Genetic and Chemical Modulation of the Immune Deficiency Pathway Influences the Immune Response and Neurodegeneration in a *Drosophila* model of Machado-Joseph Disease

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Introduction

Machado-Joseph Disease (MJD), or Spinocerebellar ataxia type 3, is the most common dominantly inherited ataxia. The disease is caused be an expanded Glutamine (CAG trinucleotide) repeat region in the Ataxin 3 gene. A normal gene has roughly 12-44 Glutamine repeats, and a disease has roughly 61-87. A well known feature of the disease is the formation of nuclear aggregates. Additionally, more repeats in the disease allele correspond to increased pathogeny (rev'd in Costa and Paulson, 2012). Common symptoms include loss of muscle control, memory deficits, and involuntary muscle movements. The Innate Immune Response (IIR) has been linked to the progression of similar neurodegenerative diseases (Peterson et al. 2001, Liu et al. 2017). In this experiment we investigate the effects of up and down regulation of the immune response on neurodegeneration in a Drosophila model of MJD. The NF-kB homolog, nuclear transcription factor Relish, mediates the immune response in Drosophila. (Fig 5, Stoven et al. 2000) Relish has an inhibitory domain that must be cleaved before it can enter the nucleus. We use transgenes, E38:Relish mutant and RelD:constituently active Relish to regulate the immune response. Additionally, we investigate the intracellular location of Relish. MJD is similar to other more prevalent neurodegenerative diseases, such as other polyglutamate repeat disorders including Huntington's Disease. Discoveries about MJD may offer insight into these related diseases.

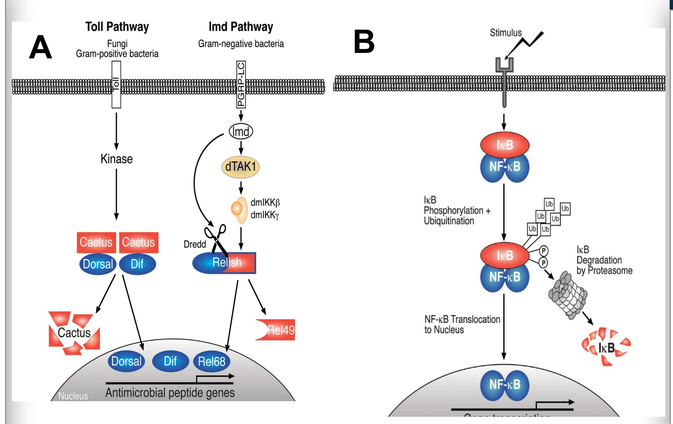

Figure 1. Relish pathway in Drosophila and homologous NF-kB pathway. Both Relish and its mammalian homologue are nuclear transcription factors that cannot translocate to the nucleus until their inhibitory domain is cleaved. A) Drosophila pathway. B) Mammalian pathway.

Results

RELISH: Increased expression of Relish increases degeneration (Fig 2-4). The exterior of the eye shows increased degeneration when the immune response is upregulated in the presence of a mutant protein (Fig 2). Increased degeneration is apparent in the RelD group over w^{1118} in a truncated Q78 disease allele (Fig 2). In these conditions qualitative degeneration is visually apparent in the rhabdomeres (Fig 3). In a non-truncated Q84 protein group, the 7 day mean rhabdomere per ommatidium count decreased from 3.12 (SD 0.87), out of a maximum of 7, in the wild type, to 2.20 (SD 0.71) in the RelD group. There was no statistically significant rescue from decreasing the immune response with the E38 group (Mean 3.43, SD 1.13) The His6 group (Mean 3.01, SD 0.59) showed no significant change in degeneration. (Fig 4)

CURCUMIN: Our results support significant progression of neurodegeneration from 1-day to 7-days in diseased Q84 flies for both curcumin and water conditions. Additionally, there were no significant differences detected in Q84 1-day curcumin (M = 6.84, SD = 0.10) versus 1-day water (M = 6.86, SD = 0.08) conditions, along with no significant differences between Q84 7-day curcumin (M = 5.92, SD = 0.18) versus 7-day water (M = 5.71, SD = 0.60) conditions (Fig 6). These results could suggest that curcumin does not have a pronounced rescuing effect on SCA3 neurodegeneration in *D. melanogaster* models. Also, there was a moderately significant difference detected in Q84 7-day ratio of Relish fluorescence in curcumin (M = 3.72, SD = 0.86) versus water conditions (M = 4.24, SD = 0.94) (Fig 5).

CURCUMIN+RELISH: Data from the constitutively activated pro-inflammatory Q84/Rel^D flies follow the same trend as histological results from Q84 flies. No significant differences were detected between Q84/Rel^D 7-day curcumin (M = 6.14, SD = 0.26) and water (M = 5.80, SD = 0.27) (Fig 6). There was not significance found in Q84/ Rel^D 7-day ratio of Relish fluorescence in curcumin (M = 3.88, SD = 0.87) versus water (M = 4.11, SD = 1.03) (Fig 5).

DREDD: Data from up and downregulation of *Dredd* showed an increase in neurodegeneration in Q84 flies. There was a statistically significant decrease in the number of photoreceptors in one-day Q84 (M=5.95, SD=0.05) when *Dredd* was downregulated as compared to the control (M=6.86, SD=0.07). Furthermore, both upregulation (M=3.49, SD=1.47), and downregulation (M=3.85, SD=1.47) of Dredd decreased the number of rhabdomeres as compared to the control (M=5.71, SD=0.57). Surprisingly, downregulating *Dredd* showed a decrease in the expected number of photoreceptors in control Q27 flies suggesting that *Dredd* may have an impact in retinal development. (Fig. 8).

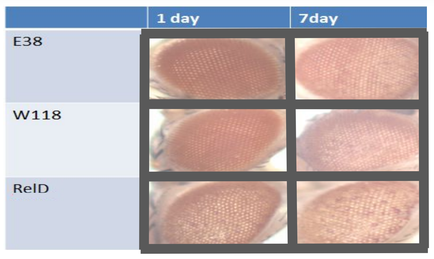

Figure 2. Increased expression of immune response transcription factor Relish increases degeneration. The exterior of the eyes of flies at 1 or 7 days old. These flies have a truncated version of the Ataxin gene with 78 CAG repeats. RelD flies show qualitative increased degeneration against the wild type for both 1 and 7 days. E38 flies show no or modest rescue of degenerative phenotype for 7 days.

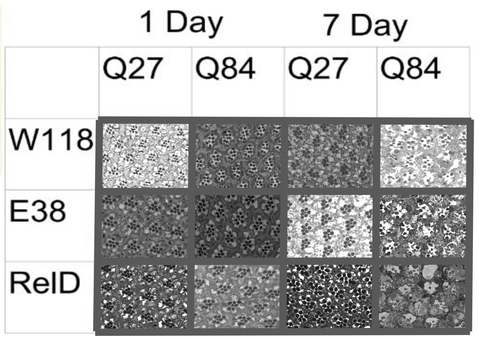

Figure 3. Increased Relish expression increases MJD mediated degeneration in photoreceptors. Histological sections of the Drosophila eye depict rhabdomeres with healthy tissues showing groupings of 7. These tissue sections were used to generate scores in Figure #. The labels on the left represent the transgenic immune response gene and the labels on top show the number of repeats in the Ataxin 3 gene.

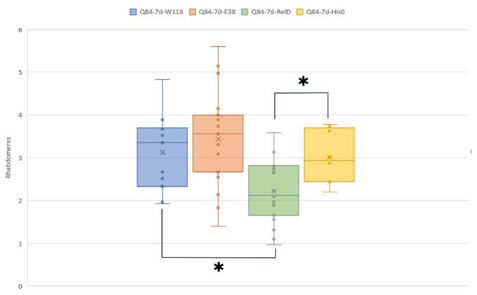

Figure 4. Relish upregulation through RelD increases neurodegeneration with a disease allele. The count of rhabdomeres per ommatidium represents the quality of the tissue with a score of 7 expected in completely wild type flies. All flies used are 7 days old. w^{1118}-Q84 (n=11), E38-Q84 (n=17), His6-Q84 (n=8), RelD-Q84 (n=19). (* : p < 0.05 with t-test)

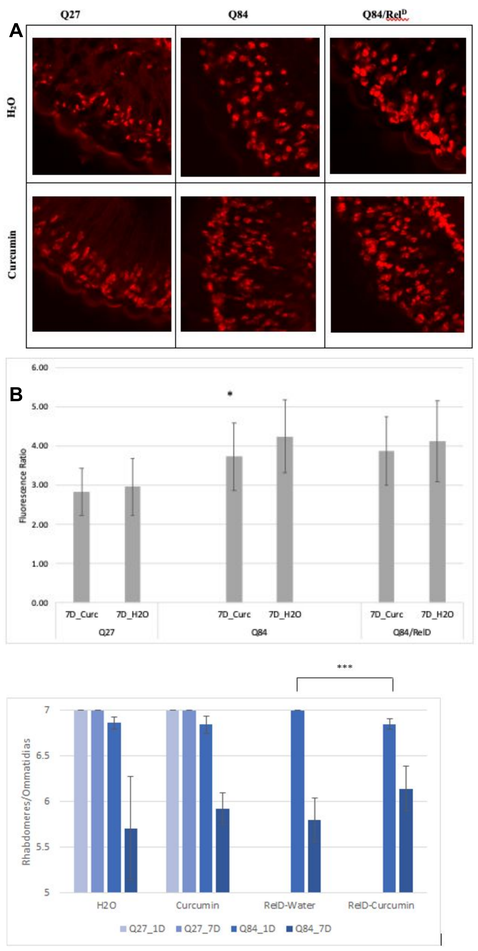

Figure 5. Effect of neurodegeneration over time (7-days) on NF-kB-like immune response in *D. melanogaster* retinas. a) Confocal microscopy representing the effect of 1mM curcumin on transcription factor Relish (red). SCA3 peptides were expressed in fly retinas. Ratios of nuclear:cytoplasmic Relish were analyzed using the Fiji version of ImageJ. Sections were imaged at 60x. b) Quantification of Relish fluorescence ratios in SCA3 Q27, Q84, and Q84/Rel^D *D. melanogaster* grown up on 1mM curcumin versus H_20 controls at 7 days. Bars represent the mean Relish nuclear: cytoplasmic ratio to measure activated Relish translocation to the nucleus. Error bars represent standard deviations. *p<0.01.

Figure 6. Average Number of Rhabdomeres per Ommatidia Demonstrating a Quantification of Neurodegeneration when Exposed to Curcumin. The average per eye was derived by the average number of rhabdomeres per ommatidia for a total of ten ommatidia. The average per condition was calculated by determining the average number of rhabdomeres for each eye cut per condition. 1-day-old H_2O_Q27 (N=9), 1-day-old Curcumin_Q27 (N=9), 7-day-old H_2O_Q27 (N=3), 7-day-old Curcumin_Q27 (N=4), 1-day-old H_2O_Q84 (N=4), 1-day-old Curcumin_Q84 (N=7), 7-day-old H_2O_Q84 (N=9), 7-day-old Curcumin_Q84 (N=7), 1-day-old Q84RelD_Water (N=5), 7-day-old Q84RelD_Water (N=5), 1-day-old Q84RelD_Curcumin (N=9), 7-day-old Q84RelD_Curcumin (N=7). Error bars represent the standard deviation of each condition. Results showed one statistically significant difference ($p<.05$) between 1-day-old Q84RelD exposed to water and 1-day-old Q84RelD exposed to curcumin, as determined by a one-way ANOVA.

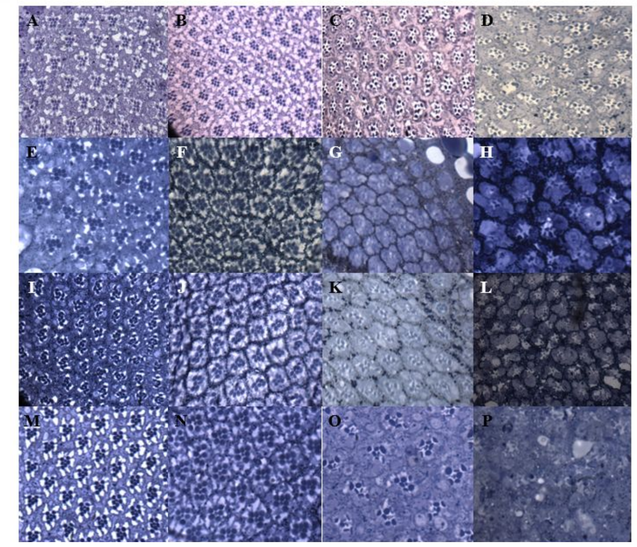

Figure 7. Regulation of *Dredd* does not Show a Suppression of Photoreceptor Neural Loss on SCA3-Q84-Induced Degeneration. (A, E, I, and M): Q27-1D flies; (B, F, J, and N): Q27-7D flies; (C, G, K, and O): Q84-1D flies; (D, H, L, and P): Q84-7D; (A-D): W^{1118}; (E-H) : Dredd-16892; (I-L): Dredd-34070; (M-P): Dredd-80924

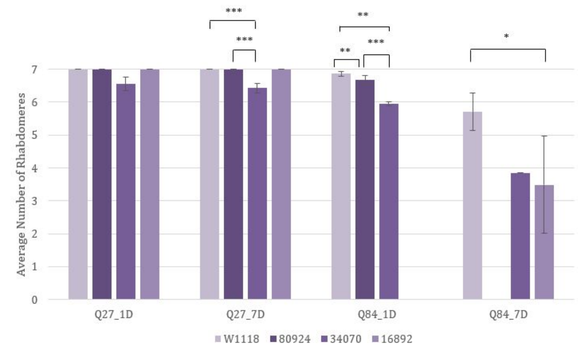

Figure 8. Average Number of Rhabdomeres per Ommatidia Demonstrating a Quantification of Neurodegeneration when Exposed to Curcumin. The average per eye was derived by the average number of rhabdomeres per ommatidia for a total of ten ommatidium. The average per condition was calculated by determining the average number of rhabdomeres for each eye cut per condition. 1-day-old H_2O_Q27 (N=9), 1-day-old Curcumin_Q27 (N=9), 7-day-old H_2O_Q27 (N=3), 7-day-old Curcumin_Q27 (N=4), 1-day-old H_2O_Q84 (N=4), 1-day-old Curcumin_Q84 (N=7), 7-day-old H_2O_Q84 (N=9), 7-day-old Curcumin_Q84 (N=7), 1-day-old Q84RelD_Water (N=5), 7-day-old Q84RelD_Water (N=5), 1-day-old Q84RelD_Curcumin (N=9), 7-day-old Q84RelD_Curcumin (N=7). Error bars represent the standard deviation of each condition. Results showed one statistically significant difference ($p<.05$) between 1-day-old Q84RelD exposed to water and 1-day-old Q84RelD exposed to curcumin, as determined by a one-way ANOVA.

Discussion

RELISH: Li et al. in 2018 also found increased degeneration with upregulation of the immune response through the NF-kB homologous pathway. Taken along with this data, strong evidence is established for pathogenic contributions of the immune response in MJD. These experiments validated multiple disease models using multiple measures of degeneration (Fig 1,2). The RelD Q84 group increased degeneration from the wild type immune response and it had a large effect size. Additionally, the regular organization of the ommatidia were disrupted in the RelD disease allele groups. This highlights the biological dysfunction caused by the disease and an increased immune response together.

CURCUMIN+RELISH:The rate of neurodegeneration in both mutant SCA3 Q84 and Q84/Rel^D significantly worsens over time in curcumin and water conditions. While the rate of neurodegeneration is significantly greater in mutant Q84 flies than non-diseased Q27 flies, curcumin did not show a statistically significant rescuing effect on the rate of neurodegeneration. Our data also suggests a slight trend in curcumin decreasing pro-inflammatory response over time in Q84 retinas, but we did not find significantly less Relish activation in Q84/Rel^D flies.

DREDD: The rate of neurodegeneration was significantly worse for both upregulation and downregulation of *Dredd*. This suggests that downregulation of *Dredd* may be insufficient to block the cleavage of Relish like previously hypothesised. Furthermore, non-diseased Q27 flies showed a statistically significant decrease in the number of photoreceptors, with rhabdomere seven missing most often. This suggests that *Dredd* may have an impact on the retinal development of photoreceptors.

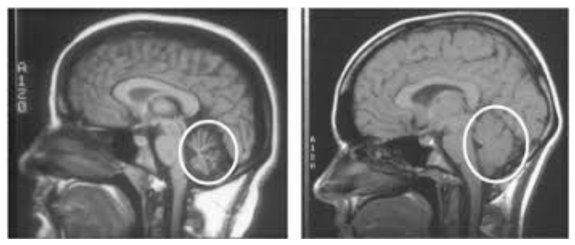

Figure 9. Midsagittal MRI Image of the Brain Showing Cerebellar Atrophy. Image on the left shows the brain of an MJD patient expressing atrophy of the cerebellum. Image on the left shows the brain of an individual not affected with MJD

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Methods

Immune response was regulated through transgenes of the proinflammatory Relish and Dredd transcription factors. Relish must be cleaved before translocation into the nucleus, while Dredd is a cytoplasmic caspase. For Relish the transgene groups were as follows: w^{1118}: wild type, E38: heterozygous Relish point mutant, His6: gene insert with native inhibitor, RelD: gene insert without native inhibitor. For Dredd the lines were as follows: 16892: upregulated EP insertion, 34070: RNAi knockdown, 80924: point mutation in initiation codon. Curcumin was administered by mixing with the food at 1mM for the entire life of the fly. Rhabdomere counts were acquired by sectioning flies embedded in plastic blocks at 900nm. All reported counts have an n of at least 3. Immunohistochemistry data was collected for Figures 4 and 5. Hoechst stained DNA to identify the nucleus and TRITC anti-mouse stained Relish transcription factor. Colocalization was determined using Image J (FIJI).