOVA on ranks with Dunn’s method, (B) and two-way repeated measures ANOVA and Holm-Sidak multiple comparisons (C) were used to analyze the data. ***P < 0.001; *P < 0.05; NS, not significant (P > 0.05).
Df(2R)BSC433
53F4-53F8
Df(3R)by10
paraShu
GstS1
Viability was significantly lower when one or two copies of GstS1 M26 were introduced into paraShu females and wild-type males. However, this could be due to a ceiling effect of severe neurological defects caused by paraShu mutation. Further investigation is required to evaluate the effect of GstS1 overexpression on paraShu phenotypes.

GstS1 M26 reduced the severity of the male paraShu phenotypes as well, including not only viability, but also courtship behavior and copulation. With respect to viability, paraShu males represented only 8.2% of the male progeny (paraShu/Y and FM7/Y) of a cross between paraShu/FM7 females and wild-type males. Viability was significantly higher when one or two copies of GstS1 M26 were introduced into paraShu males (paraShu/Y; GstS1 M26+ and paraShu/Y; GstS1 M26/GstS1 M26), with paraShu males carrying GstS1 M26 representing 31.4% and 53.1% of the total male progeny, respectively (Table 1). The effects of paraShu on male courtship behavior/copulation are a consequence of the strong morphological (down-turned wings and indented thorax) and behavioral (spontaneous tremors and uncoordinated movements) phenotypes. When paraShu males were individually placed into small tubes with four wild-type virgin females and food, only one out of 43 (2.3%) produced progeny. The introduction of GstS1 M26 improved the ability to produce progeny; 17 out of 45 paraShu males (37.8%) heterozygous for GstS1 M26, and 17 out of 44 paraShu males (38.6%) heterozygous for GstS1 M26, produced progeny under the above-mentioned conditions (Table 1).

**Table 1 Effects of GstS1 M26 on viability and fertility of paraShu males**

| Genotype                  | Viability | Fertility |
|----------------------------|-----------|-----------|
|                           | Total     | FM7/Y     | paraShu/Y | % paraShu/Y | Total     | Sterile | Fertile | % Fertile |
| paraShu/Y; +/+            | 73        | 67        | 6         | 8.2        | 43        | 42      | 1       | 2.3       |
| paraShu/Y; GstS1 M26/+    | 121       | 83        | 38        | 31.4       | 45        | 28      | 17      | 37.8      |
| paraShu/Y; GstS1 M26/GstS1 M26 | 145   | 68        | 77        | 53.1       | 44        | 27      | 17      | 38.6      |

GSTS1 M26 homozygotes (paraShu/++; GstS1 M26/GstS1 M26), where GstS1 function was completely eliminated (Figure 4A-C). Thus, GstS1 M26 is a dominant suppressor of female paraShu phenotypes. Using a ubiquitous Gal4 driver, da-Gal4, and the UAS-GstS1 transgene (Whitworth et al. 2005), we carried out a preliminary experiment to examine how overexpression of the wild-type GstS1 affects paraShu phenotypes. The phenotypic severity was not significantly increased by GstS1 overexpression (paraShu/UAS-GstS1; da-Gal4/+ vs. paraShu/++; da-Gal4/+; data not shown). However, this could be due to a ceiling effect of severe neurological defects caused by paraShu mutation. Further investigation is required to evaluate the effect of GstS1 overexpression on paraShu phenotypes.

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**Table 2 Effects of GST gene deletions on wing and thorax phenotypes of paraShu/+**

| Chromosomal deficiency | Deleted segment | Deleted GST genes | Flies scored | Down-turned wings (%) | (P-value) | Indented thorax (%) | (P-value) |
|------------------------|-----------------|-------------------|-------------|----------------------|----------|--------------------|----------|
| Df(3R)Exel6164         | 87B5-87B10      | GstD1-D11         | 72          | 95.8                 | 0.154    | 62.5               | 0.001*   |
| Df(2R)BSC335           | 55C6-55F1       | GstE1-E11         | 59          | 91.5                 | 0.741    | 86.4               | 0.550    |
| Df(2R)BSC856           | 60E1-60E4       | GstE12            | 68          | 77.9                 | 0.208    | 83.8               | 0.397    |
| Df(2R)BSC271           | 44F12-45A12     | GstE13            | 67          | 94.0                 | 0.480    | 77.6               | 0.0775   |
| Df(2R)BSC273           | 49F4-50A13      | GstE14            | 66          | 92.4                 | 0.517    | 90.9               | 1        |
| Df(3L)BSC157           | 66C12-66D6      | GstO1-O4          | 150         | 94.7                 | 0.175    | 70.0               | 0.0052   |
| Df(2R)BSC132           | 45F6-46B4       | GstT1-T2          | 51          | 68.6                 | 0.025    | 9.8                | <0.00001*|
| Df(1)Exel6254          | 19C4-19D1       | GstT3             | 34          | 91.2                 | 1        | 94.2               | 0.691    |
| Df(1)Exel6245          | 11E11-11F4      | GstT4             | 26          | 23.1                 | <0.00001*| 80.8               | 0.277    |
| Df(3R)by10             | 85D8-85E13      | GstZ1-Z2          | 57          | 89.5                 | 1        | 91.2               | 1        |
| Df(2R)BSC433           | 53F4-53F8       | GstS1             | 56          | 12.5                 | <0.00001*| 35.7               | <0.00001*|
| No deficiency          | NA              | NA                | 44          | 88.6                 | NA       | 90.9               | NA       |

Statistical significance in the severity of wing and thorax phenotypes between paraShu (paraShu/+) and paraShu with a deficiency (paraShu/++; Df+ or paraShu/Df) was assessed using Fisher’s exact test. The P-values were compared to Bonferroni adjusted type I error rate of 0.05/11 (=0.004545….) for significance (*). NA, not applicable.

**Loss of function of other glutathione S-transferase genes does not suppress paraShu phenotypes as that of GstS1**

The Drosophila melanogaster genome contains 36 genes that encode cytosolic glutathione S-transferases (GSTs). These are classified as Delta (D), Epsilon (E), Omega (O), Theta (T), Zeta (Z), or Sigma (S) based on similarities in the amino-acid sequences of the encoded proteins (Tu and Akgul 2005; Saisawang et al. 2012). GstS1 is the sole Drosophila member of the S class GST genes. To determine whether reductions in the copy number of other GST genes have significant impacts on paraShu phenotypes, we generated paraShu mutants carrying chromosome deficiencies that remove the D, E, O, T, or Z class of GST genes. Given that genes encoding GSTs of the same class tend to form gene clusters, a single chromosome deficiency often removes multiple GST genes of the same class. For example, Df(3R)Exel6164 (87B5-87B10) removes eleven GST genes of the D class (GstD1-D11) (Table 2). For GST genes on the autosomes, paraShu/ females (paraShu/FM7) were crossed to males carrying a GST deficiency on the second or third chromosome. For the two GST genes on the X chromosome (GstT3 and GstT4), females carrying the deficiency (Df/FM7) were crossed to paraShu males (paraShu/Y) because males carrying this (Df/Y) were not viable. The female progeny carrying both paraShu and a deficiency of interest were examined for their wing posture and thorax morphology. As shown in Table 2, as well as in Figure 2, removing one copy of GstS1 in the context of Df(2R)BSC433 resulted in significant suppression of both the down-turned wing and the indented thorax phenotypes of paraShu, but this ability was not shared by any of the 36 other cytosolic GST genes. In some cases, however, there was partial suppression of one or the other phenotype. For example, when one copy of GstT4 was
removed (using Df(1)Exel6245), the wing phenotype, but not the thorax phenotype, was suppressed. Similarly, the indented thorax phenotype, but not the down-turned wing phenotype, was reduced when DfD1-D11 was removed (using Df(3R)Exel6164) and when GSTT1-T2 was removed (using Df(2R)BSC132).

**GSTS1**M26 suppresses the phenotypes of other para gain-of-function mutants

We next examined whether phenotypes of other Na⁺-channel mutants are similarly affected by reduced *GstS1* function. Generalized epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome (DS) are common childhood-onset genetic epileptic encephalopathies (Claes et al. 2001; Catterall et al. 2010). Sun et al. (2012) and Schutte et al. (2014) created Drosophila para knock-in alleles, gain-of-function paraGEFS⁺ and loss-of-function paraDS, by introducing a disease-causing human GEFS+ or DS mutation at the corresponding position of the fly Na⁺-channel gene. At 40°C, paraGEFS⁺ homozygous females and hemizygous males exhibit a temperature-induced seizure-like behavior that is similar to, but milder than, that observed in paraGEFS⁺ flies (Sun et al. 2012; Kaas et al. 2016; Kasuya et al. 2019). paraDS flies lose their posture shortly after being transferred to 37°C (Schutte et al. 2014). The temperature-induced phenotype of paraGEFS⁺ was significantly suppressed when a single copy of GSTS1M26 was introduced into paraGEFS⁺ males (paraGEFS⁺/+; GSTS1M26/+ (Figure 5A)). In contrast, the severity of the phenotype in paraDS males was unaffected by a copy of GSTS1M26 (paraGEFS⁺/+; GSTS1M26/+ (Figure 5B)).

We also examined paraDS⁺, which is a hyperexcitable, gain-of-function para mutant allele that displays semi-dominant, bang-sensitive paralysis (Parker et al. 2011). The severity of the paraDS⁺ bang-sensitivity was evaluated as the time for recovery from paralysis that had been induced by mechanical stimulation (10 sec of vortexing). All paraDS⁺ flies were paralyzed immediately after this mechanical stimulation. By three minutes after mechanical stimulation, 92% of the paraDS⁺ males carrying GSTS1M26 (paraDS⁺/+; GSTS1M26/+ had recovered from paralysis and were able to right themselves, whereas only 12.6% of paraDS⁺ males had recovered. The median recovery time for paraDS⁺ males carrying GSTS1M26 was 88 sec and that for paraDS⁺ males was 160 sec (Figure 5C).

**RNA sequencing analysis revealed changes in gene expression caused by paraShu and GSTS1M26 mutations**

To gain insights into the molecular basis of the *GstS1*-dependent suppression of paraShu phenotypes, we performed RNA sequencing (RNA-seq) analysis and examined the transcriptome profiles of paraShu and wild-type females with or without GSTS1M26. Whole-body transcriptomes of one-day-old females were compared among four genotypes: (1) +/+, (2) paraShu/+; +/+, (3) +/+; GSTS1M26/+, and (4) paraShu/+; GSTS1M26/+). Each sample generated at least 21 million sequencing reads, of which >99% met the criteria of having a quality score of >20 and a length of >20 bp. Moreover, duplicate reads encompassed ~70% of total reads, which was expected from the RNA-seq data (Bansal 2017).

We found that 129 genes were differentially expressed (threshold: adjusted *P*-value (*P*adj)<0.05) between paraShu and wild-type females. Among these, 89 and 40 genes were up- and down-regulated, respectively, in paraShu vs. wild-type flies (Supplemental Table 2). Gene ontology analysis of the differentially expressed genes was performed using GOseq tools (Young et al. 2010). Genes associated with four Gene Ontology categories were found to be overrepresented within

| Gene ontology | Term | Ontology class | *P*-adj over-represented value | # of genes |
|---------------|------|----------------|------------------------------|------------|
| GO:00080010   | structural constituent of cuticle development | BP  | 2.01E-2 | 9          |
| GO:00023020   | structural constituent of cuticle | MF  | 8.39E-3 | 8          |

MF: molecular function, BP: biological process.
the dataset (P_{adj} < 0.05), each with a functional connection to the cuticle: “structural constituent of cuticle GO:0000481”), “structural constituent of cuticle GO:0000528”), and “cuticle-based cuticle development GO:0000481) (Table 3). Within this two GO categories, eight genes were differentially expressed between paraShu and wild-type flies (Table 4).

Among the genes that are differentially regulated (P_{adj} < 0.05) between wild-type and paraShu flies (Supplemental Table 2), 16 displayed a fold change of >2 and all are up-regulated in paraShu flies (Table 5). They encode: a transferase (CG32581), two lysozymes (LysC and LysD), two endopeptidases (Ion25Bi and CG32523), one endonuclease (CG3819), two cytochrome P450 proteins (Cyp4p1 and Cyp6w1), three ABC transporters (lgd, CG1494), and three transcription factors (Imd, CG1494 and Ada1-1), and two cuticle proteins (Cpr47Ef and Ccp84Ab). Of note, Gtst1 was one of the 40 genes that are significantly down-regulated in paraShu females; the average normalized sequence counts (DESeq2) were 50% reduced (15562.21 vs. 7782.01, adjusted P_{adj} = 0.00036) (Table 6, Figure 6). In general, we did not observe any significant differences in the expression of other GST genes between paraShu and wild-type flies, with the only exceptions being GstD2 and GstO2 (Table 6), down-regulated and up-regulated, respectively.

We next examined how Gtst1M26 affects gene expression profiles in paraShu mutants. The fact that Gtst1M26 is a deletion mutation that removes the entire coding region of Gtst1 (Whittworth et al. 2005) is consistent with our discovery that the levels of the Gtst1 transcript were 50% lower than those in wild-type flies when one copy of Gtst1M26 was introduced (Figure 6). Since paraShuM2 and Gtst1M26 each reduced paraShu expression by ~50%, the level of Gtst1 expression in paraShuM2 Gtst1M26 double heterozygotes (paraShu+/+; Gtst1M26+/+) was approximately one quarter of that in wild-type flies (Figure 6).

Comparison of paraShu flies to paraShuM2 and Gtst1M26 double mutants (paraShu+/+;+/+ vs. paraShu+/+; Gtst1M26+/+) revealed the differential expression of 220 genes (P_{adj} < 0.05; Supplemental Table 2). Among these, 120 were up-regulated and 100 were down-regulated in paraShuM2 plus Gtst1M26 flies. Functional enrichment analysis of the differentially expressed genes revealed that genes associated with five specific molecular functions were over-represented. These include “heme binding” (GO:00002037), “tetrapyrrole binding” (GO:00064906), “iron ion binding” (GO:0005505), “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen” (GO:0016705), and “cofactor binding” (GO:0048037) (Table 7). Thirteen differentially regulated genes were associated with all five GO terms. These all encode heme-containing enzymes CYPs (Table 8, marked with asterisks) that catalyze a diverse range of reactions and are critical for normal developmental processes and the detoxification of xenobiotic compounds (Hannemann et al. 2007; Isin and Guengerich 2007; Chung et al. 2009).

Among the 220 genes differentially regulated in paraShu in the absence or presence of Gtst1M26 (paraShu+/+;+/+ vs. paraShu+/+; Gtst1M26+/+), 25 were up-regulated and 12 were down-regulated (cutoff: fold

### Table 4 Differentially expressed genes in paraShu/M2 compared with control that are included in the enriched GO terms

| Flybase ID | Gene symbol | Fold change (log2) | Fold change | P_{adj} | Gene product |
|------------|-------------|--------------------|-------------|---------|--------------|
| FBgn0033603 | Cpr47Ef     | 1.14               | 2.20        | 2.03E-11 | Cuticular protein 47Ef |
| FBgn0004782 | Ccp84Ab     | 1.02               | 2.03        | 1.83E-10 | Ccp84Ab |
| FBgn0004783 | Ccp84Aa     | 0.92               | 1.89        | 9.93E-05 | Ccp84Aa |
| FBgn0001112 | Gld         | 0.73               | 1.66        | 3.22E-03 | Glucose dehydrogenase |
| FBgn0004780 | Ccp84Ad     | 0.72               | 1.65        | 1.02E-02 | Ccp84Ad |
| FBgn0035281 | Cpr62Bc     | 0.64               | 1.56        | 3.59E-03 | Cuticular protein 62Bc |
| FBgn0036619 | Cpr72Ec     | 0.64               | 1.55        | 3.97E-02 | Cuticular protein 72Ec |
| FBgn0036680 | Cpr73D      | 0.51               | 1.43        | 3.35E-02 | Cuticular protein 73D |
| FBgn0052029 | Cpr66D      | -0.68              | 0.62        | 9.58E-03 | Cuticular protein 66D |

Listed are genes differentially expressed in paraShu compared with control (Canton-S) with Fold change > 2 and P_{adj} < 0.01.

### Table 5 Genes most differentially expressed in paraShu/+ compared with control

| Flybase ID | Gene symbol | Fold change (log2) | Fold change | P_{adj} | Gene product |
|------------|-------------|--------------------|-------------|---------|--------------|
| FBgn0052581 | CG32581     | 3.78               | 13.75       | 5.26E-99 | uncharacterized protein |
| FBgn0010549 | (2)03659    | 2.44               | 5.43        | 3.70E-40 | lethal (2) 03659 |
| FBgn0020906 | Ion25Bi     | 1.72               | 3.29        | 4.84E-18 | Jonah 25Bi |
| FBgn0052523 | CG32523     | 1.66               | 3.17        | 3.83E-19 | uncharacterized protein |
| FBgn0004427 | LysD        | 1.47               | 2.77        | 2.68E-13 | Lysozyme D |
| FBgn0004426 | LysC        | 1.46               | 2.76        | 7.84E-14 | Lysozyme C |
| FBgn0015037 | Cyp4p1      | 1.35               | 2.55        | 6.95E-15 | Cytochrome P450-4p1 |
| FBgn0033065 | Cyp6w1      | 1.30               | 2.47        | 3.77E-15 | Cyp6w1 |
| FBgn0032286 | CG7300      | 1.20               | 2.30        | 2.47E-08 | uncharacterized protein |
| FBgn0031169 | CG1494      | 1.20               | 2.30        | 4.50E-08 | uncharacterized protein |
| FBgn0033603 | Cpr47Ef     | 1.14               | 2.20        | 2.03E-11 | Cuticular protein 47Ef |
| FBgn0039039 | Imd         | 1.11               | 2.16        | 5.21E-07 | lame duck |
| FBgn0033458 | CG18446     | 1.10               | 2.14        | 7.42E-08 | uncharacterized protein |
| FBgn0051865 | Ada1-1      | 1.09               | 2.12        | 7.74E-08 | transcriptional Adaptor 1-1 |
| FBgn0036833 | CG3819      | 1.07               | 2.11        | 1.12E-17 | uncharacterized protein |
| FBgn0004782 | Ccp84Ab     | 1.02               | 2.03        | 1.83E-10 | Ccp84Ab |

Listed are genes differentially expressed in paraShu compared with control (Canton-S) with Fold change > 2 and P_{adj} < 0.01.
The gene for which the fold-change was greatest in paraShu plus GstS1M26 flies was a member of the cytochrome P450 family, Cyp4p2; it was down-regulated 6.4-fold in the presence of GstS1M26, with \( P_{adj} = 3.5 \times 10^{-48} \). Notably, three of the top 20 genes with the greatest fold expression changes were members of this family (Cyp4p2, Cyp6a8, Cyp6a2).

**DISCUSSION**

In the present study, we performed an unbiased forward genetic screen to identify genes that can modify the severity of the phenotypes associated with paraShu, a gain-of-function variant of the Drosophila Na\(_a\) channel gene. Our key finding was that a 50% reduction of GstS1 function resulted in strong suppression of paraShu phenotypes. Glutathione S-transferases (GSTs) are phase II metabolic enzymes that are primarily involved in conjugation of the reduced form of glutathione to endogenous and xenobiotic electrophiles for detoxification (Hayes et al. 2005; Allocati et al. 2018). Reduced GST function is generally considered damaging to organisms because it is expected to lead to an accumulation of harmful electrophilic compounds in the cell and thereby disturb critical cellular processes. In fact, a previous study showed that loss of GstS1 function enhanced the loss of dopaminergic neurons in a parkin mutant, a Drosophila model of Parkinson’s disease and conversely, overexpression of GstS1 in the same dopaminergic neurons suppressed dopaminergic neurodegeneration in such mutants (Whitworth et al. 2005). Parkin has ubiquitin-protein ligase activity (Imai et al. 2000; Shimura et al. 2000; Zhang et al. 2000) and the accumulation of toxic Parkin substrates likely contributes to the degeneration of dopaminergic neurons in Parkinson’s patients and animal models (Whitworth et al. 2005). These results are consistent with the idea that GstS1 plays a role in the detoxification of oxidatively damaged products to maintain healthy cellular environments. In this regard, it seems counterintuitive that loss of GstS1 function reduces, rather than increases, the severity of paraShu phenotypes.

GstS1 is unique among Drosophila GSTs in several respects. A previous study, based on multiple alignments of GST sequences, had revealed that GstS1 is the sole member of the Drosophila sigma class of GST (Agianian et al. 2003). Unlike other GSTs, GstS1 has low catalytic activity for typical GST substrates, such as 1-chloro-2,4-dinitrobenzol (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), and ethacrynic acid (EA). Instead, it efficiently catalyzes the conjugation of harmful electrophilic compounds in the cell and thereby disturbs damaging to organisms because it is expected to lead to an accumulation of oxidatively damaged products to maintain healthy cellular environments. In this regard, it seems counterintuitive that loss of GstS1 function reduces, rather than increases, the severity of paraShu phenotypes.

### Table 6 Expression levels of GST genes in control and paraShu

| GST genes | Flybase ID  | Control average of normalized counts (DESeq2) | paraShu Fold change (log2) | Fold change Padj |
|-----------|-------------|-----------------------------------------------|---------------------------|-----------------|
| GstS1     | FBgn0063499 | 1556.21                                       | -0.76                     | 0.59            |
| GstD1     | FBgn0063495 | 1042.37                                       | -0.02                     | 0.99            |
| GstD2     | FBgn0010041 | 175.19                                        | -0.72                     | 0.61            |
| GstD3     | FBgn0037696 | 315.48                                        | -0.20                     | 0.87            |
| GstD4     | FBgn0063492 | 6.43                                          | -0.06                     | 0.96            |
| GstD5     | FBgn0063498 | 43.77                                         | -0.32                     | 0.80            |
| GstD6     | FBgn0010043 | 2.45                                          | 0.14                      | 1.10            |
| GstD7     | FBgn0063496 | 75.87                                         | 0.07                      | 1.05            |
| GstD8     | FBgn0063485 | 50.70                                         | 0.06                      | 1.04            |
| GstD9     | FBgn0010044 | 373.48                                        | 0.10                      | 1.07            |
| GstD10    | FBgn0063497 | 126.21                                        | 0.30                      | 1.23            |
| GstD11    | FBgn0050000 | 75.87                                         | 0.07                      | 1.05            |
| GstD12    | FBgn0063498 | 43.77                                         | -0.32                     | 0.80            |
| GstD13    | FBgn0010043 | 2.45                                          | 0.14                      | 1.10            |
| GstD14    | FBgn0063491 | 203.55                                        | 0.10                      | 1.07            |
| GstD15    | FBgn0038029 | 2026.31                                       | -0.02                     | 0.99            |
| GstD16    | FBgn0063497 | 34.52                                         | 0.18                      | 1.13            |
| GstD17    | FBgn0063496 | 934.78                                        | -0.05                     | 0.97            |
| GstD18    | FBgn0063495 | 126.21                                        | 0.30                      | 1.23            |
| GstD19    | FBgn0063494 | 63.43                                         | 0.18                      | 1.13            |
| GstD20    | FBgn0063493 | 679.13                                        | 0.02                      | 1.01            |
| GstD21    | FBgn0063492 | 679.13                                        | 0.02                      | 1.01            |
| GstD22    | FBgn0063491 | 203.55                                        | 0.10                      | 1.07            |
| GstD23    | FBgn0038029 | 2026.31                                       | -0.02                     | 0.99            |
| GstD24    | FBgn0063497 | 34.52                                         | 0.18                      | 1.13            |
| GstD25    | FBgn0063496 | 934.78                                        | -0.05                     | 0.97            |
| GstD26    | FBgn0063495 | 126.21                                        | 0.30                      | 1.23            |
| GstD27    | FBgn0063494 | 63.43                                         | 0.18                      | 1.13            |
| GstD28    | FBgn0063493 | 679.13                                        | 0.02                      | 1.01            |
| GstD29    | FBgn0063492 | 679.13                                        | 0.02                      | 1.01            |
| GstD30    | FBgn0063491 | 203.55                                        | 0.10                      | 1.07            |

Transcript levels of the 36 genes encoding soluble GSTs were evaluated by DEseq2 analysis of four biological replicates in control (Canton-S) and paraShu. Adjusted P-values (\( P_{adj} \)) were obtained using Benjamini-Hochberg (BH) procedure (\( P_{adj} < 0.05; **P_{adj} < 0.01, ***P_{adj} < 0.001 \)).
of glutathione to 4-hydroxynonenal (4-HNE), an unsaturated carbonyl compound derived via lipid peroxidation (Singh et al. 2001; Agianian et al. 2003). The crystal structure of GstS1 indicates that its active-site topography is suitable for the binding of amphipolar lipid peroxidation products such as 4-HNE (Agianian et al. 2003), consistent with the above-mentioned substrate specificity. 4-HNE is the most abundant 4-hydroxyalkenal formed in cells and contributes to the deleterious effects of oxidative stress. It has been implicated in the pathogenesis and progression of human diseases such as cancer, Alzheimer’s disease, diabetes, and cardiovascular disease (Shoeb et al. 2014; Csala et al. 2015). However, 4-HNE also functions as a signaling molecule and has concentration-dependent effects on various cellular processes including differentiation, growth and apoptosis (Zhang and Forman 2017). GstS1 plays a major role in controlling the intracellular 4-HNE concentration to balance its beneficial and damaging effects; one study estimated that GstS1 is responsible for ~70% of the total capacity to conjugate 4-HNE with glutathione in adult Drosophila (Singh et al. 2001). It is thus possible that in paraShu flies the reduction of GstS1 activity enhances the strength of 4-HNE-dependent signaling, leading to changes in neural development and/or function that compensate for the defect caused by the paraShu mutation.

Notably, GSTs are not limited to conjugating glutathione to potentially harmful substrates for their clearance, and it is possible that another such function accounts for our observations. Specifically, some GSTs catalyze the synthesis of physiologically important compounds. With respect to its primary amino acid sequence, Drosophila GstS1 is more similar to the vertebrate hematopoietic prostaglandin D2 synthases (HPGDSs) than to other Drosophila GSTs (Agianian et al. 2003). Indeed, the sequence identity/similarity between Drosophila GstS1 and human HPGDSs are 37%/59%, respectively. The Drosophila Integrative Orthology Prediction Tool (DIOP; http://www.flyrnai.org/diop) (Hu et al. 2011), as well as a recent bioinformatics analysis (Scarpati et al. 2019), classified GstS1 as a fly ortholog of HPGDS, a sigma-class member of the GST family that catalyzes the isomerization of prostaglandin H\(_2\) (PGH\(_2\)) to prostaglandin D\(_2\) (PGD\(_2\)). Mammalian HPGDS is a critical regulator of inflammation and the innate immune response (Rajakarui et al. 2007; Joo and Sadikot 2012). In light of this observation, findings implicating GstS1 in the development and function of the innate immune system in insects are of interest. For example, in a lepidopteran Spodoptera exigua, the ortholog of Drosophila GstS1, SePGDS, was identified as PGD\(_2\) synthase because the addition of PGD\(_2\), but not its precursor (arachidonic acid) could rescue immunosuppression in larvae caused by SePGDS knockdown (Sajjadian et al. 2020). Consistent with this finding, previous studies in Drosophila had revealed that overexpression of GstS1 in hemocytes (the insect blood cells responsible for cellular immunity) leads to increases in the number of larval hemocytes (Stofanko et al. 2008) and that GstS1 in hemocytes is increased ~10-fold at the onset of metamorphosis (Regan et al. 2013). These results strongly support a significant role for GstS1 in the insect innate immune system. In addition, we previously found that genes involved in innate immune responses were up-regulated in the adult head of paraShu mutants (Kaas et al. 2016), suggesting that the neuronal hyperexcitability induced by gain-of-function paraShu Na\(_\alpha\) channels might lead to activation of the innate immune system. In light of these observations and our current findings it is possible that the reason that loss of GstS1 function reduces the severity of paraShu phenotypes is that it suppresses the innate immune response through hemocytes and prostaglandin-like bioactive lipids.

Another connection to the innate immune system is the discovery, based on our transcriptome analysis, that CYP genes are over-represented among the genes that are differentially expressed in the paraShu with a GstS1 mutation (Table 8). CYP enzymes play vital roles in the activation and suppression of inflammation, an essential mechanism of innate immune responses, by synthesizing or metabolizing bioactive mediators. In particular, CYP enzymes are involved in the oxygenation of a wide range of compounds, including eicosanoids such as prostaglandins. In mammals, activation of the innate immune response alters CYP expression and eicosanoid metabolism in an isoform-, tissue-, and time-dependent manner (Theken et al. 2011). GstS1 loss of function may affect paraShu phenotypes by changing the activities of CYP

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**Table 7** Enriched GO terms that are overrepresented in differentially expressed genes in paraShu/+; GstS1M26/+ compared with paraShu/+; +/+  

| Gene ontology | Term | Ontology class | \(P_{\text{adj}}\) over-represented value | # of genes |
|---------------|------|----------------|-----------------------------------------|------------|
| GO:0020037    | heme binding | MF | 7.31E-4 | 14 |
| GO:0046906    | tetrapyrrole binding | MF | 7.31E-4 | 14 |
| GO:0005506    | iron ion binding | MF | 1.53E-3 | 14 |
| GO:0016705    | oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | MF | 2.83E-3 | 14 |
| GO:0048037    | cofactor binding | MF | 4.60E-3 | 21 |

MF: molecular function.
enzymes. Further studies are required to elucidate whether and how CYP genes, as well as the genes involved in innate immune response and bioactive lipid signaling, contribute to GstS1-mediated modulation of paraShu phenotypes.

To obtain insight into functional significance of changes in gene expression, we classified differentially expressed genes. For the 89 genes that were up-regulated by paraShu+/+ (paraShu+/+ vs. +/+), it is notable that 13 were down-regulated when GstS1M26 was also introduced (paraShu+/+ vs. paraShu+/+; GstS1M26/) and that all of the GO categories associated (Padj < 0.05) with this group of genes were related to the chtin-based cuticle (Table 3). On the other hand, among the 40 genes down-regulated by paraShu, only 2 (CG3966 and CG5770) were up-regulated by GstS1M26. Although CG3966 is an uncharacterized gene, CG3966 encodes proteins that are highly expressed in the larval and adult fat bodies and predicted to be involved in lipid catabolism. A human CG3966 homolog encodes pancreatic lipase, which hydrolyzes triglycerides in the small intestine and is essential for the efficient digestion of dietary fat (Davis et al. 1991). Notably, changes in the expression of these cuticle-associated and fat metabolism-associated sets of genes appear to correlate with the phenotypic severity of paraShu in that a change in the phenotype or gene expression induced by paraShu is reversed by GstS1M26. It is possible that changes in the expression of these genes is causative and contributes to the severity of paraShu phenotypes. Alternatively, these changes in gene expression could be a consequence of phenotypic changes caused by other factors. Further functional analysis is required to determine the significance of these genes in controlling paraShu phenotypes.

In contrast to the expression of the above-mentioned genes, that of 24 genes was changed in the same direction by paraShu and GstS1M26. Among these, 17 were up-regulated and 7 were down-regulated. No GO category was identified for any of the gene sets with Padj < 0.05. Interestingly, GstS1 itself is one of the genes whose expression is down-regulated by both paraShu and GstS1M26. The observed reduction in levels of GstS1 expression in the GstS1M26 mutant is consistent with it being a deletion allele. However, its down-regulation in paraShu mutants was unexpected. One possible explanation for this finding is that homeostatic regulation at the level of gene expression counteracts the defects caused by hyperexcitability. It will be important to elucidate the mechanisms by which a gain-of-function mutation in a Na+ channel gene leads to down-regulation of the expression of its modifier gene and to reduction of the severity of the phenotype.

A previous genetic screen that was similar to ours revealed that loss of the function of gilgamesh (gish) reduces the severity of the seizure phenotypes of paraShu mutant. gish encodes the Drosophila ortholog of casein kinase CK1γ3, a member of the CK1 family of serine/threonine kinases (Howlett et al. 2013). Another modifier of seizure activity was discovered by Lin et al. (2017); this group identified pumilio (pum) based on transcriptome analyses of Drosophila seizure models, with pum significantly down-regulated in both the genetic (paraShu) and pharmacological (picrotoxin-induced) models. It was shown that pan-neuronal overexpression of pum is sufficient to dramatically reduce seizure severity in paraShu as well as other seizure-prone Drosophila mutants, easily shocked (es) and slamdance (sda) (Lin et al. 2017). pum encodes RNA binding proteins that act as homeostatic regulators of action potential firing, partly by regulating the translation of para transcripts (Lin et al. 2017). In addition, we recently discovered that the seizure phenotypes of paraShu and other seizure-prone fly mutants are significantly suppressed when the flies are fed a diet supplemented with milk whey (Kasuya et al. 2019). Many seemingly disparate genetic and environmental modifiers of hyperexcitable phenotypes of Drosophila mutants have been identified, demonstrating a wealth of complexity. It remains unclear how these factors interact with one another in complex regulatory networks and how they modify the neurological phenotypes of mutants. A mechanistic understanding of such functional interactions is expected to reveal the molecular and cellular processes that are critical for the manifestation of hyperexcitable phenotypes in Drosophila mutants, and to provide
useful insights into the corresponding processes in vertebrate animals, including humans.

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LITERATURE CITED
Aganian, B., P. A. Tucker, A. Schosten, K. Leonard, B. Bullard et al., 2003 Structure of a Drosophila sigma class glutathione S-transferase reveals a novel active site topography suitable for lipid peroxidation products. J. Mol. Biol. 326: 151–165. https://doi.org/10.1016/S0022-8053(02)01327-X
Allocati, N., M. Masulli, C. Di Ilio, and L. Federici, 2018 Glutathione transferases: substrates, inhibitors and pro-drugs in cancer and neurodegenerative diseases. Oncogenesis 7: 8. https://doi.org/10.1038/s41389-017-0025-3
Bansal, V., 2017 A computational method for estimating the PCR duplication rate in DNA and RNA-seq experiments. BMC Bioinformatics 18: 43. https://doi.org/10.1186/s12859-017-1471-9
Benzer, S., 1967 Behavioral mutants of Drosophila isolated by countercurrent distribution. Proc. Natl. Acad. Sci. USA 58: 1112–1119. https://doi.org/10.1073/pnas.58.3.1112
Besson, M., and J. R. Martin, 2005 Centrophobism/thigmotaxis, a new role for the mushroom bodies in Drosophila. J. Neurobiol. 62: 386–396. https://doi.org/10.1002/neu.20111
Catterall, W. A., 2012 Voltage-gated sodium channels at 60: structure, function and pathophysiology. J. Physiol. 590: 2577–2589. https://doi.org/10.1113/jphysiol.2011.224204
Catterall, W. A., F. Kalume, and J. C. Oakley, 2010 NaV1.1 channels and epilepsy. J. Physiol. 588: 1849–1859. https://doi.org/10.1113/jphysiol.2010.187484
Chung, H., T. Sztal, S. Pasricha, M. Sridhar, P. Batterham et al., 2009 Characterization of Drosophila melanogaster cytochrome P450 genes. Proc. Natl. Acad. Sci. USA 106: 5731–5736. https://doi.org/10.1073/pnas.0812141106
Claes, L., J. Del-Favero, B. Ceulemans, L. Lagae, C. Van Broeckhoven et al., 2001 De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. Am. J. Hum. Genet. 68: 1327–1332. https://doi.org/10.1086/320609
Cook, R. K., S. J. Christensen, J. A. Deal, R. A. Coburn, M. E. Deal et al., 2012 The generation of chromosomal deletions to provide extensive coverage and subdivision of the Drosophila melanogaster genome. Genome Biol. 13: R21. https://doi.org/10.1186/gb-2012-13-3-r21
Csala, M., T. Kardon, B. Legeza, B. Lizak, J. Mandl et al., 2015 On the role of 4-hydroxynonenal in health and disease. Biochem. Biophys. Acta 1852: 826–838. https://doi.org/10.1016/j.bbadis.2015.01.015
Jen, J. C., T. Ashizawa, R. C. Griggs, and M. F. Waters, 2016 Rare neuro-

Kaas, G. A., J. Kasuya, P. Lansdon, A. Ueda, A. Iyengar

Isin, E. M., and F. P. Guengerich, 2007 Complex reactions catalyzed by

Imai, Y., M. Soda, and R. Takahashi, 2000 Parkin suppresses unfolded

Hu, Y., I. Flockhart, A. Vinayagam, C. Bergwitz, B. Berger

Hodgkin, A. L., and A. F. Huxley, 1952 A quantitative description of

Hannemann, F., A. Bichet, K. M. Ewen, and R. Bernhardt,

Ganetzky, B., and C. F. Wu, 1982 Indirect Suppression Involving

Dib-Hajj, S. D., J. A. Black, and S. G. Waxman, 2015 NaV1.9: a sodium

Kroll, J. R., A. Saras, and M. A. Tanouye, 2015 Drosophila sodium

Lilly, M., R. Kreber, B. Ganetzky, and J. R. Carlson, 1994 Evidence that the Drosophila olfactory mutant smellblind defines a novel class of sodium channel mutation. Genetics 136: 1087–1096.

Lin, W. H., C. N. Giachello, and R. A. Baines, 2017 Seizure control through genetic and pharmacological manipulation of Pumilio in Drosophila: a key component of neuronal homeostasis. Dis. Model. Mech. 10: 141–150. https://doi.org/10.1242/dmm.027045

Lindsay, H. A., R. Baines, R. ffrench-Constant, K. Lilley, H. T. Jacobs et al., 2008 The dominant cold-sensitive Out-cold mutants of Drosophila melanogaster have novel missense mutations in the voltage-gated sodium channel gene paralytic. Genetics 180: 873–884. https://doi.org/10.1534/genetics.108.099051

Love, M. I., W. Huber, and S. Anders, 2014 Moderated estimation of fold change and dispersion for RNA-seq data with DSESeq2. Genome Biol. 15: 550. https://doi.org/10.1186/s13059-014-0550-8

Martin, R. L., B. Pittendrigh, J. Liu, R. Reeman, R. ffrench-Constant et al., 2000 Point mutations in domain III of a Drosophila neuronal Na channel confer resistance to alethrin. Insect Biochem. Mol. Biol. 30: 1051–1059. https://doi.org/10.1016/S0965-1748(00)00080-1

Parker, L., M. Padilla, Y. Du, K. Dong, and M. A. Tanouye, 2012 Drosophila as a model for epilepsy: bss is a gain-of-function mutation in the para sodium channel gene that leads to seizures. Genetics 187: 523–534. https://doi.org/10.1534/genetics.111.123299

Rajakariar, R., M. Hilliard, T. Lawrence, S. Trivedi, P. Colville-Nash et al., 2007 Hematopoietic proaglandin D2 synthase controls the onset and resolution of acute inflammation through PGD2 and 15-deoxyDelta12 14 PGJ2. Proc. Natl. Acad. Sci. USA 104: 20979–20984. https://doi.org/10.1073/pnas.0707394104

Rajakulendran, S., D. Kaski, and M. G. Hanna, 2012 Neuronal P/Q-type calcium channel dysfunction in inherited disorders of the CNS. Nat. Rev. Neuro: 8–96. https://doi.org/10.1038/nrneur.2011.228

Regan, J. C., A. S. Brandao, A. B. Leitao, A. R. Mantas Dias, E. Sucena et al., 2013 Steroid hormone signaling is essential to regulate innate immune cells and fight bacterial infection in Drosophila. PLoS Pathog. 9: e1003720. https://doi.org/10.1371/journal.ppat.1003720

Rosseto, J., and S. Russell, 2012 Toward a complete Drosophila deficiency kit. Genome Biol. 13: 149.

Saisawang, C., J. Wongsantichon, and A. J. Ketterman, 2012 A preliminary characterization of the cystosolic glutathione transferase proteome from Drosophila melanogaster. Biochem. J. 442: 181–190. https://doi.org/10.1042/BJ20111747

Sajjadian, S. M., S. Ahmed, M. A. Al Baki, and Y. Kim, 2020 Prostaglandin D2 synthase and its functional association with immune and reproductive processes in a lepidopteran insect, Spodoptera exigua. Gen. Comp. Endocrinol. 287: 113352. https://doi.org/10.1016/j.ygcen.2019.113352

Scarpati, M., Y. Qi, S. Govind, and S. Singh, 2019 A combined computational strategy of sequence and structural analysis predicts the existence of a functional eicosanoid pathway in Drosophila melanogaster. PLoS One 14: e0211897. https://doi.org/10.1371/journal.pone.0211897

Schutte, R. J., S. S. Schutte, J. Alagara, E. V. Barragan, J. Gilligan et al., 2014 Knock-in model of Dravet syndrome reveals a constitutive and conditional reduction in sodium current. J. Neurophysiol. 112: 901–912. https://doi.org/10.1152/jn.00135.2014

Shimura, H., N. Hattori, S. Kubo, Y. Mizuno, S. Asakawa et al., 2000 Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. 25: 302–305. https://doi.org/10.1038/ng.1007

Shoeb, M., N. H. Ansari, S. K. Srivastava, and K. V. Ramana, 2014 4-Hydroxynonenal in the pathogenesis and progression of human diseases. Curr. Med. Chem. 21: 230–237. https://doi.org/10.2174/1022129X105300315

Singh, S. P., J. A. Coronella, H. Benes, B. J. Cochrane, and P. Zimniak, 2001 Catalytic function of Drosophila melanogaster glutathione S-transferase DmGSTS1–1 (GST-2) in conjugation of lipid peroxidation end products. Eur. J. Biochem. 268: 2912–2923. https://doi.org/10.1046/j.1432-1031.2001.01279.x

Stefanko, M., S. Y. Kwon, and P. Badenhorst, 2008 A misexpression screen to identify regulators of Drosophila larval hemocyte development. Genetics 180: 253–267. https://doi.org/10.1534/genetics.108.089094

Sun, L., J. Gilligan, C. Staber, R. J. Schutte, V. Nguyen et al., 2012 A knock-in model of human epilepsy in Drosophila reveals a novel cellular
mechanism associated with heat-induced seizure. J. Neurosci. 32: 14145–14155. 
Suzuki, D. T., T. Grigliatti, and R. Williamson, 1971 Temperature-sensitive mutations in Drosophila melanogaster. VII. A mutation (para-ts) causing reversible adult paralysis. Proc. Natl. Acad. Sci. USA 68: 890–893. https://doi.org/10.1073/pnas.68.5.890
Theken, K. N., Y. Deng, M. A. Kannon, T. M. Miller, S. M. Poloyac et al., 2011 Activation of the acute inflammatory response alters cytochrome P450 expression and eicosanoid metabolism. Drug Metab. Dispos. 39: 22–29. https://doi.org/10.1124/dmd.110.035287
Tu, C. P., and B. Akgul, 2005 Drosophila glutathione S-transferases. Methods Enzymol. 401: 204–226. https://doi.org/10.1016/S0076-6879(05)01013-X
Ugur, B., K. Chen, and H. J. Bellen, 2016 Drosophila tools and assays for the study of human diseases. Dis. Model. Mech. 9: 233–244. https://doi.org/10.1242/dmm.023762
Venetucci, L., M. Denegri, C. Napolitano, and S. G. Priori, 2012 Inherited calcium channelopathies in the pathophysiology of arrhythmias. Nat. Rev. Cardiol. 9: 561–575. https://doi.org/10.1038/nrcardio.2012.93
Waxman, S. G., and G. W. Zamponi, 2014 Regulating excitability of peripheral afferents: emerging ion channel targets. Nat. Neurosci. 17: 153–163. https://doi.org/10.1038/nn.3602
Whitworth, A. J., D. A. Theodore, J. C. Greene, H. Benes, P. D. Wes et al., 2005 Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a Drosophila model of Parkinson’s disease. Proc. Natl. Acad. Sci. USA 102: 8024–8029. https://doi.org/10.1073/pnas.0501078102
Williamson, R. L., 1982 Lithium stops hereditary shuddering in Drosophila melanogaster. Psychopharmacology (Berl.) 76: 265–268. https://doi.org/10.1007/BF00432558
Williamson, R. L. M., 1971 The isolation and study of two mutants affecting motor activity in Drosophila melanogaster., University British Columbia, Vancouver.
Young, M. D., M. J. Wakefield, G. K. Smyth, and A. Oshlack, 2010 Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11: R14. https://doi.org/10.1186/gb-2010-11-2-r14
Zhang, H., and H. J. Forman, 2017 4-hydroxynonenal-mediated signaling and aging. Free Radic. Biol. Med. 111: 219–225. https://doi.org/10.1016/j.freeradbiomed.2016.11.032
Zhang, H., J. Tan, E. Reynolds, D. Kuebler, S. Faulhaber et al., 2002 The Drosophila slamdance gene: a mutation in an aminopeptidase can cause seizure, paralysis and neuronal failure. Genetics 162: 1283–1299.
Zhang, Y., J. Gao, K. K. Chung, H. Huang, V. L. Dawson et al., 2000 Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc. Natl. Acad. Sci. USA 97: 13354–13359. https://doi.org/10.1073/pnas.240347797

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