Title: A Genetic Manipulation of Motor Neuron Excitability Does Not Alter Locomotor Output in Drosophila Larvae

Author: Erin C. McKiernan

(Author comments marked in blue)

Notable changes made apart from reviewer suggestions:

- During revisions, I noticed that WT and EKI labels for one animal had been inadvertently flipped in one spreadsheet. The labels were corrected, analysis redone, Figure 3 replotted, and Table 1 revised. Figure 2B labels were also corrected. The results did not change, as activity recorded from WT and EKI channels in this prep was nearly identical.
- I made all data, code, and instructions for analyzing and plotting the data publicly available in a GitHub repository (github.com/emckiernan/eki-study) and archived the repository via figshare (DOI: 10.6084/m9.figshare.1437747). This information is provided in the manuscript.

Reviewer Comments (from submission to PeerJ)

Reviewer 1 (Andreas Prokop)

My thanks go to this reviewer for his detailed feedback and for signing his review.

Basic reporting
Insufficient validation of used tools (see below)

Experimental design
Good design, but insufficient use of complementary strategies (see below).

Validity of the findings
Lack of tool validation (see below).

Comments for the author
This paper is well written with impressive knowledge of the background of the topic. The experimental strategy and data presented are of high quality and support the statements the authors make. However, unfortunately, this work is not ready for publication.

First, the authors have not provided good controls which would convince me that the experimental manipulation used was functional.

It is important to point out that EKI is an established genetic manipulation. EKI has been shown previously to increase motor neuron excitability in both *Drosophila* larvae (Hartwig *et al.*, 2008) and adults (Duch *et al.*, 2008). The individual dominant-negative transgenes forming the EKI recombinant, eag-DN and Shaker-DN, have also been characterized and shown to increase neuronal excitability

(Broughton et al., 2004; Duch et al., 2008; Mosca *et al.*, 2005). Citing previous results, EKI (sometimes also referred to as the UAS-eag-DN, UAS-Sh-DN construct) has subsequently been used in several published studies to increase excitability without additional confirmation of its effects on neural activity (Chiang et al., 2009; Hindle et al., 2013; Singh et al., 2010; Timmerman *et al.*, 2013; Vonhoff et al., 2013). A review by Hodge et al., 2009 lists both eag-DN and Shaker-DN as established tools for increasing neural activity. For these reasons, I did not include data reconfirming the functionality of EKI. To clarify the established nature of EKI, I expanded on its description and added citations to the Introduction (lines 42-50) and Methods (lines 70-78) sections.

In the Discussion a list of possible further tests is given that could and should have been tested within the presented work.

Unfortunately, I am unable to perform these experiments at this time. However, I believe there is value in the study as it stands and outline some reasons below.

First, it is important to combat the 'file drawer problem' in science, in which studies with negative results or open questions do not get published. It is possible other *Drosophil*a labs are considering conducting the same experiments I report on in this article, and it is important they know that my results were negative and can read my discussion of why mosaic transgenics may not be the best choice for answering questions about larval locomotion (starting on line 294).

Second, I propose hypotheses and outline future experiments. I have put forward a clear work plan that can be adopted by other labs. This work plan is based on my knowledge of the subject area and my experience working with this preparation. One of my goals with this paper is to share insights and thereby speed up the experimentation process for other labs so they can move on to the next studies that will advance the field further.

Finally, there is almost exclusive mentioning of synaptic input to the MNs but little mentioning of synaptic output and potential compensation at the NMJ, although there is a huge body of work about homeostasis at the Drosophila NMJ. Certainly, this would not explain timing issues of MN activity, but the amplitude of responses and provide validation that the used tools are functional. Easy tests can be performed to image NMJs and their receptor clusters, and also miniature analyses could have been provided.

A new section on 'Compensation in motor systems' has been added to the Discussion (starting on line 241). Information on compensation at the *Drosophila* NMJ, along with relevant references, is included (lines 242-266). Excitability at the NMJ in larvae panneuronally expressing the Shaker dominant-negative (SDN) transgene – a component of the EKI recombinant – has been studied previously by others and suggests that the NMJ is not able to fully compensate for changes in neuronal excitability. This study (Mosca et al., 2005) is cited and discussed in this section. NMJ imaging and miniature analysis in EKI animals are now mentioned as future directions.

Second, the authors provide an impressive and knowledgeable list of published data in the Discussion, but most of them point out that their own findings are unusual and unexpected, even including experiments using the exact same genetic tools. Their own experimental data are therefore conflictive and require a more rigorous validation to make this a convincing manuscript.

In retrospect, I think the results are not that unexpected and coincide with the large body of literature on compensation in motor systems. I rewrote several sections in the Discussion to clarify this and cited studies on compensation in *Drosophila* and other motor systems (e.g. starting on line 241). A section on the usefulness of mosaic transgenics (starting on line 294) addresses why sensory feedback and muscle coupling in the larva may explain the motor pattern was unchanged in response to EKI expression in MNs. To the author's knowledge, no previous published study has examined the effects of EKI expression on locomotor behavior in larval *Drosophila*. Similar studies were performed in larvae expressing *slowpoke* RNAi (McKiernan, 2013), and adult flies expressing EKI (Duch et al., 2008). Both studies found motor behavior was altered by expressing genetic manipulations in MNs. However, I added comments to the Discussion (lines 355-366), with possible explanations for why their results differ from the current study, including the use of driver lines that target larger groups of MNs and the different organization of the adult motor system.

Many other tools for complementary approaches to manipulate Drosophila neurons are available and, in my view, restricting to one single and uncontrolled manipulation is insufficient. In conclusion, I do unfortunately not feel that this paper is ready for publication because it does not yet make a valid scientific statement.

I appreciate the reviewer's concern, but do not agree that the manipulation was “uncontrolled”. In each animal, muscles innervated by EKI-expressing and wildtype MNs were recorded and activity compared. Thus, each animal served as its own control. The valid scientific statement made by this study is that expressing a genetic manipulation know to affect MN excitability (EKI) does not affect rhythmic motor output as recorded from target muscles. The data presented support this statement.

Minor comments:
l. 74-6: EKI needs a more thorough explanation either in the introduction or results section.

I added a more detailed description of EKI and cited additional relevant references in the Introduction (lines 42-50) and Methods (line 70-78) sections.

l. 162ff.: The MN1-Ib nomenclature is precise, yet little used. Certainly, the statement in the Discussion that the old nomenclature describes embryonic predecessors of the larval MN neurons is misleading. To make this article easier accessible, please, provide the traditional names at least once to explain what neurons your are using. Similarly, for muscle 1, please, mention the Bate nomenclature which is more precise.

I added mention of the aCC/RP2 nomenclature throughout, with accompanying citations. However, I chose to stick with the MN1-Ib/MNISN-Is nomenclature because of its preciseness in describing the target and type of muscle innervation. To avoid confusion, I removed the statement about the embryonic predecessors of the larval MNs, but would like to note that several studies (e.g. Choi et al., 2004) do maintain that aCC/RP2 are the embryonic origins of the MN1-Ib/MNISN-Is neurons.

I also added mention of the Bate muscle nomenclature (dorsal acute muscle 1; DA1) and cited relevant work. However, for shorthand, I stuck with muscle 1 (M1), as done in other studies (e.g. Hoang & Chiba, 2001; Choi et al., 2004).

l. 168ff.: Authors should consider to take out statements about non-included larvae since they do not provide helfpul information and are even confusing (I am not clear about whether 2 of four or 2 of six larvae were discarded). Take out similar statements in l. 193f. and l. 207.

I think these statements do provide useful information to the reader, as they indicate how many larvae were excluded due to lack of rhythmic activity. This is important, since the manipulation could have increased the likelihood that animals were unable to produce rhythmic activity. However, I agree that the statements were unclear as written, so I revised them to be clear about the total number of recordings versus how many were not included in the analyses (e.g. see lines 184-187).

l. 168ff: Please, indicate that you measure postsynaptic responses in muscles and not the neurons themselves; please, state what technique was used (intracellular recordings? one/two electrode? Patch?)

In the Methods subsection 'Electrophysiology' (starting on line 96), it is indicated that recordings are intracellular and made using sharp electrodes. In the Methods subsection 'Experimental design' (starting on line 111), it is indicated that recordings are made from muscles not neurons. I explain why these recordings serve as a proxy for MN activity (lines 118-120). I also added mention that muscle recordings are a proxy for neural activity in the Discussion (lines 249-251).

l. 181-90: The authors should consider giving the information about the physiological differences between aCC and RP2 together with similar information at the beginning of the first results section.

As suggested, I moved the description of the physiological differences between the two MNs to the beginning of the Results section (lines 158-182).

Reviewer 2

My thanks to the reviewer for their feedback.

Basic reporting
No comment

Experimental design
No comment

Validity of the findings
In this manuscript by McKiernan, an FLP/FRT system is used to drive the expression of transgenes encoding dominant negative alleles of two Drosophila K channel subunits: ether-a-go-go and Shaker. The activity of muscles innervated by these motor neurons versus muscles that were innervated by unaffected motor neurons is then measured. Surprisingly, they found no difference in motor activity between affected muscles and controls. The authors then propose and discuss several scenarios that could account for the observed result.

Although this result was unexpected and therefore potential interesting there is no attempt to experimentally test at least some of their hypotheses.

In retrospect, the findings may not have been that unexpected, given knowledge of compensation in motor systems and the sensory feedback involved in producing coordinated peristaltic waves. I revised the Discussion section to clarify this and explained in more detail in multiple sections (e.g. starting on line 241 and again on line 279).

I also revised the Discussion section to explain why some of the suggested experiments are either highly technically challenging or not possible to perform at this time. For example, hypotheses about compensation within the motor network cannot be tested until the synaptic partners of the MNs are identified. These synaptic partners and the organization of the upstream network is unknown.

In addition, the authors may want to consider the possibility that although this genetic manipulation has been shown to alter neuronal excitability, may not be effective to see a difference in muscle activity in their experimental paradigms.

It is possible changes at the NMJ compensate for altered MN excitability, leading to normal motor output. I added mention of this possibility in the Discussion section (starting on line 249), and summarized results from previous studies on one of the EKI transgenes (Shaker dominant-negative; SDN) which indicate any such compensation is insufficient. Even so, I listed future studies that could be done to check for compensation at the NMJ in larvae expressing EKI.

MN expression of EKI may also result in changes in firing frequency that affect, for example, the force but not the overall timing of muscle contractions. I acknowledge that such an effect, if present, is below the threshold for detection with the current preparation due to problems with analyzing intraburst firing frequency. This information is included in the Discussion (lines 339-354) and Supplemental Fig. 2.

In my opinion, this paper in its actual version raises more problems than it can solve and therefore it does not represent a sufficient advancement of the field to warrant publication at this stage. The authors should consider the possibility to test some of their hypotheses and only then a revised version of the manuscript may be re-considered for publication.

I responded to a similar critique from reviewer Andreas Prokop. I've reproduced that answer below:

Unfortunately, I am unable to perform these experiments at this time. However, I believe there is value in the study as it stands without additional experiments and outline some reasons below.

First, it is important to combat the 'file drawer problem' in science, in which studies with negative results or open questions do not get published. It is possible other *Drosophil*a labs are considering conducting the same experiments I report on in this article, and it is important they know that my results were negative and can read my discussion of why mosaic transgenics may not be the best choice for answering questions about larval locomotion (starting on line 294).

Second, I propose hypotheses and outline future experiments. I have put forward a clear work plan that can be adopted by other labs. This work plan is based on my knowledge of the subject area and my experience working with this preparation. One of my goals with this paper is to share insights and thereby speed up the experimentation process for other labs so they can move on to the next studies that

will advance the field further.

Reviewer 3

My thanks to the reviewer for their feedback.

Basic reporting
Figures 6A and 6B do not correlate with the description.

This was a copy-paste error in the text and has been corrected.

Experimental design
In the present manuscript authors investigate a possible effects of altering MN intrinsic properties on the rhythmic motor behavior in Drosophila larvae.

I have some concerns about the experimental design of this work:
1. Mosaic expression of EKI in MN1-Ib and MNISN-Is in created mosaic animals should be demonstrated.

In all larvae, I confirmed expression of EKI by checking for co-labeling of GFP and RFP tags (attached to EKI and the promoter, respectively) in both MN cell bodies within the ventral ganglia and their axon terminals at the NMJ. Neighboring MNs in adjacent ventral ganglia segments and NMJ terminals did not show fluorescent labeling, thereby confirming mosaic expression. This is described in the Methods section (lines 79-87) and schematized in Fig.1. Unfortunately, my recording equipment did not have a camera setup that would have allowed me to capture images of the mosaic expression, and I am not able to carry out these experiments at this time. However, I should note that the laboratory of Matthias Landgraf (from whom we obtained the FRT/FLP driver) generated this line, characterized it, and confirmed it produces mosaic expression of downstream targets (Roy et al., 2007; Zwart et al., 2013).

2. Altered excitability of MN1-Ib and MNISN-Is in created mosaic animals should be demonstrated. For the best control, excitability of motor neurons and motor activity of muscle innervated by these neurons should be recorded from the same animal.

I responded to a similar critique from reviewer Andreas Prokop. I have reproduced that answer below and added additional points:

EKI is an established genetic manipulation. EKI has been shown previously to increase motor neuron excitability in both *Drosophila* larvae (Hartwig *et al.*, 2008) and adults (Duch *et al.*, 2008). The individual dominant-negative lines included in the EKI recombinant, eag-DN and Shaker-DN, have also been characterized and shown to increase neuronal excitability (Broughton et al., 2004; Duch et al., 2008; Mosca *et al.*, 2005). Citing previous results, EKI (sometimes also referred to as the UAS-eag-DN, UAS-Sh-DN line) has subsequently been used in several published studies to increase excitability without additional confirmation of its effects on neural activity (Chiang et al., 2009; Hindle et al., 2013; Singh et al., 2010; Timmerman *et al.*, 2013; Vonhoff et al., 2013). A review by Hodge et al., 2009 lists both eag-DN and Shaker-DN as established tools for increasing neural activity. For these reasons, I did not include data reconfirming the functionality of EKI. To clarify the established nature of EKI, I

expanded on its description and added the above references to the Introduction (lines 42-50) and Methods (lines 70-78) sections.

If I understand the reviewer's suggestion, the proposed experiment would require dual MN patch and dual intracellular muscle recordings (4 electrodes in total). The muscle recordings described herein are difficult to obtain themselves because the muscles move substantially during waves of contractions. I dealt with this by making long, flexible electrodes that move with the muscle during contractions. However, it was still often difficult to obtain two simultaneously stable muscle recordings. If we add to that challenges of obtaining two stable MN patch recordings in an active prep, things become more complicated. In addition, spontaneous rhythmic activity tends to run down fairly quickly; the majority of spontaneous activity is seen within 15 minutes of opening up the larva. It typically takes longer than 15 minutes to treat the nervous system with enzyme and clear MNs for patching, meaning that most spontaneous activity is gone by the time these recordings are established. In sum, although the suggested experiment would be great to perform, it would be very challenging and is beyond the scope of the current study.

3. It would be good to check crawling behavior of not dissected mosaic larvae to avoid the possible effects of larval preparation on the muscle activity.

Previous studies have analyzed crawling activity in wildtype intact larvae and shown that the motor pattern is very similar to that recorded in the dissected preparation, except faster (e.g. Heckscher et al., 2013). The dissected preparation is therefore considered a good model for studying crawling despite its semi-intact nature (see studies by Barclay & Atwood, 2002; Cattaert & Birman, 2001; Cooper & Neckameyer, 1999; Fox et al., 2006 and more).

4. Although authors found no effect of changing motor neurons excitability on spontaneous motor behavior, they have planned several experiments for possible explanation. Authors could direct more efforts toward identification of possible mechanisms in this study.

I outlined several future directions throughout the Discussion section, as well as highlighted the challenges inherent to some of the proposed experiments. I am unfortunately not able to perform these experiments at this time. However, I hope other researchers working in this field will be able to build on the techniques, results, and insights provided herein.

Validity of the findings

It would be good to have recordings from more than two animals in experiments described in Fig. 6.

The sample size for this group was admittedly low. The reason was the low probability of several necessary events occurring. The 'flip' events leading to expression of EKI were relatively rare to begin with at room temperature. It was even rarer to find animals in which both motor neurons innervating the same abdominal segment expressed EKI while none of the motor neurons innervating neighboring segments did. Increasing the temperature would have increased the probability of the 'flip' events occurring, increasing the likelihood of finding more MN1-Ib/MNISN-Is pairs expressing EKI. However, this would have unfortunately also increased the likelihood of 'flips' in neighboring segments. A brief explanation for the low sample size was added to the Results (lines 218-221).