Hyperthermic seizures and aberrant cellular homeostasis in *Drosophila* dystrophic muscles

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Supplementary Information

Supplementary Table 1: ROS levels seen in *Dys*, *Dg* and *Cam* mutants

Genotype	ROS ([AU]/µg/ml protein)	P-Value
OregonR [2]	1.00 ± 0.26	-
DysDf/+	1.25 ± 0.83	0.091
Dg^{O86}/+	1.04 ± 0.14	0.37
Cam^{n339}/+	0.88 ± 0.10	0.22
DysDf	4.66 ± 2.54	$3.0X10^{-8}$***
$Dg^{O86/O55}$	0.61 ± 0.37	0.0074**
Dg^{O86}/+; *DysDf/+*	0.66 ± 0.22	0.0034**
Cam^{n339}/+; *DysDf/+*	0.49 ± 0.17	$2.0X10^{-5}$***
$cora^{k08713}$/+; *DysDf/+*	0.86 ± 0.07	0.18

[1] P-Values are relative to control animals of genotype *OregonR*,
*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001
[2] All values are normalized relative to *OregonR*
[3] errors reported represent the standard deviation

Supplementary Table 2: Decrease in *Dystrophin* mRNA level

Genotype	***Dys* Average C_T**	***RpL32* Average C_T**	**ΔC_T *Dys* – *RpL32*[1]**	**$\Delta\Delta C_T$ (ΔC_T-$\Delta C_{T,control}$[2])**	**Average *Dys* relative to control[3]**	***Dys* mRNA fold reduction relative to control[4]**
tub-Gal4/+	22.40±0.09	18.35±0.20	4.05±0.22	0.00±0.32	1.00±0.22	1.00±0.22
tub-Gal4::dsDys/+	22.26±0.06	16.35±0.04	5.91±0.08	1.86±0.24	0.28±0.05	3.63±0.60

[1] the ΔC_T value is determined by subtracting the average *RpL32* C_T value from the average *Dys* C_T value. The standard deviation of the difference is calculated from the standard deviation of the *Dys* and *RpL32* values using the formula "$s=\sqrt{(s_1^2+s_2^2)}$, where s=stdev

[2] the calculation of the $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This standard deviation is determined the same as in '1'

[3] the range given for *Dys* relative to Control is determined by evaluating the expression: $2^{-\Delta\Delta CT}$ where the error is determined using regressional analysis

[4] the fold reduction given for *Dys* relative to Control is determined by evaluating the expression: $2^{\Delta\Delta CT}$ where the error is determined using regressional analysis

Supplementary Table 3: Decrease in *Dystrophin* mRNA level using *tub-Gal80^{ts}* system

Genotype	*Dys* Average C_T	*RpL32* Average C_T	ΔC_T *Dys* – *RpL32*[1]	$\Delta\Delta C_T$ (ΔC_T-$\Delta C_{T,cont}$[2])	T Index[3]	T corrected *Dys* mRNA fold reduction relative to control[4]
tub-Gal80^{ts}/+; tub-Gal4/+ pupae at 18˚C	24.46±0.16	16.61±0.03	7.86±0.17	0.00±0.24	-	1.00±0.16
tub-Gal80^{ts}/+; tub-Gal4/+ pupae 3d. at 29˚C	21.87±0.02	16.53±0.27	5.34±0.27	-2.52±0.32	5.72	1.00±0.31
tub-Gal80^{ts}/+; tub-Gal4::dsDys/+ pupae at 18˚C	23.47±0.17	16.20±0.06	7.27±0.17	0.00±0.25	-	1.00±0.17
tub-Gal80^{ts}/+; tub-Gal4::dsDys/+ pupae 3d. at 29˚C	22.60±0.12	16.28±0.13	6.32±0.17	-0.95±0.25	5.72	2.97±0.27
tub-Gal80^{ts}/+; tub-Gal4::dsDys/+ flies at 18˚C	23.88±0.17	18.19±0.29	5.70±0.34	0.00±0.48	-	1.00±0.33
tub-Gal80^{ts}/+; tub-Gal4::dsDys/+ flies 3d. at 29˚C	24.76±0.23	17.28±0.03	7.48±0.24	1.78±0.41	-	3.44±0.99

[1] the ΔCT value is determined by subtracting the average *RpL32* CT value from the average *Dys* C_T value. The standard deviation of the difference is calculated from the standard deviation of the *Dys* and *RpL32* values using the formula "$s=\sqrt{(s_1^2+s_2^2)}$, where s=stdev

[2] the calculation of the $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This standard deviation is determined the same as in '1'

[3] the temperature dependent scaling factor was determined because expression levels of *Dys* in control pupae varied greatly depending on the temperature. Where applicable this value is calculated as $2^{-\Delta\Delta CT}$. This scaling factor is then used to determine the relative expression levels in mutant animals of the same life stage at 29˚C relative to the same animals at 18˚C (Supplementary Figure 2)

[4] the fold reduction given for *Dys* relative to Control is determined by evaluating the expression: $2^{\Delta\Delta CT}$ where the error is determined using regressional analysis. The temperature related scaling index has been multiplied by this value where applicable

Supplementary Table 4: Frequency of muscle degeneration in *Dys RNAi* mutants with and without developmentally restricted expression of *RNAi*

Genotype	***Dys* down regulated**	**Condition[1]**	**Age, days**	**n**	**% of muscle degene-ration**	**P-Value[2]**	**Normalized[3] % of muscle degeneratio**	**P-Value[2]**
tub-Gal4/+	Through-out lifetime	25˚C	16	121	9.1	0.021*	2.4	0.11
tub-Gal4:dsDys				73	21.9			
tub-Gal80^{ts}/+; tub-Gal4/+	As adult	18˚C development 29˚C adulthood	19	84	21.4	0.0081 **	2.0	
tub-Gal80^{ts}/+; tub-Gal4:dsDys/+				108	42.6			

[1] Note that higher temperature (29˚C) accelerates aging and muscle degeneration.

[2] P-value determined from the χ^2 statistic

[3] Normalized to the control value under the same conditions

Supplementary Table 5: Relative Dg immunofluorescence intensities

Genotype	Relative Intensity	P-Value[1]
OregonR [2]	1.00 ± 0.37[3]	-
DysDf	0.49 ± 0.27	0.042*
Dg^{O55}	0.02 ± 0.02	1.2×10^{-4}***
$cora^{k08713}$	0.11 ± 0.11	1.4×10^{-3}**
Dg^{O86}/+; *DysDf*/+	0.06 ± 0.05	1.9×10^{-5}***
$cora^{k08713}$/+; *DysDf*/+	0.07 ± 0.06	1.1×10^{-4}***
$cora^{k08713}/Dg^{O86}$	0.10±0.12	$9{,}9 \times 10^{-5}$***

[1] P-Values are relative to control animals of genotype *OregonR*,
*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001
[2] All values are normalized relative to *OregonR*
[3] errors reported represent the average deviation

Supplementary Table 6: Decrease in *Dystroglycan* mRNA level

Genotype	***Dg* Average C_T**	***RpL32* Average C_T**	**ΔC_T *Dg* – *RpL32*[1]**	**$\Delta\Delta C_T$ (ΔC_T-$\Delta C_{T,control}$[2])**	**Average *Dg* relative to control[3]**	***Dg* mRNA fold reduction relative to control[4]**
tub-Gal4/+	23.33±0.07	18.35±0.20	4.98±0.21	0.00±0.30	1.00±0.21	1.00±0.21
tub-Gal4::dsDg/+	25.94±0.13	18.37±0.11	7.57±0.18	2.59±0.28	0.17±0.03	6.02±1.16
tub-Gal80^{ts}/+; tub-Gal4::dsDg/+ flies at 18˚C	20.97±0.05	16.09±0.05	4.87±0.07	0.00±0.10	1.00±0.07	1.00±0.07
tub-Gal80^{ts}/+; tub-Gal4::dsDg/+ flies 3d. at 29˚C	25.26±0.07	19.09±0.03	6.17±0.08	1.29±0.11	0.41±0.03	2.45±0.18

[1] the ΔC_T value is determined by subtracting the average *RpL32* C_T value from the average *Dg* C_T value. The standard deviation of the difference is calculated from the standard deviation of the *Dg* and *RpL32* values using the formula "$s=\sqrt{(s_1^2+s_2^2)}$, where s=stdev

[2] the calculation of the $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This standard deviation is determined the same as in '1'

[3] the range given for *Dg* relative to Control is determined by evaluating the expression: $2^{-\Delta\Delta CT}$ where the error is determined using regressional analysis

[4] the fold reduction given for *Dg* relative to Control is determined by evaluating the expression: $2^{\Delta\Delta CT}$ where the error is determined using regressional analysis

Supplementary Table 7: Impact of Ca^{2+} channel blockers on seizure activity of dystrophic animals (*DysDf*)

Drug	**n**	**%Seized^{T}**	**Avg. T_s±sd (˚C)***	**Avg. A_{max}±sd (mV)***	**Avg. Area±sd (pixel x 10^{-3})***	**Median S_i** (25^{th}/75^{th} percentiles)**	**P-Value**
Sucrose	5	80	37.8±2.1	6.6±5.7	2.4 ±1.9	1.9 (1.8/2.1)	-
3.6 mM Nifedipine	5	100	37.8±3.3	3.1±2.2	1.2 ± 0.5	1.8 (1.8/1.9)	0.50
0.5 mM 2-APB	5	60	35.7±5.7	2.8±1.2	0.8 ± 0.4	1.5 (0.0/1.7)	0.21
20 µM Ryanodine	6	33	34.4±0.6	2.8±0.6	0.9 ±0.2	0.0 (0.0/1.4)	0.078

n = number of animals fed the indicated drug

Ŧ percent of animals measured that had a seizure

* Calculated using data from animals that seized only

** Index calculated by integrating the area of the graph of voltage vs. temperature during a seizure, taking the natural logarithm of this number, dividing by the temperature that the seizure started, and then multiplying times ten.

Supplementary Figure Legends

Supplementary Figure 1:

Control animals do not have temperature-sensitive seizures as can be seen by monitoring the output voltage from the IFMs as the temperature increases. b) All tested *Dys* mutant alleles exhibit hyperthermic seizures.

Supplementary Figure 2:

Average *Dys* expression relative to appropriate controls in *RNAi* knock-down mutants that are under the control of the *tub-Gal80^{ts}* driver. a) Control pupae showed a vast increase in *Dys* expression upon being shifted from 18˚C to 29˚C indicating a natural increase in protein expression with temperature increase. Thus, to determine adequately the effectiveness of *RNAi* down-regulation this change in expression had to be taken into consideration using a temperature index (Supplementary Table 5). b) Average *Dys* expression levels relative to control in pupae (after the temperature index was considered) and in adults after being shifted to 29˚C for four days.

Supplementary Figure 3:

Seizure indices showing that animals with *RNAi* directed against muscle (*24B-Gal4*) and mononeuron (*D42-Gal4*) *Dg* mRNA do not have temperature-sensitive seizures. Endogenous (*tub-Gal4*) expression results in a reduced amount of seizures that are not significant over controls, but this is reversed when *Dg* is down-regulated after development. Refer to Table 1 for exact P-values compared to control animals.

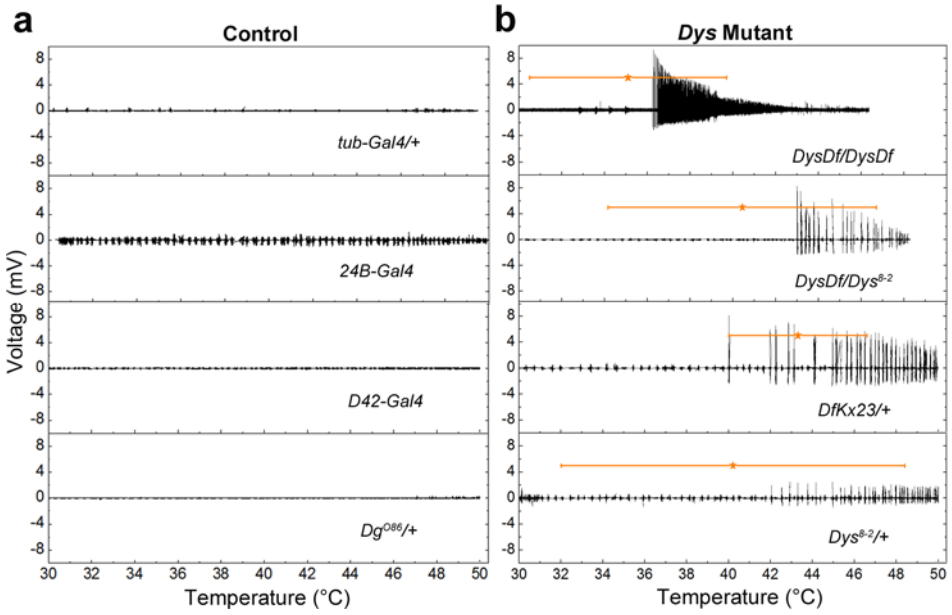
Supplementary Figure 4:

a) β_{PS} integrin subunit loss of function allele heterozygouts (*mys*^{1}*/+)* had seizures when heated, most likely due to compromised stability of the sarcolemma and/or NMJ. Introducing a mutant copy of *Dg* into the genome of these animals does not alter the seizure character. b) Seizure indices of *mys*^{1}/+ heterozygous and *mys*^{1}*/+; Dg/*+ trans-heterozygous animals where a Kruskal-Wallis test show that there are no significances in the indices (χ^2 = 2.93, P = 0.231). The following P-values were determined upon comparison to wild type animals: *mys*^{1}/+ (***P = 2.0X10^{-4}), *mys*^{1}*/+; Dg*^{O86}/+ (*P = 0.013), *mys*^{1}*/+; Dg*^{O55}/+ (*P = 0.082) using a one-tailed Mann-Whitney U-test. 100% of *mys*^{1}/+ animals tested had seizures (n=8) with T_s = 39.2 ± 2.6, A_{max} = 3.6 ± 1.7 and Avg. Area = 1.5 ± 1.1 x 10^3. 80% of *mys*^{1}*/+;Dg*^{O86}/+ animals tested had seizures (n=5) with T_s = 36.0 ± 0.7, A_{max} = 3.8 ± 2.4 and Avg. Area =1.2 ± 0.5 x 10^3. 83% of *mys*^{1}*/+;Dg*^{O55}/+ animals tested had seizures (n=6) with T_s = 40.3 ± 3.7, A_{max} = 4.3 ± 3.0 and Avg. Area =1.2 ± 1.0 x 10^3.

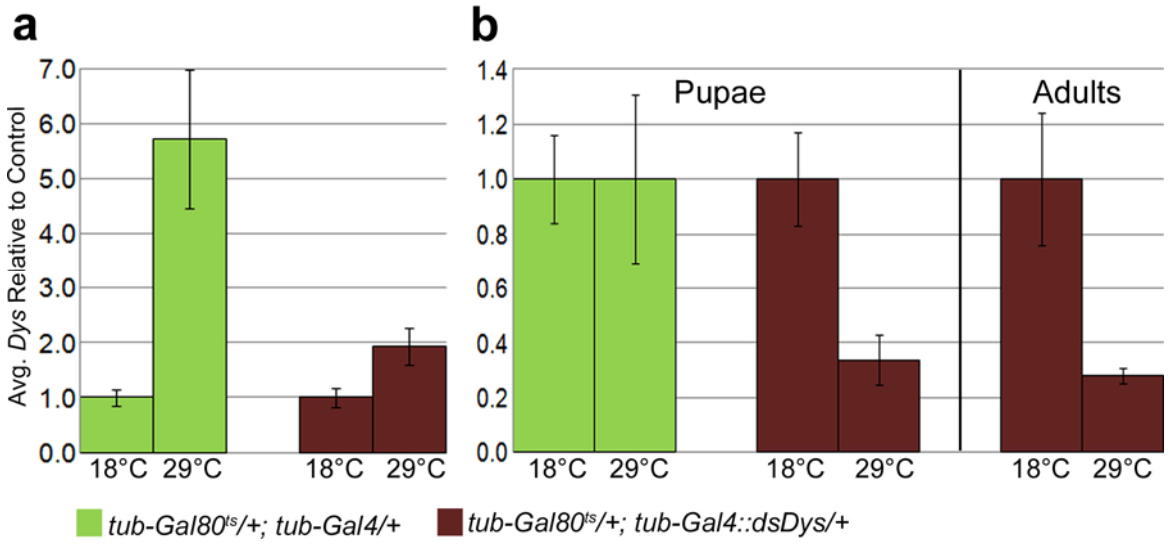
Supplementary Figure 5:

a) TGF-β pathway mutants, *tkv* (type-I receptor) and *Mad* (receptor-regulated Smad) have seizures similar to what is seen in *Dys* mutants, however; when animals are missing one copy of *Dys* and one copy of each of these genes there is no significant increase in seizures over what is seen in *Dys.* Down-regulation of *tkv* after NMJ development *(tub-Gal80*^{ts}*)* does not alleviate seizures implying that there is an additional need for *tkv* after development is complete. Animals that express *RNAi* against *tkv* prior to pupae formation die supporting an important role for TGF-β signaling during pupation. Transheterozygous animals when compared to *Dys* heterozygous

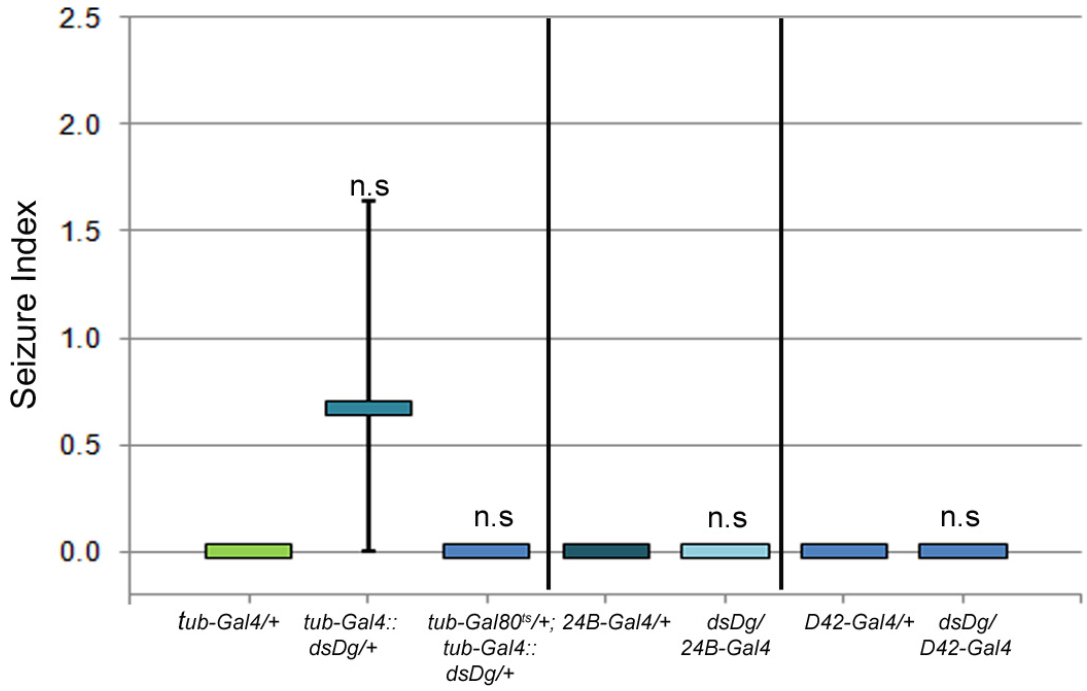
animals using a Mann-Whitney U-test had P-values of 0.25 and 0.46 for *tkv/Dys* and *Mad/Dys* respectively and P-values of 0.099 and 0.040 when compared to control animals respectively. Animals of genotypes tkv^{1}, $tub\text{-}Gal80^{ts}/act\text{-}Gal4;tkv^{RNAi}$ and $Mad^{12}/+$ have P-values of 1.9×10^{-3}, 0.012 and 0.072 when compared to controls respectively. 100% of tkv^{1} animals tested had seizures (n=9) with $T_s = 39.4 \pm 4.7$, $A_{max} = 4.8 \pm 3.6$ and Avg. Area = $1.3 \pm 0.8 \times 10^{3}$. 60% of $tkv^{1}/+;DysDf/+$ animals tested had seizures (n=5) with $T_s = 44.2 \pm 2.1$, $A_{max} = 7.4 \pm 1.3$ and Avg. Area $=1.9 \pm 1.3 \times 10^{3}$. 67% of $Mad^{12}/+;DysDf/+$ animals tested had seizures (n=6) with $T_s = 42.2 \pm 4.0$, $A_{max} = 15 \pm 8.7$ and Avg. Area $=2.0 \pm 1.0 \times 10^{3}$. 80% of $Mad^{12}/+$ animals tested had seizures (n=5) with $T_s = 42.8 \pm 3.2$, $A_{max} = 8.8 \pm 6.3$ and Avg. Area $=1.7 \pm 1.4 \times 10^{3}$. 100% of $tub\text{-}Gal80^{ts}/act\text{-}Gal4;\ tkv^{RNAi}/+$ animals tested had seizures (n=5) with $T_s = 40.0 \pm 3.4$, $A_{max} = 7.3 \pm 2.8$ and Avg. Area $=4.4 \pm 2.8 \times 10^{3}$. b) Electrical output from IFM vs. temperature of TGF-β pathway mutants exhibiting seizures. *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001



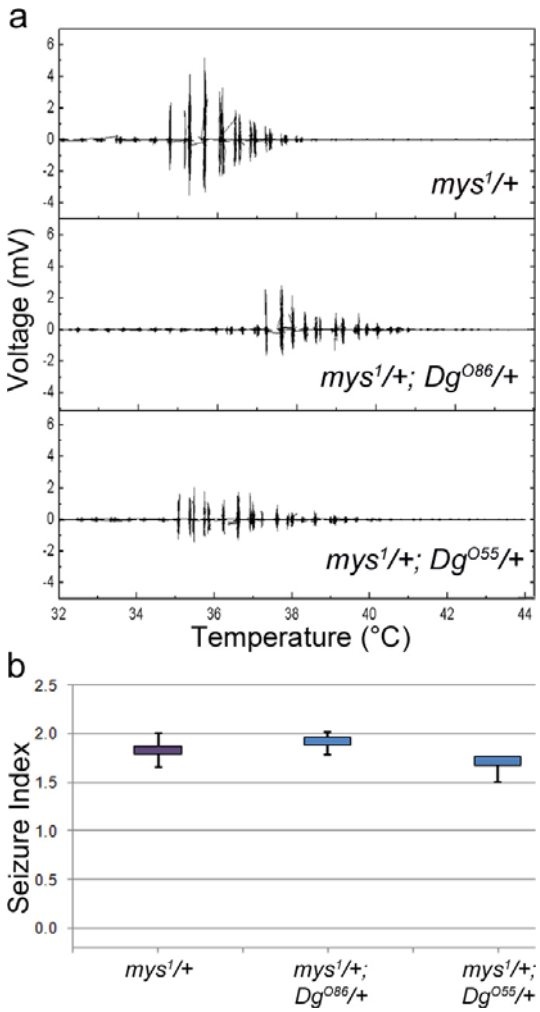
Supplementary Figure 1



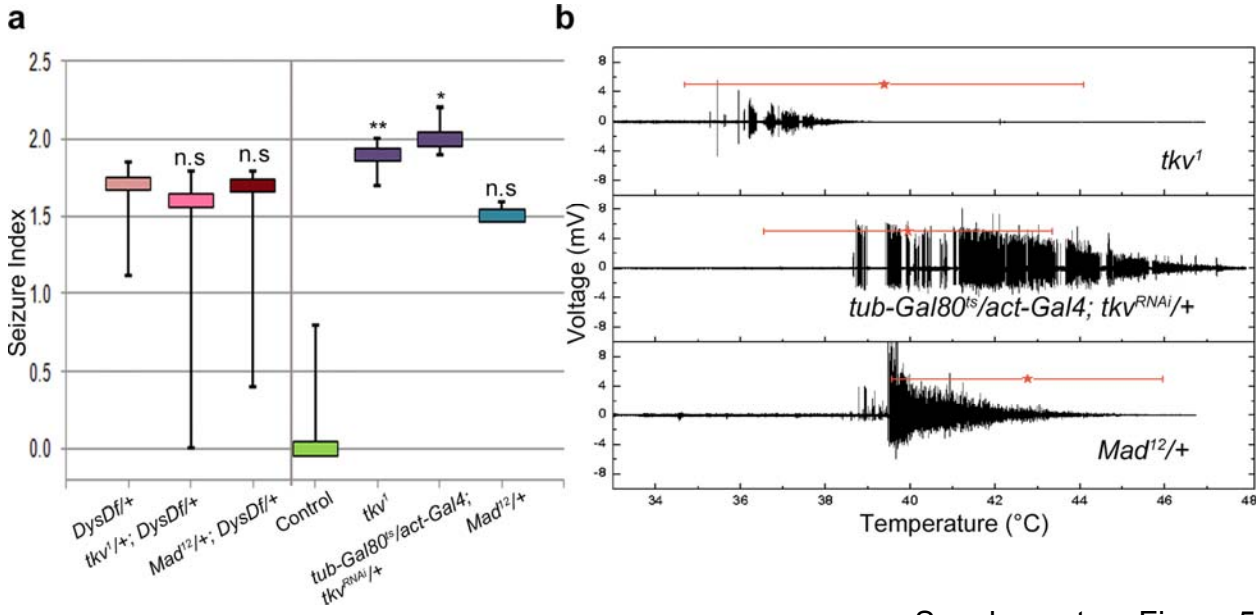
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5