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Deleterious effects of neuronal accumulation of glycogen in flies and mice

Jordi Dura, María Florencia Tevy, Mar Garcia-Rocha, Joaquim Calbó, Marco Milán, Joan J. Guinovart

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 December 2011

Thank you for the submission of your manuscript "Deleterious effects of neuronal accumulation of glycogen in flies and mice" to EMBO Molecular Medicine and please accept my apologies for the delayed reply. We have now finally heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #2 highlights that possible mechanisms by which glycogen accumulation leads to neuronal death should be discussed and some insight provided. This reviewer also notes that quantitative assessments of glycogen granule formation and TUNEL staining should be provided.

On a more editorial note, the description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

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I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

Several studies have demonstrated that there is a causal relationship between the accumulation of neuronal glycogen and neurodegenerative diseases such as LD. In the current study, the authors provide strong genetic evidence that abnormal accumulation of glycogen in nervous system due to constitutive (and chronic) activation of GS [but not to accumulation of (inactive) enzyme] sufficiently causes neurodegenerative phenotype in both flies and mice in vivo. This study is significant in that it clearly links abnormal regulation of GS/glycogen metabolism and pathogenesis of certain neurodegenerative disease.

Specific comments:

1. Some key information/data is missing in the current manuscript, which needs to be addressed and included in the revised manuscript by the authors.
Although it is clear that aberrant glycogen accumulation is due to over-expression of constitutively active MGS-9A mutant, but not to enzyme-dead 9A(D) mutant, it is important to show that the magnitude of over-expression/activation of MGS achieved in the mouse model used. There is a Western blot (MGS expression in transgenics) data in the Supporting data section, however since there is no visible band (MGS) in control mouse lane and therefore over-expression of active mutant relative to endogenous GS is not clear. The authors should show GS activity data and address relative expression and activation between control and transgenic mice. Based on these data, the authors should discuss if the over-expression/hyper activation of GS achieved in this study mimics the pathogenic state of LD and/or other neurodegenerative diseases.

2. A recent study published by the authors group (Valles-Ortega et al. EMBO Mol Med, 2011) reported that in the LD mouse model (Malin KO mice) aberrant accumulation of glycogen was predominantly observed in insoluble fraction, but not in soluble fraction. It would be informative if the authors measured glycogen in these two fractions from their transgenic mouse tissues and address the importance of particular localization of glycogen for the induction of neuronal disease.

3. In Introduction, the authors describe that "MGS is regulated by phoshorylation in the N and C terminal domains of the enzyme.", however as they would definitely know MGS is also critically regulated by allosteric effectors such as glucose-6-phosphate (G6P) in vivo. A sentence describing the importance of allosteric regulation should be included in Introduction. MGS-9A is described as "non-inhibitable" mutant, but it is not mentioned if it is "activatable" by G6P at least in cell-free assays. This referee thinks that it is more appropriate to describe MGS-9A mutant as "constitutively active".

Referee #2 (Comments on Novelty/Model System):

The pathogenic mechanisms of Lafora disease and other related polyglucosan diseases are unknown. Based on a original finding made by the authors in 2007 (Vilchez ez al 2007), fly and mouse models of the disease have been generated. These models are entirely novel and will be useful to understand the mechanisms of the disease.

Referee #2 (Other Remarks):

This article is a follow up of an original observation made by the authors showing that mutations in two genes that encode molecules involved in glycogen synthase processing cause glycogen accumulation in neurons. They have generated two models that present some neurological abnormalities. While these models will be useful, there are a number of points that need to be addressed before the article can be considered for publication.

The main criticism is that the principal clinical observation of Lafora disease, namely myoclonus epilepsy, does not seem to have been reproduced. The authors should discuss this.
The second principal criticism is that the article does not provide any indication of the mechanism by which accumulation of glycogen in neurons leads to neuronal death. This may be the object of another study, but still the authors should discuss possibilities. In this context the authors should discuss the work of Moncada and Bolanos' laboratories which shows that neurons cannot upregulate glycolysis because they use glycolsyl units to produce NADPH via the pentose phosphate pathway. Inability to do so leads to oxidative stress. The authors should look at the possibility that oxidative stress indeed occurs in the affected neurons.
The authors indicate increased glycogen granules as revealed by EM in neurons and increased TUNEL-positve neurons. In both cases the authors should provide a quantitative assessment of these findings comparing pathological versus control animals.
It is unclear why the wt of MGS is also overexpressed and produces a (milder) phenotype.
Given the fact that locomotor impairments are seen, one wonders what is the status of glycogen in skeletal muscles?
The nestin promoter is also expressed in glia. What is the status of glycogen in astrocytes?
Minor : abstract last line : add "glycogen accumulation in neurons" .

Referee #3 (Comments on Novelty/Model System):

The medical condition of Lafora disease had a tremendous breakthrough several years ago showing that neurons from affected patients had altered activity of glycogen synthase and increased levels of glycogen. This was followed by the finding of mutations in Laforin, a negative regulator of GS in neurons. Although impactful, these findings left the field with a direct mechanism of disease onset. Was it really glycogen accumulation, or some other aspect of GS regulation that leads to disease onset.

This is a very nice body of work because it gets to the heart of the matter at hand. By directly manipulating gain of function and loss of function forms of GS, the authors have made two models of the disease that clearly show that glycogen accumulation is the CAUSE of the disease. Now the field can move forward and determine methods to rid neurons of this excess glycogen and the only way it can be done is by using the models outlined in this manuscript.

The description of the models and techniques used and reported is robust. The results are solid and fully support the conclusions drawn. I would recommend publication without further experimentation.

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The description of the models and techniques used and reported is robust. The results are solid and fully support the conclusions drawn. I would recommend publication without further experimentation.

The only technical question is whether the fly model with GS expressed in the dopanergic neurons has a short lifespan and any observable phenotype?

1st Revision - Authors' Response 12 March 2012

***** Reviewer's comments *****

Referee #1:

Several studies have demonstrated that there is a causal relationship between the accumulation of neuronal glycogen and neurodegenerative diseases such as LD. In the current study, the authors provide strong genetic evidence that abnormal accumulation of glycogen in nervous system due to constitutive (and chronic) activation of GS [but not to accumulation of (inactive) enzyme] sufficiently causes neurodegenerative phenotype in both flies and mice in vivo. This study is significant in that it clearly links abnormal regulation of GS/glycogen metabolism and pathogenesis of certain neurodegenerative disease.

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Following the reviewer's suggestions, we have:

- improved the WB analysis by using a different antibody with a wider dynamic range. This antibody allows the detection of both basal (control) and overexpressed (transgenic, *mMGS-9A*^{Nestin}) levels (new Supporting Information Figure 3B).
- added data from samples of *Epm2b* KO mouse brains (a model of Lafora disease) in order to compare the levels of MGS accumulation in each case (new Supporting Information Figure 3B).
- quantified total GS activity in control and in transgenic (*mMGS-9A*^{Nestin}) mice (new Supporting Information Figure 3C).
- quantified the GS activation state (–G6P/+G6P activity ratio) (new Supporting Information Figure 3C).
- added the description of the corresponding methods to the Supporting Information.
- commented on the results in the Results section (subheading "A form of mMGS resistant to inactivation leads to accumulation of glycogen in mouse neurons").
- further discussed the results in the Discussion section.

As a more direct answer to the reviewer, we wish to underline that the models presented in this work are of glycogen-induced neurodegeneration rather than specific models of Lafora disease. Thus, we generated fly and mouse models in which glycogen accumulation is highly accelerated in order to have a more direct way to test glycogen toxicity and avoid side-effects caused by interference with other pathways. These systems express high levels of an active MGS, way above the basal levels and even higher than the levels accumulated in Lafora disease models. In the Nestin-driven transgenic mouse, all cells in the brain express the active form of MGS, whereas this is not the case in the mouse model of Lafora disease: MGS becomes progressively accumulated in many but not all cells and with distinct degree of penetrance depending on the brain region and age (Vallès-Ortega et al, EMBO Mol Med 2011). This feature explains the difference in the levels of MGS seen in the two models. Moreover, the fly models provided the appropriate controls, in the sense that we observed the following: 1) a catalytically dead MGS (*hMGS-9A(D)*) does not affect the phenotype; 2) overexpression of the endogenous GS (*dGS*) is also harmless to flies (new Supporting Information Figure 2, see response to reviewer #2); and 3) WT *hMGS* and *hMGS-9A* give a dose-response relation between glycogen accumulation and impact on phenotype.

2. A recent study published by the authors group (Valles-Ortega et al. EMBO Mol Med, 2011) reported that in the LD mouse model (Malin KO mice) aberrant accumulation of glycogen was predominantly observed in insoluble fraction, but not in soluble fraction. It would be informative if the authors measured glycogen in these two fractions from their transgenic mouse tissues and address the importance of particular localization of glycogen for the induction of neuronal disease.

The reviewer is correct regarding this appreciation. We have measured glycogen in the brain of nestin-driven transgenic animals and also the distribution between the supernatant (soluble) and pellet (insoluble) fractions after a low speed centrifugation. Our results, presented in Supporting Information Figure 3D and commented in the Results section, show that indeed there is a considerable accumulation of insoluble glycogen, similar to that seen in *Epm2b* KO mice (Valles-Ortega et al., EMBO Mol Med, 2011). It is tempting to speculate that accumulation of glycogen as insoluble aggregates causes the phenotype in *mMGS-9A*^{Nestin} transgenics and *Epm2b* KO mice; but we consider that we do not have sufficient evidence to support this hypothesis, and the collection of conclusive data in this regard is experimentally challenging.

3. In Introduction, the authors describe that "MGS is regulated by phoshorylation in the N and C terminal domains of the enzyme.", however as they would definitely know MGS is also critically regulated by allosteric effectors such as glucose-6-phosphate (G6P) in vivo. A sentence describing the importance of allosteric regulation should be included in Introduction.

Again, the reviewer is right mentioning the need to stress the relevance of the allosteric regulation of GS by G6P. Thus we have included a sentence in the Introduction section, with reference to a recent report demonstrating the importance of G6P in the *in vivo* regulation of glycogen synthase activity:

"High levels of glucose-6-phosphate (G6P) allosterically activate MGS even when the enzyme is phosphorylated, this being the primary mechanism by which insulin promotes glycogen accumulation in the muscle (Bouskila et al, 2010)".

3.b MGS-9A is described as "non-inhibitable" mutant, but it is not mentioned if it is "activatable" by G6P at least in cell-free assays. This referee thinks that it is more appropriate to describe MGS-9A mutant as "constitutively active".

In this case, we consider that the differences between the term used in the manuscript and the one proposed by the reviewer are mainly semantic. We feel more comfortable with the original term used (note, it is "non-inactivatable" or "resistant to inactivation" and NOT "non-inhibitable" as stated by the reviewer). The reason being is that phosphorylation causes inactivation (not inhibition) and the mutant cannot be phosphorylated. One expression that falls closer to the unequivocal description of the mutant

would be just "non-phosphorylable" but this one implies other interpretational problems: other non-described residues may be phosphorylated with or without impact on activity; furthermore, it may be difficult for the non-familiarized broad readership to understand at a glance the functional consequence of a non-phosphorylable mutant. We leave this point to be decided by the Reviewer and the Editor in the last instance.

However, regarding whether these mutants can be further activated by G6P, our results presented in Supporting Information Figure 3C show that, indeed, the mMGS-9A mutant is activated by high concentrations (6.6 mM) of G6P, as deducted by the observation that the activity measured in the presence of G6P is higher than that measured in its absence. The –G6P/+G6P ratio (0.565) measurement is consistent with the one previously published for the same mutant (human homologue) transduced to COS-1 cells (0.57, Cid et al. FEBS J. 2005).

Referee #2 (Comments on Novelty/Model System):

The pathogenic mechanisms of Lafora disease and other related polyglucosan diseases are unknown. Based on a original finding made by the authors in 2007 (Vilchez ez al 2007), fly and mouse models of the disease have been generated. These models are entirely novel and will be useful to understand the mechanisms of the disease.

Referee #2 (Other Remarks):

This article is a follow up of an original observation made by the authors showing that mutations in two genes that encode molecules involved in glycogen synthase processing cause glycogen accumulation in neurons. They have generated two models that present some neurological abnormalities. While these models will be useful, there are a number of points that need to be addressed before the article can be considered for publication.

The main criticism is that the principal clinical observation of Lafora disease, namely myoclonus epilepsy, does not seem to have been reproduced. The authors should discuss this.

We agree that those proposed here are not specific models for the main clinical manifestation of Lafora disease (LD), and thus we avoided referring to the models as "LD models" thorough the manuscript. Instead, we present these systems as models of glycogen-induced neurodegeneration, which address an aspect of LD, but also of several other pathologies that course with neuronal glycogen accumulation. That said, the main manifestation of LD, namely progressive myoclonus epilepsy (EPM), pinpoints the cells or the functions that are more susceptible to malin/laforin deficiency. Many other cells, tissues and functions are altered in LD patients and animal models, but their clinical relevance is obscured by the prominent and visible impact of epilepsy on the lives of patients. With the progression of the disease, patients also suffer a rapid cognitive decline, resulting in dementia. In widespread models (nestin-driven in mice and elav-driven in flies), all neurons (and astrocytes in the former) are affected and the main manifestations are, not surprisingly, more severe than EPM. In fact, we realized that these widespread models were too aggressive and thus we cell-subtype specific systems (Pcp2-driven in mice and TH-driven in flies) in which the processes initiated by glycogen accumulation can be longitudinally studied.

We have thoroughly discussed this issue in the discussion section, as follows:

"The level of GS expression obtained in this model is substantial compared to the basal levels of GS expression in the brain, and even higher than the elevated levels observed in diseased Epm2b KO mice. The observation that each cell in the brain of the nestin-driven transgenic mice expresses the transgene, together with the high activity of the recombinant promoter, may account for such a dramatic increase in MGS and glycogen levels, and for the profound impact in the phenotype of the mice. Thus, we have generated a highly accelerated model of glycogen-induced neurodegeneration. This acceleration might also explain the fact that the main clinical observation of LD, namely myoclonus epilepsy, is not reproduced in these mice. Glycogen-induced neurodegeneration addresses a specific aspect not only of LD but also of several other pathologies that course with neuronal glycogen accumulation. Myoclonus

epilepsy pinpoints the cells or functions that are more susceptible to malin or laforin deficiency. Many other cells, tissues and functions are altered in LD patients and animal models, but their clinical relevance is obscured by the prominent and visible impact of epilepsy on the life of patients. It is interesting to note in this context that with the progression of the disease, LD patients also suffer a rapid cognitive decline, which results in dementia."

The second principal criticism is that the article does not provide any indication of the mechanism by which accumulation of glycogen in neurons leads to neuronal death. This may be the object of another study, but still the authors should discuss possibilities. In this context the authors should discuss the work of Moncada and Bolanos' laboratories, which shows that neurons cannot upregulate glycolysis because they use glycolsyl units to produce NADPH via the pentose phosphate pathway. Inability to do so leads to oxidative stress. The authors should look at the possibility that oxidative stress indeed occurs in the affected neurons.

Once more, the reviewer is right both regarding the fact that the mechanism by which glycogen induces apoptosis in neurons is not mentioned in the manuscript and the perception that this may be the object of another study. We are addressing this topic using *in vitro* culture of neurons and also using the fly model system (with the latter, preparing genome-wide RNAi screenings to identify genes that interfere with the neurodegenerative phenotype). More specifically, we are addressing the hypothesis that oxidative stress caused by interference of glycogen synthesis deregulation with the pentose-phosphate pathway is at the base of glycogen-induced apoptosis (again both *in vitro*, and in flies using an *in vivo* reporter of oxidative stress (Albrecht et al, Cell Met. 2011)); however, this work will be performed over the next 12-18 months and we consider that it falls beyond the scope of the present study. However, we coincide with the reviewer about the need to discuss the possible mechanisms involved in glycogen-induced apoptosis. Thus, we have introduced the following paragraph in the Discussion section:

"Energy shortage caused by excessive pulling of glucose into glycogen and/or impaired capacity to degrade glycogen in the case of demand, interference with the unfolded protein response or with autophagy, or damage to the architecture of complex cells such as neurons might contribute to glycogen-induced neuronal cell death. Interestingly, a recent report proposes that a shortage in glucosyl units required for the production of NADPH through the pentose phosphate pathway leads to oxidative stress and cell death in neurons (Herrero-Mendez et al. 2009). Further research is required to identify the relevant molecular mechanisms linking excessive or aberrant glycogen accumulation to neuronal cell death."

The authors indicate increased glycogen granules as revealed by EM in neurons and increased TUNEL-positve neurons. In both cases the authors should provide a quantitative assessment of these findings comparing pathological versus control animals.

We are convinced that EM is a technique that provides important qualitative data but the use of this approach for quantification is almost impossible and of controversial value. Thus, we use EM in this study and we report the EM data in this manuscript to support qualitative observations such as the presence or absence of glycogen aggregates in the neurons of flies. A similar case is PAS staining: it offers relevant qualitative data but offers little if any quantitative value. To make this point clear in the manuscript, we have added the statement *"Glycogen accumulation was monitored by two qualitative methods: Periodic acid-Schiff (PAS) staining and by electron microscopy (EM)"* in the Results section. In order to address this particular issue, we have quantified the amount of glycogen accumulated in the brains of *MGS-9A*^{Nestin} neurons (Supporting Information Figure 3D).

Since the concern may arise from doubts on how representative of a robust observation the images are, we stated in each case, in the corresponding figure legend, how many individuals per group were analyzed, taking into account that for each individual, several preparations were observed under the microscope. The images presented in the text are representative of the phenomena observed in each group.

With regards to TUNEL staining, this is indeed an easily quantifiable technique, and in fact we already offered quantitative data in the original submission of the manuscript (Figure 7B). However, these data were not specifically referred to in the narrative, which may have confused this reviewer. Thus, we have included a reference to the quantitative data in the Results section, and we have introduced a detailed description of the method used for quantification and the number of individuals and sections/fields per individual analyzed.

It is unclear why the wt of MGS is also overexpressed and produces a (milder) phenotype.

The expression of a WT MGS in cultured neurons results in complete inactivation of the enzyme by phosphorylation (Vilchez et al., Nat. Neurosci. 2007). Similarly, expression of *Drosophila* GS (dGS) in flies had no effect on phenotype (these data have now been added to the manuscript as Supplementary Information Figure 2). In order to force glycogen accumulation in the neurons of flies, we then generated the transgenic lines expressing the non-phosphorylable mutant of the human MGS (MGS-9A). In order to rule out inter-specific differences in stability, folding, activity and so forth, we considered that the proper controls for this experiment were the WT form of human MGS and a catalytically dead form of the non-phosphorylable enzyme. With these controls, we confirmed that all trangenes were expressed at similar levels (Supporting Information Figure 1). Surprisingly though, the WT MGS showed a phenotype on lifespan and locomotion, but this was milder than that observed for the MGS-9A mutant. Our interpretation is that the regulatory mechanisms controlling endogenous GS activity in flies fails to completely block the activity of a foreign enzyme like the human homolog (with only 59% overall protein sequence identity) and thus some glycogen is produced, thus causing the mild phenotype. By serendipity, we generated a dose-response system confirming glycogen (and not protein) accumulation as the cause of neurodegeneration. These notions are now further clarified with the introduction of the dGS data in the Results and Discussion sections.

In Results:

"So far, our data indicate that hMGS-9A and to a lesser extent hMGS-wt have the capacity to drive glycogen accumulation when expressed in Drosophila neurons. We next analyzed the impact of these transgenes on adult lifespan and locomotion."

...

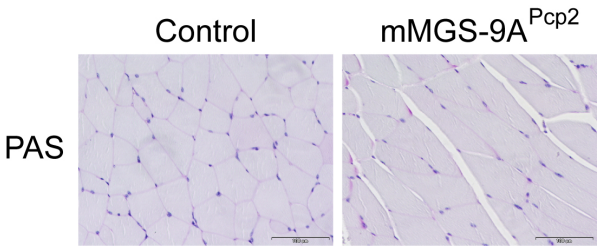
"In contrast, pan-neuronal overexpression of Drosophila GS (dGS), which has low sequence similarity to hMGS, was not able to induce glycogen accumulation or cause lifespan defects (mean survival=34.9, n=62, p=0.233, maximum survival = 50 days) when compared to control GFP-expressing flies (mean survival=36.1, n=75, maximum survival = 56 days, Supporting Information Fig 2). These data support the notion that hMGS-wt, but not dGS, escapes downregulation by phosphorylation in flies, probably because of sequence differences between the hMGS and dGS isoforms as well as differences in the regulatory machineries between Drosophila and mammalian neurons."

In Discussion:

"In Drosophila, the expression of human MGS-9A induced the accumulation of large glycogen deposits which correlated with a dramatic reduction in lifespan and clear locomotion defects. Interestingly, expression of a wild-type version of MGS led to mild glycogen accumulation and moderate changes in lifespan and locomotion, whereas expression of the Drosophila homolog of GS had no impact on the variables studied. Control flies expressing a catalytically dead form of MGS-9A did not show neuronal glycogen and had an almost normal lifespan. All together, these results support the idea that it is the glycogen dose, hence the protein activity and not its accumulation in adult neurons that leads to neurodegeneration."

Given the fact that locomotor impairments are seen; one wonders what is the status of glycogen in skeletal muscles?

The promoters used to drive the expression of the transgene for the mouse model showing locomotor impairment (*mMGS-9A*^{Pcp2}) is not considered to be active in the skeletal muscle, hence we have verified the possibility of organism- wide effects suggested by the referee by testing for the accumulation of glycogen aggregates in skeletal muscle of this mouse. No glycogen aggregates nor altered histological signs were detected in the skeletal muscle of these animals (see attached figure showing PAS staining of quadriceps muscle sections obtained from control and *mMGS-9A*^{Pcp2} mice as indicated). Therefore, the effects of locomotion in *mMGS-9A*^{Pcp2} mice can be solely attributed to altered CNS functioning, in this specific case, to Purkinje cell degeneration.



The nestin promoter is also expressed in glia. What is the status of glycogen in astrocytes?

Indeed, since the nestin-promoter is expressed in neuronal and glial progenitors, cre-mediated recombination takes place in these cells in Nestin-cre mice. Astrocytes in *MGS-9A*^{Nestin} mice are then expected to express the recombinant protein and accumulate glycogen. However, the damage caused to astrocytes is most likely negligible compared to that made to neurons. This is because astrocytes tolerate high levels of glycogen accumulation compared to neurons, at least *in vitro* (Vilchez et al, Nat. Neurosci. 2007), and in contrast to neurons, astrocytes can divide and repopulate.

As mentioned above, we have generated other models driving the expression of the transgene to more specific sets of cells, in particular to all neurons (in flies using the elav driver), dopaminergic neurons (in flies using the TH driver) or Purkinje neurons (in mice using the Pcp2 driver). All these models allowed us to dissect the impact of glycogen accumulation in neurons surrounded by normal, non-recombined astrocytes.

Minor : abstract last line : add "glycogen accumulation in neurons" .

We have added the sentence as indicated by the reviewer.

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The medical condition of Lafora disease had a tremendous breakthrough several years ago showing that neurons from affected patients had altered activity of glycogen synthase and increased levels of glycogen. This was followed by the finding of mutations in Laforin, a negative regulator of GS in neurons. Although impactful, these findings left the field with a direct mechanism of disease onset. Was it really glycogen accumulation, or some other aspect of GS regulation that leads to disease onset.

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move forward and determine methods to rid neurons of this excess glycogen and the only way it can be done is by using the models outlined in this manuscript.

The description of the models and techniques used and reported is robust. The results are solid and fully support the conclusions drawn. I would recommend publication without further experimentation.

The only technical question is whether the fly model with GS expressed in the dopaminergic neurons has a short lifespan and any observable phenotype?

Indeed, flies expressing MGS-9A (and to a lesser extent flies expressing MGS WT) driven by the TH-Gal4 (in dopaminergic neurons) show a shortened lifespan (already reported in the original submission, in Figure 3B) and a locomotion defect, as shown in the climbing assay (added to the current version of the manuscript as Figure 3D). These results are now adequately commented in the Results section, as follows:

"Interestingly, flies expressing hMGS-9A or hMGS-wt under the control of the elav-gal4 or TH-gal4 drivers showed locomotion defects when compared to hMGS-9A(D)-expressing flies, as monitored by measuring the mean speed in a climbing assay (Fig 3C, D)."

2nd Editorial Decision 19 March 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendment:

- In the Supporting Information file, please include a Table of Contents as the first page and remove the shading of the revised text.

In addition, we would need the following information:

- You included a figure in your point-by-point response. This would be included in the Review Process File. If you do NOT want the file to be published or would like to exclude figures, please immediately inform the editorial office via e-mail.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1:

The authors appropriately and thoroughly addressed all my concerns and in my opinion the manuscript is now acceptable for publication.