Gender differences in a *Drosophila* transcriptomic model of chronic pentylenetetrazole induced behavioral deficit

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Abstract

A male *Drosophila* model of locomotor deficit induced by chronic pentylenetetrazole (PTZ), a proconvulsant used to model epileptogenesis in rodents, has recently been described. Antiepileptic drugs (AEDs) ameliorate development of this behavioral abnormality. Time-series of microarray profiling of heads of male flies treated with PTZ has shown epileptogenesis-like transcriptomic perturbation in the fly model. Gender differences are known to exist in neurological and psychiatric conditions including epileptogenesis. We describe here the effects of chronic PTZ in *Drosophila* females, and compare the results with the male model. As in males, chronic PTZ was found found to cause a decreased climbing speed in females. In males, overrepresentation of Wnt, MAPK, TGF-beta, JAK-STAT, Cell communication, and Dorso-Ventral axis formation pathways in downregulated genes was previously described. Of these, female genes showed enrichment only for Dorso-Ventral axis formation. Most significant, ribosomal pathway was uniquely overrepresented in genes downregulated in females. Gender differences thus exist in the *Drosophila* model. Gender neutral, Dorso-Ventral axis formation may be considered as the candidate causal pathway in chronic pentylenetetrazole induced behavioral deficit. Prior evidence of developmental mechanisms in epileptogenesis underscores the usefulness of fly model. Gender specific pathways may provide a lead for understanding brain dimorphism in neuropsychiatric disorders.

Introduction

The prevalence and course of various neurological and psychiatric disorders are known to differ between the sexes (*1, 2*). Sexually dimorphic CNS structure and function are known in animals as well (*3*). The gender-specific differences have been observed at gene expression, metabolic and cytogenic levels (*4, 5*). For example, sex-specific differences in brain metabolism have been observed in epileptic patients (*4*). Similarly, seizures have been found to cause gender-specific effect on cell proliferation and survival in rats (*5*). Differences in patterns of gene expression have been suggested as a possible contributing factor in sexual dimorphism in neuropsychiatric disorders (*6*).

A male *Drosophila* model of locomotor deficit induced by chronic pentylenetetrazole (PTZ), a proconvulsant used to model epileptogenesis in rodents, has recently been described (*7*). Antiepileptic drugs (AEDs) have been found to ameliorate development of this behavioral abnormality. Time-series of microarray profiling of heads of male flies treated with PTZ has shown that gene expression changes in the fly model resemble, to some extent, that known in epileptogenesis (*7*). Given this, we developed a female model and examined sex differences, if any. We describe here the effects of chronic PTZ in *Drosophila* females, and compare the results with the male model.

Results

Chronic treatment with PTZ for seven days was earlier described to result in a decreased climbing speed in males (*7*). We similarly treated females and measured the climbing

speed. Whereas the control NF treated flies (n=24) climbed with a speed of 1.23 cm/sec, PTZ treated ones (n=24) showed a climbing speed of 0.86 cm/sec. Pair-wise Student's t-test, two-tailed, heteroscedastic, showed the gender difference in climbing speed as significant (p=0.001). Females were thus found comparable to males in terms of behavioral effect of chronic PTZ.

Time-series of microarray expression profiles of heads of male flies at 0 hr and after PTZ treatment for 12 hrs, two days and seven days have previously been described (*7*). Numbers of SAM (Significant Analysis of Microarrays) analyzable genes in male microarrays at these time-points were 4369, 4637, 5259 and 4297, in that order (*7*). At 0 hr, no gene was detected as differentially expressed below 96% FDR (false Discovery rate). At 12 hrs, 2nd day and 7th day time-points, 23, 2439 and 265 genes were found to be downregulated at 22.76%, 13.74% and 23.03% FDR, in that order (*7*). No upregulated gene was detected at these FDRs (*7*). Given small number of genes at 12 hrs, these FDRs were considered as the best compromise between uniformity across time points and acceptability in terms of incorporating false positives. To compare with males, we generated microarray gene expression profiles of heads of female flies after 12 hrs, 2 days and 7 days of PTZ treatment using the same materials and methods. Analyzable genes in SAM were found to be 5307, 5346 and 6995, in that order. At 12 hrs, 2nd day and 7th day time-points, 1, 3 and 1 genes were upregulated, and 1, 68 and 800 genes were downregulated at 0%, 16% and 19% FDR, in that order (**Table S1, supporting material**). Given that the next FDR jumped to 43% at 12 hrs time point, we considered

the above FDRs as comparable to male profiles. It was notable that in females, like males, PTZ's overall effect on gene expression was inhibitory.

Earlier, clustering of male microarrays was found to be consistent with the time-series (*7*). We clustered the female microarrays and examined their consistency. Like males, female microarrays also clustered according to the time-series (**Fig. 1a**). To examine possible gross level sex differences, we clustered both male and female time-series together. Except 2nd day time-point where some discrepancy was noted, males and females were found to be similar in their chronological response to PTZ (**Fig. 1b**). Next, we identified overrepresented pathways in genes downregulated in females at all the three time-points combined (**Table 1**). In males, overrepresentation of Wnt, MAPK, TGF-beta, JAK-STAT, Cell communication, and Dorso-Ventral axis formation pathways in downregulated genes was previously described (*7*). Of these, female genes showed enrichment only for Dorso-Ventral axis formation (**Fig. 2**). Remarkably, ribosomal pathway was most significantly overrepresented only in females (**Fig. 3**).

Discussion

Our results show that gender differences do exist in the *Drosophila* model at transcriptomic level. Whereas Dorso-Ventral axis formation pathway has been found to be common in both sexes, other pathways showed gender bias. Considering similar behavioral effect of PTZ, the gender neutral Dorso-Ventral axis formation may be considered as the pathway causally associated with development of behavioral deficit caused by the chemoconvulsant. It is interesting to note here that gene expression studies

have previously implicated developmental mechanisms in epileptogenesis (*8*). This underscores the relevance of the fly model in disease genomics. The gender specific pathways that we have identified may provide a lead for understanding brain dimorphism. Known translational control of long-lasting brain plasticity (*9, 10*), for example, is notable in the context of ribosomal pathway association with the female fly model. The *Drosophila* systems model seems to offer an excellent opportunity to unravel the gender differences in neuropsychiatric disorders at cellular and molecular level.

Materials and Methods

Previously described (*7*) methods were used. In brief, 3-4 days old virgin females were treated with 8 mg/ml of PTZ for varying length of time. Flies treated with normal food (NF) were used as controls. Total cellular RNA was isolated from fly heads belonging to four biological replicates. Microarray -cDNA Synthesis Kit, -Target Purification Kit, and -RNA Target Synthesis Kit (Roche) were used to generate labeled antisense RNA. Starting with 10 µg of total cellular RNA, Eberwine method (kits from Roche) was used to generate cDNA and thereafter Cy^3 and Cy^5 (Amersham) labeled antisense RNA. The Cy^3 and Cy^5 labeled aRNAs (control and treated) were pooled together and precipitated, washed, air-dried, and dissolved in 18MΩ RNAase free water. A total of 12 microarrays (12Kv1, CDMC) were hybridized, four each for 12 hrs, 2^{nd} day and 7^{th} day of PTZ treatment. Out of four, two slides were dye-swaps. Slides were scanned at 10 µm resolution using GenePix 4000A Microarray Scanner (Molecular Devices) and the images preprocessed and quantified using Gene Pix Pro 6.0 (Molecular Devices). The full microarray data set has been deposited in the Gene Expression Omnibus

(http://www.ncbi.nlm.nih.gov/geo/) under accession series GSE10852. Ratio based data normalization and selection of features were performed using Acuity 4.0 (Molecular Devices). All Spots with raw intensity less then 100U and less then twice the average background was ignored during normalization. Normalized data was filtered for the selection of features before further analysis. Only those spot were selected which contained only a small percentage (<3) of saturated pixels, were not flagged bad or found absent (flags>0), had relatively uniform intensity and uniform background [Rgn R2 (635/532)>0.6] and were detectable above background (SNR>3). Analyzable spots in at least three of four biological replicates performed were retrieved for downstream analysis using SAM (v.3.0, Excel Add-In) (*11*), under the conditions of one class response and 100 permutations. DAVID (*12*) was used for pathway enrichment analysis (http://david.abcc.ncifcrf.gov/home.jsp). KEGG pathway (http://www.genome.jp/kegg/tool/color_pathway.html) was used for mapping of fly genes.

Author's contributions

The research was conceived and planned by A.S. Experiments were performed and the data collected by F.M. and P.S. The data was interpreted and the manuscript written by A.S.

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Figure legends

Figure 1.

Time-series of microarray expression profiles. Profiles were generated from heads of PTZ treated of flies. Hierarchical clustering of female profiles alone (a), and both male and female profiles together (b). Male microarrays were described previously [9]. City Block similarity metric and average linkage methods were used for clustering of arrays. Each profile represents mean of normalized $\log_2$ ratio (635/532) of four biological replicates with balanced dye-swaps. The cluster was generated using Acuity 4.0.

Figure 2.

Downregulated genes related to Dorso-ventral axis formation pathway. Green boxes indicate genes downregulated in females after chronic PTZ. Differentially expressed genes in all the three time-points combined were used for pathway mapping. Dorso-ventral axis formation pathway was previously found to be enriched in males also [9].

Figure 3.

Downregulated genes related to ribosomal pathway. Green boxes indicate genes downregulated in females after chronic PTZ. Differentially expressed genes in all the three time-points combined were used for pathway mapping. Ribosomal pathway was previously not found to be enriched in males [9]. Grey and white boxes indicate presence and absence of *Drosophila melanogaster* genes in the pathway, respectively.

Table S1. KEGG pathways enriched in genes downregulated in females after chronic PTZ

Term	P-Value	Benjamini adjusted P-Value
Ribosome	2.60E-16	2.80E-14
Dorso-ventral axis formation	3.90E-02	8.20E-01
Pyruvate metabolism	4.70E-02	7.80E-01

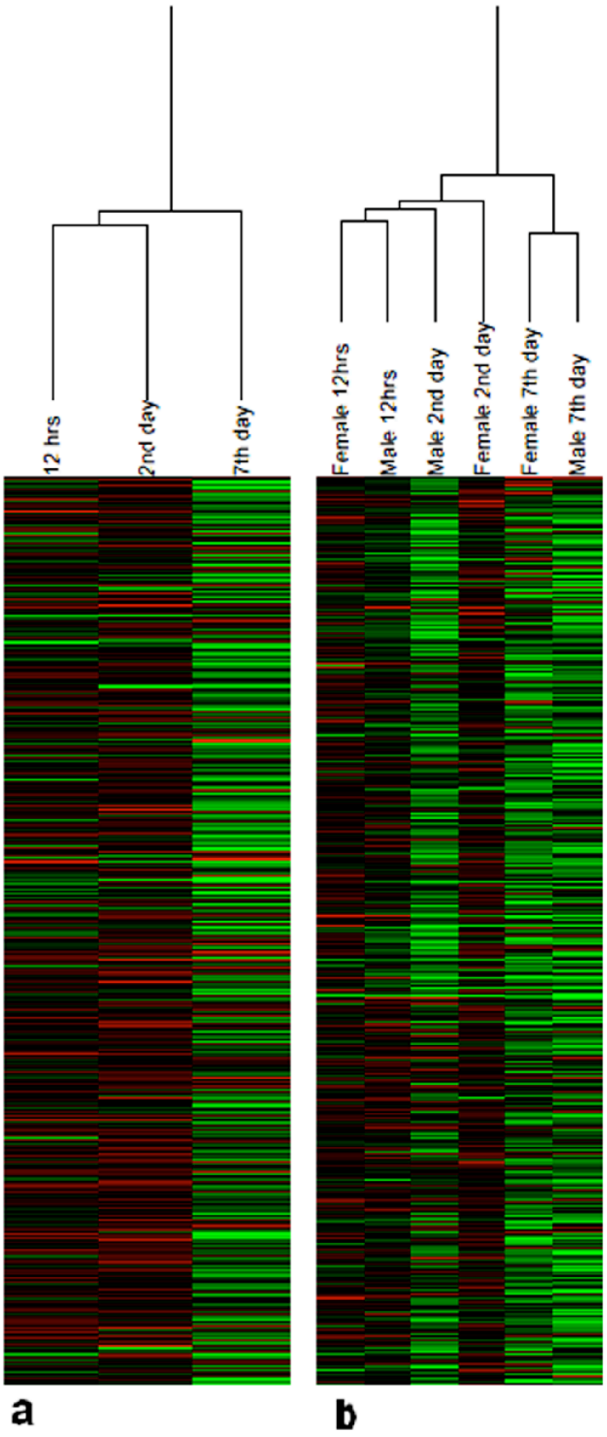
12 hrs
2nd day
7th day
a
Female 12hrs
Male 12hrs
Male 2nd day
Female 2nd day
Female 7th day
Male 7th day
b

Figure 1

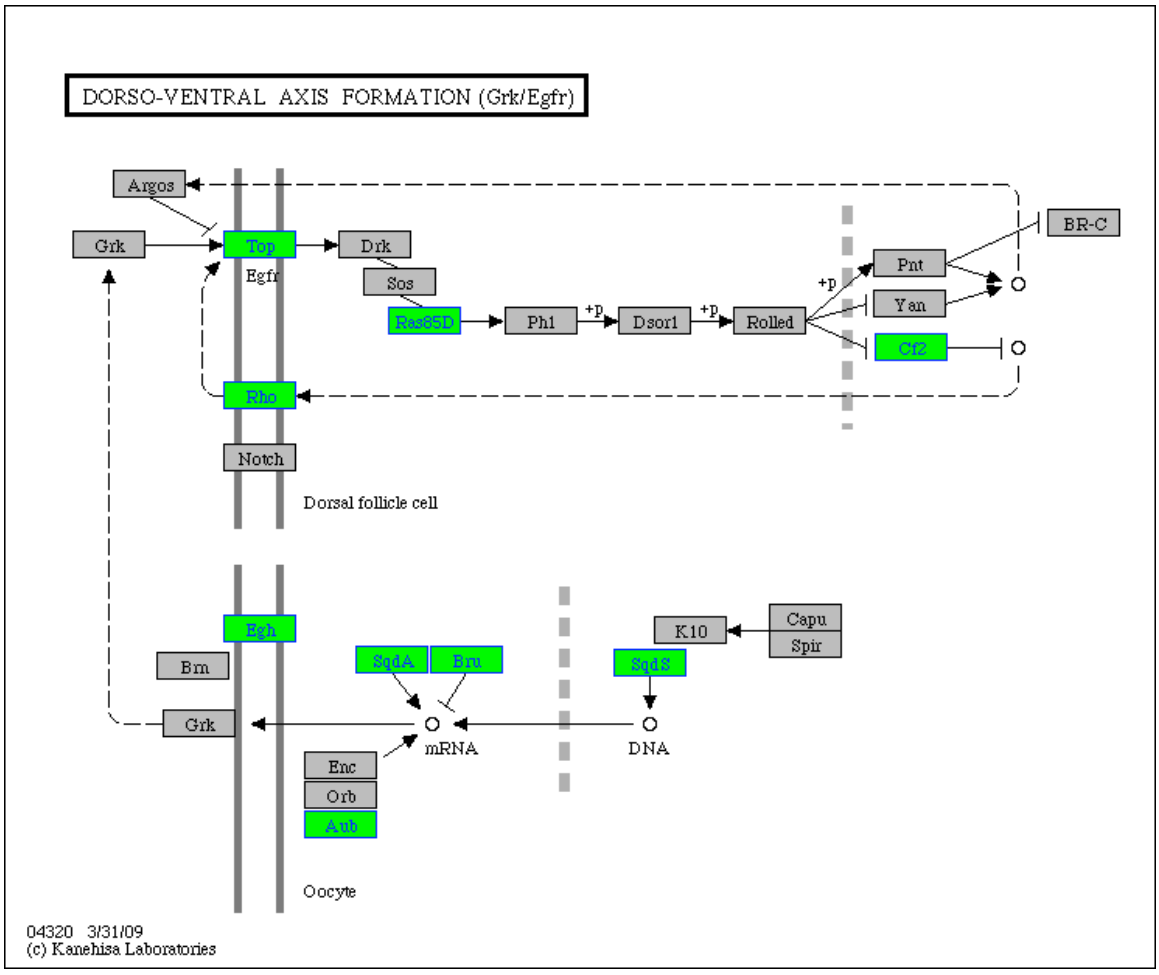
DORSO-VENTRAL AXIS FORMATION (Grk/Egfr)
Argos
Grk
Top
Egfr
Drk
Sos
Ras85D
Phl
+p
Dsor1
+p
Rolled
+p
Pnt
Yan
Clz
BR-C
Rho
Notch
Dorsal follicle cell
Egh
Brn
Grk
SqdA
Bru
mRNA
Enc
Orb
Aub
SqdS
DNA
K10
Capu
Spir
Oocyte
04320 3/31/09
(c) Kanehisa Laboratories

Figure 2

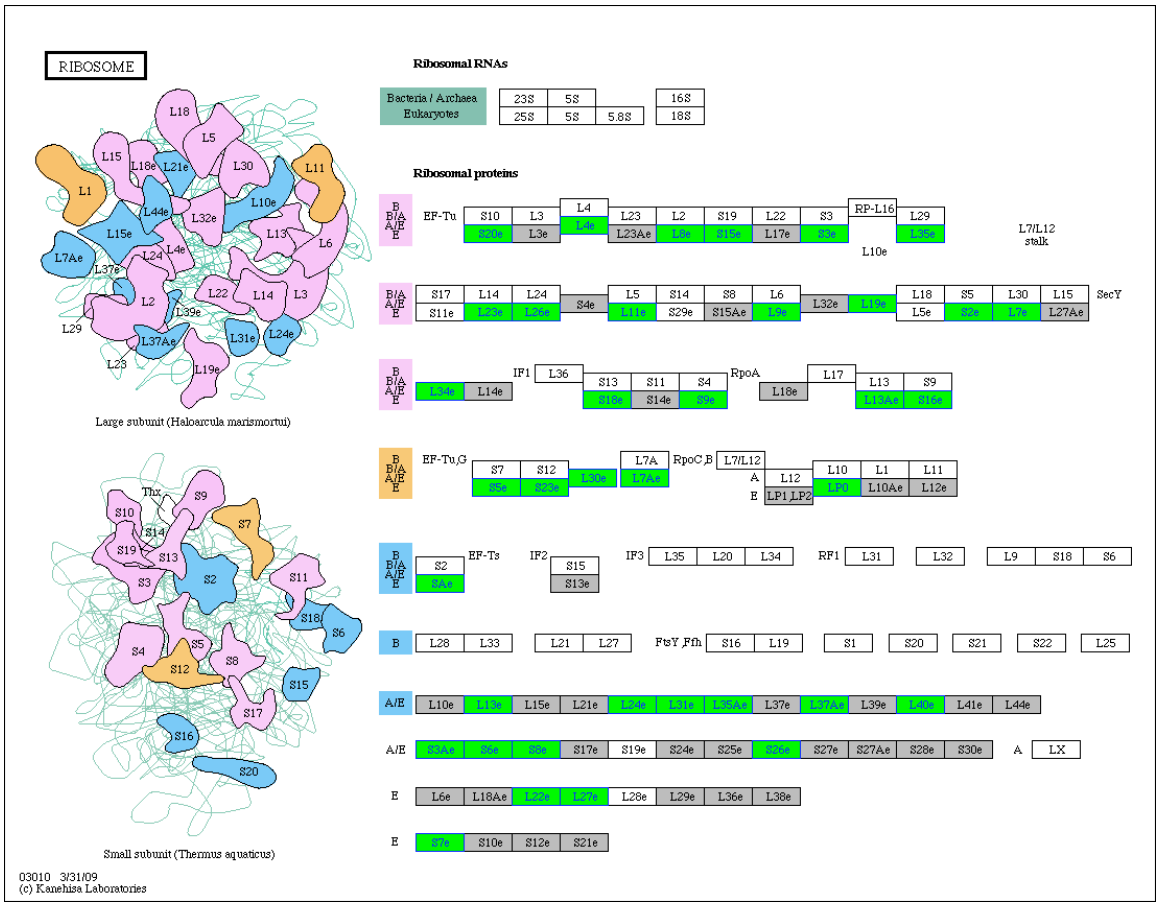
RIBOSOME
Large subunit (Haloarcula marismortui)
Small subunit (Thermus aquaticus)
Ribosomal RNAs
Bacteria / Archaea
Eukaryotes
23S
5S
5.8S
25S
16S
18S
Ribosomal proteins
EF-Tu
RP-L16
L10e
L7/L12 stalk
SecY
RpoA
EF-Tu,G
RpoC,B
L7/L12
EF-Ts
IF1
IF2
IF3
RF1
FtsY ,Ffh
03010 3/31/09
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Figure 3