Figures 1A and 1B reveal that *Z. indianus* are most prevalent at the baits in the morning, and their numbers decrease as the day progresses, becoming almost absent at night at either the rural or urban sites. We cannot exclude the possibility that exhaustive sampling at the urban site on the first day of collection (unpublished data) explains the very low numbers of *Z. indianus* on the second day of collections.

Initially, we speculated that *Z. indianus* might displace *D. melanogaster* with its invasion, but our results indicate otherwise. We observed a continuous presence of *D. melanogaster* while *Z. indianus* is almost absent at night. The low numbers of *Z. indianus* at the urban site is consistent with the preference of this fly for open habitats (Tidon *et al*., 2003; Silva *et al*., 2005). Species composition at the urban site almost entirely consisted of *D. melanogaster* at any time of the day, consistent with its position as a cosmopolitan species with humans. Although *Z. indianus* does not appear to alter the diurnal activity of *D. melanogaster*, there may be other effects from this invasion on native species. Habitats in Alamos seem to provide ample resources to support the coexistence of these species.

As observed previously, male *D. melanogaster* spend considerable time courting at the feeding site, and copulating pairs are only observed infrequently (Partridge *et al*., 1987; Gromko and Markow, 1993). Our observations suggest that females of both species either experience a higher mortality and are thus less abundant, or that they spend considerable time at other sorts of habitats or resources. Our understanding of the impact of *Z. indianus* on other Drosophilids would be enhanced by monthly monitoring of species composition at both sites coupled with determination of the actual operational sex ratio at different times of year.

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Additional phenotypes of a myotonic dystrophy *Drosophila* model.

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Introduction

Myotonic dystrophy type 1 (DM1) is a dominant autosomal genetic disease with a broad range of symptoms. Clinically, it affects up to 11 systems including muscular, nervous, ocular, digestive, respiratory, and cardiovascular tissues. Characteristic features are loss of muscle strength (with a distal to proximal pattern), myotonia, excessive daytime sleepiness, excessive fatigue, abdominal pain, as well as dysphagia (Harper, 2001). The genetic basis of DM1 is the expansion of unstable CTG repeats in the 3´ untranslated region of the *Myotonic Dystrophy Protein Kinase*

(*DMPK)* gene (Aslanidis, Jansen *et al.* 1992; Brook *et al.,* 1992; Fu *et al.,* 1992; Mahadevan *et al.,* 1992). An RNA gain-of-function model of myotonic dystrophy pathogenesis has been proposed. The expanded CUG tracts in the mutant *DMPK* transcripts fold into hairpins and accumulate forming nuclear foci that sequester nuclear factors with high RNA binding affinity, such as transcription factors and alternative splicing regulators including Muscleblind-like 1 proteins (MBNL1) (Lin *et al.,* 2006).

Aberrant binding of MBNL1 to the expanded CUG repeats causes loss-of-function of the protein, and the concomitant misregulation of the alternative splicing of at least 126 specific pre-mRNAs (Du *et al.*). The splicing factor CUG-Binding Protein 1 (CUGBP1), a protein that antagonizes MBNL1 in the regulation of alternative splicing of a number of transcripts, is also altered in DM1. CUGBP1 is abnormally upregulated in DM1, further contributing to missplicing (Jiang *et al.,* 2004; Mankodi *et al.,* 2005).

In our laboratory, a fly model of DM1 was generated using the Gal4/UAS system. This model expresses 480 non-coding CTG repeats, (CTG)480 in different tissues producing visible phenotypes that reproduce multiple aspects of the disease, such as ribonuclear CUG foci that colocalize with Muscleblind (Mbl, *Drosophila* MBNL1 ortholog), missplicing of specific Mbl-regulated transcripts, and muscle degeneration of the indirect flight muscles (IFMs) (Garcia-Lopez *et al.,* 2008). These and other results (Monferrer and Artero, 2006) support the functional conservation between human MBNL1 and fly Muscleblind proteins and validate the use of *Drosophila* as a model to study DM1 pathogenesis.

DM1 model flies were previously used in our laboratory to identify new components of the pathogenesis pathway, as well as potential therapeutic compounds. In particular, we confirmed that (CUG)480 expression in different fly tissues was toxic and originated characteristic and quantifiable phenotypes. Using *Myosin heavy chain (mhc)-Gal4*, muscle degeneration and reduction of lifespan were detected. Moreover, targeted expression of expanded CUG repeats to eye (using the *sevenless (sev)-Gal4 driver*) and mushroom bodies (using the *103Y-Gal4* driver) originated reproducible and easy-to-score phenotypes, which were used to search for chemical and dominant genetic modifiers (Garcia-Lopez *et al.,* 2008).

In the current study we describe additional phenotypes resulting from CTG repeat expression in *Drosophila* eye, skeletal and visceral muscle, and cholinergic and dopaminergic neurons. Our new data contribute a more detailed description of phenotypes resulting from CTG expression in the eye and brain and provide a new tool to study CTG-induced defects in smooth muscle, which could be used in genetic screens in order to identify new components of the CTG toxicity in this tissue, as the causes for smooth muscle disfunction in DM1 have not been explored in depth to date.

Materials and Methods

Drosophila stocks

UAS-(CTG)480 flies were generated in our laboratory (Garcia-Lopez *et al.,* 2008). We obtained *bap-Gal4* from Dr. S. Zaffran (Mount Sinai School of Medicine, New York, EEUU). *mhc-Gal4* was kindly provided by Dr. G. Davis (University of California San Francisco, California, EEUU). s*ev-Gal4* flies were a gift from Dr. M. Mlodzik (Mount Sinai School of Medicine, New York). *Cha-Gal4* were obtained from the Bloomington *Stock* Center (Indiana University), and *Dcd-Gal4* was provided by Dr. Feany (Dartmouth College, New Hampshire EEUU).

Visceral muscle function assays

Ingestion and excretion assays were performed as described in (Gao *et al.,* 2004; Sullivan *et al.,* 2000) with minor modifications. All the experiments were performed at 28ºC on first instar (L1) larvae.

Morphometric studies of imaginal eye discs

Eye-antenna discs from third instar larvae were dissected out in cold PBS, fixed for 30 min at room temperature in a 1:1 mixture of 8% formaldehyde: PEM2x (0.2 M PIPES, 2 mM $MgCl_2$, 2 mM EGTA). 30 discs of control flies (*y w*) and 41 of *sev-Gal4 UAS-(CTG)480* flies were mounted in glycerol, and bright field microscopy images were taken with a Leica DM 2500 microscope. Measurements of eye disc surface (not including the antenna discs) were performed using the image processing software IM500 from Leica.

Statistics

Morphometric measures of eye imaginal discs and differences in viability were statistically compared using two tailed Student's t-test. The results obtained in the ingestion-excretion assays were statistically analyzed using Fisher's exact test. $p < 0.05$ was used as threshold of significance.

Results and Discussion

Expanded CUG repeat RNA reduces eye imaginal disc size

We previously reported that expression of CTG expansions in eye photoreceptor precursors using the *sevenless (sev)-Gal4* driver line produces a rough eye phenotype, with ommatidia and mechanosensory bristle disorganization and reduction in adult eye size (Garcia-Lopez *et al.,* 2008). In order to study further the origin of this phenotype, we analyzed changes in imaginal eye disc morphology of *sev-Gal4 > UAS-(CTG)480* third instar (L3) larvae. We found that the size reduction we previously described in adult eyes is already present in L3 eye discs, with a decrease of about 14% compared to control flies of the same genetic background (*y w*) (Figure 1).

These results demonstrate that the adult eye size reduction phenotype is already prefigured during larval development.

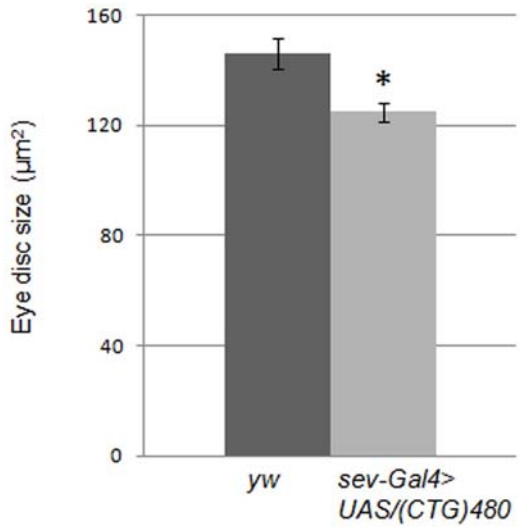

Figure 1. Bar graph showing imaginal eye disc size in flies overexpressing CTG repeats (sev-Gal4>UAS-(CTG)480) and control flies (yw). (*) indicates significance in a two tailed Student's t-test. Error bars are standard deviations.

Expression of expanded CTG repeats is toxic to cholinergic and dopaminergic neurons in Drosophila

Mushroom bodies (MB) are the most complex structure of *Drosophila* brain and are involved in learning and memory functions, including associative memory and motor control. MB function has been compared to the cerebral cortex of mammals, a brain

region which is largely affected in DM1 patients (Masse *et al.,* 2009; Gauthier, 2010).

We previously described that targeted expression of (CUG)480 to the MB using the X-linked *103Y-Gal4* driver produces a temperature-sensitive pupal lethal phenotype, which was successfully used to screen chemical modifiers of the phenotype. In this screen, we found that cholinergic and monoamine inhibitory compounds improved viability of flies expressing CTG repeats in the MB (Garcia-Lopez *et al.,* 2008). Three major groups of neurons have been described in the MB: cholinergic and dopaminergic neurons, which are excitatory, and gabaergic neurons, which are inhibitory. Taken together, our previous observations suggest that (CUG)480 RNA may be toxic to cholinergic and dopaminergic cells. In order to confirm this hypothesis, in this work we expressed (CTG)480 in both neuronal types independently, using specific Gal4 driver lines: *dopa decarboxylase* (*Dcd*)-*Gal4* (Li *et al.,* 2000), which targeted expression to dopaminergic neurons, and *choline acetyl transferase* (*Cha*)-*Gal4* (Salvaterra and Kitamoto, 2001), which targeted expression to cholinergic neurons. The study of fly viability revealed a decrease in viability to the 82% and 68% in the number of emerged flies upon (CTG)480 expression, respectively, compared to control flies of the same genetic background (w^{1118}) (Figure 2). In both cases, lethality occurred before pupa formation.

These results confirm that CUG repeats can be toxic to different neuron populations and indicate that cholinergic neurons could be more sensitive to CUG transcripts than cholinergic cells. Since little is known about the effects of CUG repeats in the brain of DM1 patients, our observation contributes new insight in understanding CUG toxicity in the brain.

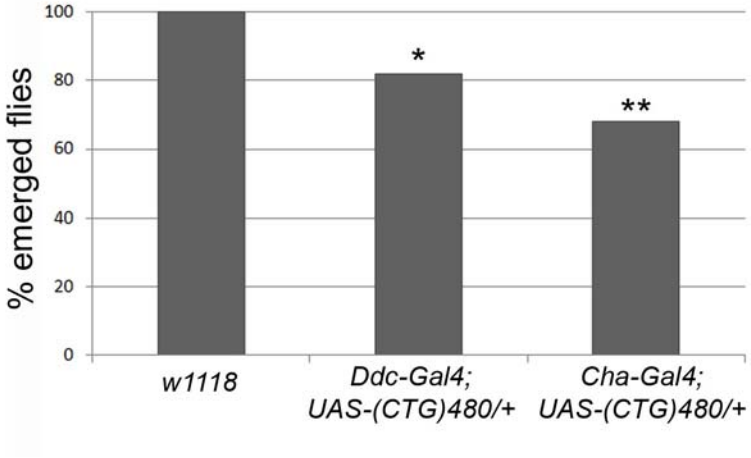

Figure 2. CTG repeats are toxic in different neuronal groups. Expression of (CTG)480 in dopaminergic (Ddc-Gal4; UAS-(CTG)480/+) and cholinergic (Cha-Gal4; UAS-(CTG)480/+) neurons reduces fly viability demonstrating that these cells are sensitive to CTGs repeats expression. (*) indicates significance ($p < 0.05$) or (**) highly significance ($p < 0.001$) in a two tailed Student's t-test.

The expression of CTG repeat expansions impairs visceral muscle function

Smooth muscle function is impaired in DM1 patients, with symptoms that affect visceral motility, such as diarrhea, constipation, and swallowing problems (Bellini *et al.,* 2006). However, none of the DM1 animal models available to date have been used to study the effects of CTG repeat expression in smooth muscles. In order to shed light onto the origin of these alterations, we expressed (CTG)480 in *Drosophila* visceral muscles using a *bag pipe* (*bap)-Gal4* line and performed an *in vivo* visceral muscle function assay to assess ingestion and excretion impairment.

In this assay, we fed L1 larvae for 40, 50, and 60 min with yeast containing a green commercial food dye, and then transfered them to a new plate with white-color yeast. The rate of ingestion was quantified as the percentage of larvae with green colorant in the first section of their midguts. Excretion rate was then analyzed by scoring the percentage of larvae that still contained green colorant in the gut after 70, 90, 180, 190, and 200 min in the new white-yeast food. The

ingestion experiment was repeated at least twice for each time point, whereas excretion studies were repeated four times for each time point.

In *bap-Gal4>UAS-(CTG)480* larvae, the ingestion rate was significantly faster compared with the control genotype (*UAS-(CTG)480/+*) (Figure 3A). At 40 min after exposure to the green food, 96% of the CUG expressing individuals were positive for ingestion compared to 90% in the control group. This difference was smaller at the later time points assayed (50 min and 60 min) due to a saturation effect.

The excretion rate was also increased in *bap-Gal4>UAS-(CTG)480* larvae compared to the control (Figure 3B). This difference was significant at 70 and 90 min after transferring green-positive larva to non-colored agar, although it was reduced for the later time points (180, 190, 200 min), for which a saturation behavior was again observed.

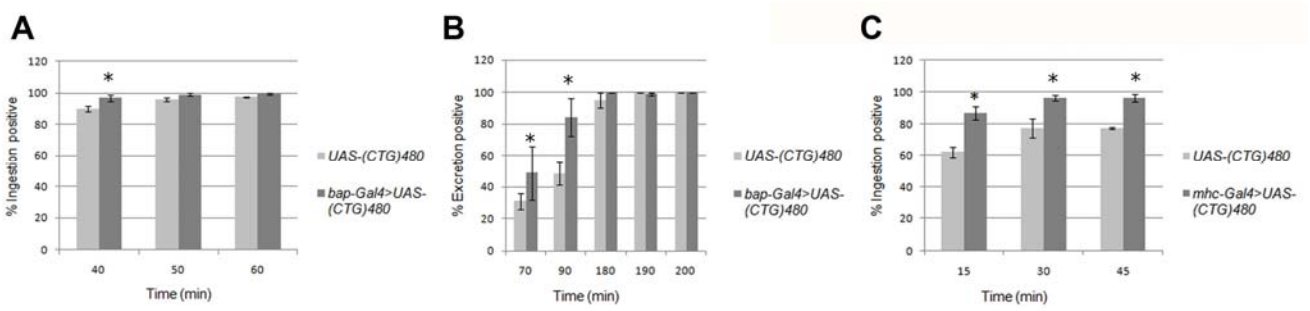

Figure 3. Visceral muscle function assays using *bap-Gal4* (A and B) and *mhc-Gal4* drivers (C). (A) Percentage of larvae positive for ingestion after 40, 50, and 60 minutes eating in the green dyed yeast plates. (B) Percentage of positive for excretion larvae 70, 90, 180, 190, and 200 min after the transference to not dyed yeast plates. (C) Percentage of positive for ingestion larvae after 15, 30, and 45 min in green dyed yeast plates (*) indicates significance ($p < 0.05$) in a Fisher exact test and the error bar is the standard deviation.

All together, these results indicate that the expression of expanded CTG repeats in *Drosophila* causes impairment of the visceral muscle function by increasing its activity.

To confirm this effect, we expressed (CTG)480 in the visceral muscle of L1 larvae using a different Gal4 line, *mhc-Gal4*. As higher differences between genotypes were observed at shorter time points, we assumed that ingestion assays at 40, or less, minutes might be suitable and easier to perform.

For the ingestion assay using *mhc-gal4*, L1 larvae were incubated 15 minutes in the green dyed yeast and assayed for ingestion. Those larvae negative for ingestion were transferred to new molasses agar plates covered by green dyed yeast and incubated for 15 min (this is, 30 min after the assay started). Then, the positive and negative for ingestion larvae were quantified and the negative were transferred for another 15 min to new green dyed yeast plates (45 min after the beginning) were they were scored again. Every experiment was repeated at least twice.

In this case, even higher differences in the ingestion rate were observed compared to the control at all the time points analyzed (15, 30, and 45 min) (Figure 3C). Worth noting, we previously reported that CTG repeat expression under the control of the *mhc* promoter induces skeletal muscle degeneration in the indirect flight muscles (IFMs). Therefore, it is possible that the ingestion impairment detected in these larvae was the synergistic result of visceral motility impairment and tracking defects. However, *mhc* driven expression of (CTG)480 to skeletal mucles does not cause

climbing or motor effects in adult flies (not shown), indicating that the ingestion phenotype described here could be solely caused by visceral muscle dysfunction.

From a methodological stand point, we found that the protocol used for ingestion assays with the *mhc-Gal4* gives higher differences between genotypes, was more robust (smaller errors), easier to perform, and less time consuming.

In conclusion, the identification and characterization of sensitized and easy-to-score phenotypes is crucial in basic research to study the mechanism of pathogenesis of a disease and to perform genetic and chemical screens when a wide range of mutations or compounds are tested. In the current study, we describe three additional phenotypes in DM1 model flies; imaginal eye disc size reduction, decreased viability, and ingestion rate impairment, resulting from CTG repeat expression in eye, dopaminergic and cholinergic neurons, and visceral muscle, respectively. These phenotypes might prove useful in subsequent studies aimed at testing molecular defects in the pathogenesis pathways of the DM1.

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Remating behavior in a few closely related species of *Drosophila nasuta* subgroup.

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Introduction

In insects, successful copulation does not ensure paternity as competition between sperm coming from different males to fertilize the ovum continues even within the female reproductive tract. Hence, males have evolved various mechanisms to alter female's physiology and behavior to ensure his paternity. In *Drosophila*, the females that are once mated can store sperm in her storage organs that are enough to fertilize the eggs laid in her life time. Nevertheless, she often remates even in the presence of stored sperm in her reproductive tract (Lefevre and Jonsson, 1962) creating a competitive milieu for sperm coming from different males. In this conflict between interests of males and females during reproduction, female remating acts as a key determinant of the pattern of sexual selection (Singh and Singh, 2004) leading to the genetic heterogeneity and divergence. The time