# A Genetic Manipulation of Motor Neuron Excitability Does Not Alter Locomotor Output in *Drosophila* Larvae

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## Abstract

Are motor neurons just passive relayers of the signals they receive? Or, do motor neurons shape the signals before passing them on to the muscles, thereby influencing the timing of motor behavior? Few direct tests of the role of motor neuron intrinsic properties in shaping motor behavior have been carried out, and many questions remain about the role of specific ion channel genes in motor neuron function. In this study, two potassium channel transgenes were expressed in *Drosophila* larval motor neurons to increase their excitability. Mosaic animals were created in which some identified motor neurons expressed the transgenes while others did not. Motor output underlying crawling was compared in muscles innervated by control and experimental neurons in the same animals. No effect of the transgenic manipulation on motor output was seen. Possible explanations for these results are discussed, and future experiments are outlined that could shed light on how the larval nervous system produces normal motor output in the face of altered motor neuron excitability.

## Introduction

To produce essential motor behaviors like breathing and walking, muscles must contract in the same order, for roughly the same duration, each time a breath or step is taken. There is a pattern of activity, called a motor pattern, that must be reliably produced by the nervous system [1–6]. However, the system also must be flexible enough to respond to changing internal and external conditions [7, 8]. Open questions remain about how the nervous system controls rhythmic movements, permitting reliability and flexibility. What determines the timing of the motor pattern? Under what conditions can the timing be altered, and how?

Rhythmic motor behaviors are controlled by networks of neurons which communicate electrically and chemically [1–6]. Since motor neurons (MNs) represent the direct connection between neurons in those networks and the muscles, it is important to understand how MNs receive, integrate, and generate signals [9–11]. What happens as signals pass from the MNs to the muscles? Do MNs transmit a temporally similar pattern of activity to the one they received, or do they change the pattern? If the latter, to what extent do MNs contribute to shaping the final timing of motor behavior?

MNs express channels that allow ions such as $Ca^{2+}$, $K^{+}$, and $Na^{2+}$ to cross the membrane, producing currents that change electrical activity [12, 13]. Studies have demonstrated that persistent inward currents (PICs) carried by $Na^{2+}$ and $Ca^{2+}$ can



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shape MN responsiveness to synaptic input and firing output [14–17]. A recent study combining modeling and experimental approaches showed that MN currents, not necessarily just PICs, can shape the phasing of the motor pattern [18]. However, the common drawback of many of these studies is that the role of MN currents was not examined during ongoing, spontaneous motor behavior. Questions remain about the extent to which intrinsic properties of MNs contribute to the timing of rhythmic motor output and which ion channel genes are involved in MN responsiveness.

The aim of this study was to examine the effects of altering MN excitability on the timing of spontaneous rhythmic motor behavior. Two dominant-negative $K^+$ channel transgenes were expressed in *Drosophila* larval MNs using a recombinant construct known as 'Electrical Knock-In' (EKI) [19]. The transgene components of EKI reduce functional expression of $K^+$ channels encoded by the *eag* and *Shaker* genes, and have each been shown to increase neural excitability [20–22]. Expression of the recombinant EKI decreases both transient and sustained $K^+$ currents and increases the excitability of larval [19] and adult [21] MNs. In response to somatic current injection, larval MNs expressing EKI spike sooner, faster, and at lower amplitudes [19]. EKI has been used in other previous studies to increase neural activity [23–27].

EKI was expressed in two identified MNs, MN1-Ib and MNISN-Is [28, 29], which display different levels of excitability and contribute in distinct ways to rhythmic locomotor activity [30]. MN1-Ib, also known as aCC, innervates dorsal acute muscle 1 in the body wall [28, 29, 31] and appears to be the primary contributor to rhythmic motor activity recorded in that muscle [30]. MNISN-Is, also known as RP2, innervates dorsal muscles 1-4, 9, 10, and 18-20 [28, 29]. It activates later and spikes less during rhythmic activity than MN1-Ib [30], but may be important for coordinating muscles in the dorsal group and providing extra drive during the late phase of locomotor cycles. Mosaic animals were generated using the FLP/FRT system [32, 33] in which some MNs expressed EKI while others did not, permitting the comparison of control and experimental conditions in the same animal and the examination of the relative effects of changing excitability in MN1-Ib or MNISN-Is. The results show that expressing EKI in MN1-Ib, MNISN-Is, or both MNs had no effect on the pattern of rhythmic activity recorded from their target muscle. These results raise important questions about how networks maintain locomotor behaviors in the face of perturbations.

# Materials and Methods

## Fly lines and genetics

*Drosophila melanogaster* were reared at 25 °C under 12-hour light-dark cycles on standard yeast-sugar-cornmeal media. Wandering third-instar larvae were used for all experiments. To increase MN excitability, UAS-*ether-a-go-go* [20] and UAS-*Shaker* [22] dominant-negative (DN) transgenes were expressed using a recombinant construct (+;UAS-eagDN932,USDT-207/Cyo), also known as 'Electrical Knock-In' (EKI) [19]. Each of the DN transgenes have previously been shown to increase neural excitability [20–22]. Larval MNs expressing the recombinant EKI have reduced transient and sustained $K^+$ currents, fire action potentials at lower levels of current injection, show a decreased latency to first spike, and fire at higher frequencies than control neurons [19]. EKI expression in adult *Drosophila* converts MNs from single to repetitive spikers [21]. Other studies have also used EKI to increase neural activity [23–27].

EKI expression in MN1-Ib and MNISN-Is, also known as aCC and RP2 [28, 31], was driven by RN2-GAL4 [34] (w-;UAS::mRFP;RN2::FLP,Tub<FRT<GAL4,UAS::mRFP) [35]. A FLP/FRT recombinase cassette was included in the driver to create mosaic animals [32, 33] in which MN1-Ib and/or MNISN-Is neurons in some nervous system

hemisegments expressed EKI, while the same identified MNs in other hemisegments did not (Fig. 1). Thus, control and experimental cells could be compared in the same animal. MNs expressing EKI were identified by co-expression of a red fluorescent protein (RFP) tag attached to the promoter and a green fluorescent protein (GFP) tag attached to the EKI construct [22].

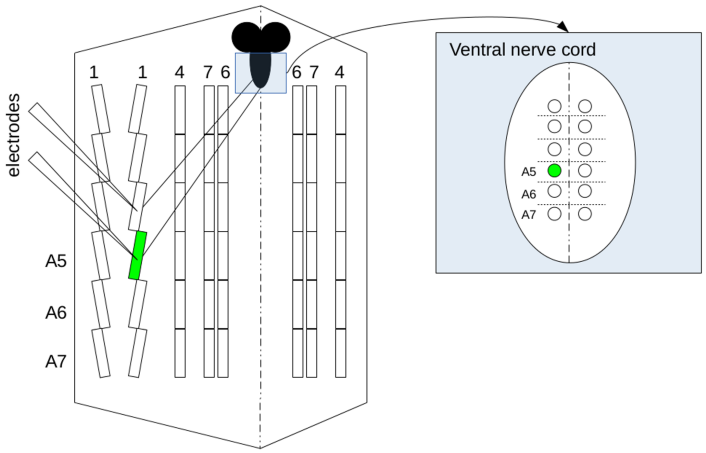


**Figure 1. Schematic of the larval preparation.** Larvae were prepared for recording using the off-midline dissection [36]. A posterior-to-anterior cut was made to the right of the midline (dashed line) near muscle 4. Larvae were pinned and cleaned to expose the muscles (rectangles) and central nervous system (solid black). Motor neurons are found in the ventral nerve cord (zoom at right) and were screened for GFP (green) to determine if they expressed EKI. Dual intracellular recordings were made from muscles innervated by EKI-expressing MNs (green) or wildtype MNs. Muscles 1, 2, 4, 6, 7 and abdominal segments A5–A7 are labeled. For clarity, not all muscles, segments, nerves, or neurons are shown.

## Larval preparation

Larvae were dissected using the off-midline preparation (Fig. 1), as described previously [36]. Briefly, a cut was made from the tail to the head near muscle 4, and larvae were opened and pinned out flat. Organs and trachea were removed to expose the muscles and nervous system. This preparation minimizes damage to dorsal-most muscles on the recording side. Larvae dissected in this way generate spontaneous crawling-related motor activity comparable to larvae dissected with the more common dorsal-midline preparation, although at a faster rate [36].

## Electrophysiology

Larval preparations were bathed and recorded in HL3.1 saline [37] containing (in mM): 70 NaCl, 5 KCl, 1.5 $CaCl_2$, 4 $MgCl_2$, 10 $NaHCO_3$, 5 Trehalose, 115 Sucrose, 5 HEPES, pH 7.1-7.3. All chemicals were obtained from Sigma (St. Louis, MO). Dual intracellular recordings were made at $21 - 23\,^{\circ}C$ from dorsal acute muscle 1 [31] in abdominal segments 2-6, as described previously [36]. Sharp electrodes were pulled from thin-walled borosilicate glass using a filament puller (Sutter Instrument Co., P-87 Flaming/Brown) to a 30-50 MΩ resistance. This produced a long, flexible electrode tip that moved with the muscle during contractions. Electrodes were filled with 3 M KCl for recording. Recordings were amplified using a Axoclamp 2B amplifier (Molecular



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Devices) in bridge mode and digitized at a sampling rate of 10 kHz by Digidata 1320A (Axon Instruments). Recordings were stored using PClamp 8.2 (Molecular Devices) and were imported into Spike2 (Cambridge Electronic Design) for processing. All recordings are publicly available via a GitHub repository (github.com/emckiernan/eki-study), which is archived via figshare (DOI: 10.6084/m9.figshare.1437747).

## Experimental design

Forward locomotion in *Drosophila* larvae is produced by peristaltic waves of muscle contractions that travel from the back to the front of the animal and can be recorded in the dissected preparation [36, 38–43]. The body of a *Drosophila* larva comprises multiple segments, and in each hemisegment is a repeated set of 30 muscles [29, 44]. This organization is mirrored across the midline. The central nervous system, where the MNs are located, has the same repeated and mirrored organization, with the same group of identified neurons found in each segment. Since the relationship between MN action potentials and muscle excitatory junctional potentials (EJPs) is one-to-one [28], muscle activity can be recorded as a proxy for MN activity. One of the muscles involved in locomotion is dorsal acute muscle 1, referred to herein simply as muscle 1 (M1), which is innervated by two MNs known as MN1-Ib and MNISN-Is [28, 29]. When M1s in neighboring abdominal segments are recorded, the activity is very similar, only with a short temporal delay. Thus, two samples of M1 activity and the MNs that innervate it, can be obtained from the same animal. Tests were conducted to establish that there is no gap-junctional coupling between M1s in adjacent segments (S1 Fig), as reported for ventral muscles [45]. Therefore, the activity recorded from each muscle should only arise from its innervating neurons. If one muscle receives signals from a MN expressing EKI and the other from a control MN, their activity can be compared to see how it differs due to the genetic manipulation.

## Data Analysis

Preparations were observed through an Olympus BX51WI microscope, and the occurrence of peristaltic waves recorded manually and/or marked with electronic timestamps to restrict analysis only to electrical activity underlying forward locomotion. For more details on inclusion criteria, see [36]. Burst start and end times were marked manually using cursors in Spike2 and exported to csv files. Analysis code was written in Python (version 2.7.6) to extract burst durations (time from the start to the end of a burst), cycle durations (time from the start of one burst to start of the next), duty cycles (burst duration divided by cycle duration), and quiescence intervals (time from the end of one burst to the start of the next) from the recordings. Although intraburst firing frequency was of interest due to the effects of EKI on MN firing [19], recordings showed it could not be effectively analyzed due to problems with event detection and separation of units coming from individual MNs (S2 Fig). All animals exhibited multiple bursts, though not necessarily the same number. As such, pooling bursts would constitute pseudoreplication and give more weight to some animals in the sample than others. To avoid this, the distributions of each measure for the control and experimental conditions were first calculated in single animals. Then, distributions from multiple animals were averaged to generate group distributions in which each animal was represented only once for each condition. The minimum, maximum, and quartile ($Q_1$, $Q_2$, $Q_3$) values for each measure are reported to give a complete description of the averaged group distributions. To statistically test for differences between control and experimental conditions, the Wilcoxon rank-sum test was used with an alpha of 0.05. The Python SciPy library was used for analysis [46]. Graphs were generated using Python Matplotlib [47]. Other figures were created using GIMP 2.8. Analysis code is



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publicly available via a GitHub repository (github.com/emckiernan/eki-study), which is archived via figshare (DOI: 10.6084/m9.figshare.1437747).

# Results

The target muscle recorded in these experiments was dorsal acute muscle 1 [31], referred to herein as M1. M1 is innervated by two MNs, MN1-Ib and MNISN-Is [28, 29], also referred to in the literature as aCC and RP2, respectively [28, 31]. The Hoang and Chiba [29] nomenclature is used herein due to its preciseness regarding the target and type of muscle innervation.

MN1-Ib innervates M1 with Type I glutamatergic terminals, forming big ('b' designation) synaptic boutons [28, 29]. Whole-cell patch clamp recordings during spontaneous locomotor activity have shown that MN1-Ib fires sooner and with a greater number of action potentials during a single burst than MNISN-Is [30]. Thus, MN1-Ib may be the primary contributor to locomotor activity recorded from M1.

MNISN-Is innervates muscles 1-4, 9, 10, and 18-20 via the intersegmental nerve (ISN) with Type I glutamatergic terminals ending in small ('s') boutons [28, 29]. Whole-cell patch clamp recordings have shown that MNISN-Is activates later and fires less than MN1-Ib during locomotor activity and may not be recruited during every cycle [30]. However, at least in ventral muscles, Type Is boutons are associated with larger excitatory junctional currents (EJCs) and potentials (EJPs) than Type Ib boutons, and have been likened to the "phasic" or "fast" motor axons found in crustaceans [48, 49]. The multiple innervation provided by Type Is MNs could also be important for coordinating the activity of muscle groups [28].

MN1-Ib and MNISN-Is thus show different electrophysiological properties and may serve different functions during crawling. To explore the effects of changing excitability in each MN, recordings were made from three groups of larvae in which: (1) EKI was expressed in MN1-Ib, (2) EKI was expressed in MNISN-Is, or (3) EKI was expressed in both MNs innervating the same segment. The results from each set of experiments are described in turn.

## EKI expression in MN1-Ib

Recordings were obtained from a total of six larvae in which EKI was expressed in MN1-Ib in one hemisegment while adjacent hemisegments were innervated by wildtype (WT) MNs. Recordings were analyzed from four larvae, while two larvae (33%) were excluded due to a lack of rhythmic activity. Representative recordings are shown in Fig. 2. Histograms comparing burst duration, cycle duration, duty cycle, and quiescence interval for WT versus EKI segments are shown in Fig. 3. Minimum, maximum, and quartile values are compared in Table 1. Although in some larvae measures were significantly different between WT and EKI recordings, no consistent effect was observed. For example, in one animal, quiescence interval was shorter in the WT than the EKI segment, while in another animal, the opposite was seen. In two other larvae, the recorded muscles showed no difference in quiescence interval. This suggests any differences were due to variability in measures across segments, rather than induced by EKI. Such variability has also been observed in fully WT animals [50]. Comparing the averaged group distributions revealed no significant differences between WT and EKI recordings on any measure ($p>0.05$).

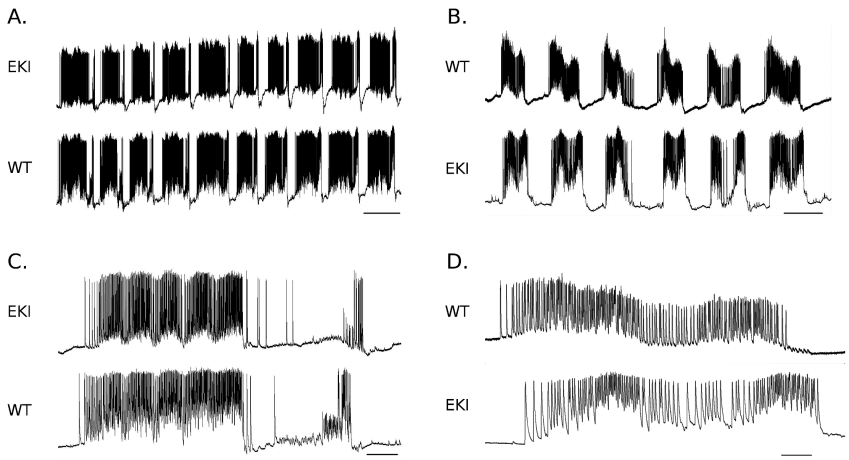


**Figure 2. Simultaneous intracellular recordings from muscles innervated by WT MNs or MN1-Ib expressing EKI.** A: Recording from one larva. M1 in A3 was innervated by MN1-Ib expressing EKI (top trace), while M1 in A4 was innervated by WT MNs (bottom trace). Scale bar is 20s. B: Recording from second larva. M1 in A4 was innervated by MN1-Ib expressing EKI (bottom), while M1 in A5 was innervated by WT MNs (top). Scale bar is 6s. C,D: Single bursts from recordings in A and B, respectively. Scale bar is 2s in C and 0.5s in D.

## EKI expression in MNISN-Is

Recordings were obtained from a total of fourteen larvae in which EKI was expressed in MNISN-Is in one hemisegment while adjacent hemisegments were innervated by WT MNs. Recordings were analyzed from seven larvae, while recordings from seven other larvae (50%) were excluded due to a lack of rhythmic activity or electrode instability in one of the channels. Representative recordings are shown in Fig. 4. Histograms comparing burst duration, cycle duration, duty cycle, and quiescence interval for WT versus EKI segments are shown in Fig. 5. Minimum, maximum, and quartile values are compared in Table 2. As with MN1-Ib, in some larvae, select measures were significantly different between WT and EKI recordings. However, no consistent effect was observed, again suggesting these differences were due to inherent variability across segments. Comparing the averaged group distributions revealed no significant differences between WT and EKI recordings on any measure ($p>0.05$).

## EKI expression in MN1-Ib and MINISN-Is

Since M1 is innervated by both MN1-Ib and MNISN-Is [28, 29], it is possible the effect of EKI expression in one MN could be compensated for by the other. To test this, recordings were obtained from three larvae in which both MN1-Ib and MNISN-Is innervating a given segment expressed EKI, while MNs in adjacent segments were WT. Recordings from two of these larvae were analyzed, while the third (33%) was excluded due to a lack of rhythmic activity. The size of this sample is small due to the low probability of several necessary events occurring - it was extremely rare to find larvae in which both MN1-Ib and MSISN-Is innervating the same segment expressed EKI without any expression in the MNs innervating neighboring segments. Recordings from both larvae are shown in Fig. 6. Histograms comparing burst duration, cycle duration, duty cycle, and quiescence interval for WT versus EKI segments are shown in Fig. 7. Minimum, maximum, and quartile values are compared in Table 3. Neither of the two



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larvae showed a significant difference in burst duration or cycle duration between muscles innervated by WT or EKI MNs. In one larva, quiescence intervals were longer and duty cycles were smaller in muscles innervated by EKI versus WT MNs ($p<0.05$). However, no differences in either duty cycle or quiescence interval were seen between muscles recorded in the second larva. Comparing the averaged group distributions revealed no significant differences between WT and EKI recordings on any measure ($p>0.05$).

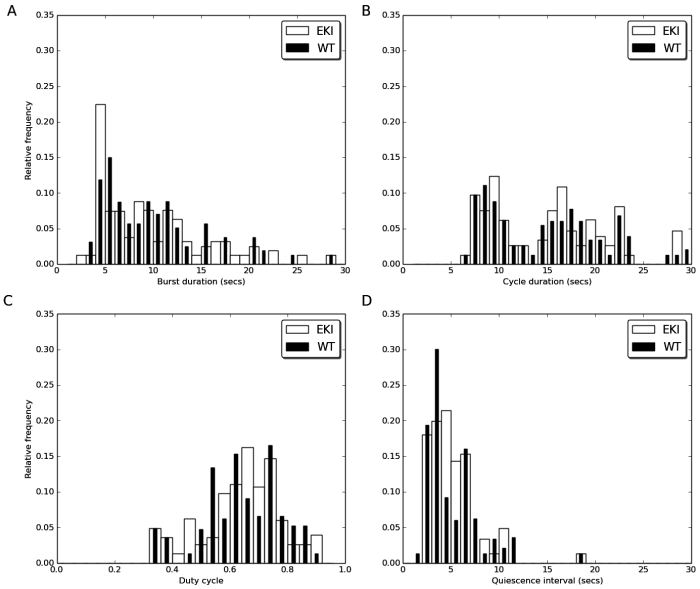


**Figure 3. Quantification of motor activity.** Histograms of burst durations (A), cycle durations (B), duty cycles (C), and quiescence intervals (D) as calculated from recordings of muscles innervated by WT MNs (black) and MN1-Ib expressing EKI (white). N=4. Bins: A,B,C = 1s; D = 0.04.

# Discussion

This study used mosaic transgenic animals to study the role of MN excitability in shaping the timing of rhythmic motor behavior. The most obvious advantage of this approach is that animals act as their own controls, reducing the confounding effects of extraneous variables and permitting smaller sample sizes. Furthermore, the model system (*Drosophila* larva) allowed for examining the effects of changing MN excitability on spontaneous, ongoing behavior. Although previous studies have shown that EKI expression alters the firing properties of MNs [19, 21], no difference was seen in the patterned activity of muscles innervated by EKI-expressing MNs relative to controls.

## Compensation in motor systems

In retrospect, the results are not necessarily surprising, given that motor systems are known to compensate for differences in excitability to preserve function [51–54]. At the *Drosophila* neuromuscular junction (NMJ), changes in ion channel expression lead to homeostatic upregulation of other channels to control excitability. For example, larvae expressing mutations of the $K^+$ channel genes *Shal* or *slowpoke* show increased *Shaker* expression that helps regulate EJP or EPSP amplitudes [55, 56]. A variety of other homeostatic mechanisms regulate excitability at the *Drosophila* NMJ [57].



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**Table 1. Bursting measures in M1s innervated by WT MNs or MN1-Ib expressing EKI**

| | WT | | | | |
|---|---|---|---|---|---|
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 3.90 | 5.87 | 8.93 | 12.01 | 28.52 |
| cycle duration | 6.99 | 9.00 | 15.04 | 18.85 | 31.34 |
| duty cycle | 0.34 | 0.55 | 0.65 | 0.73 | 0.91 |
| quiescence interval | 1.73 | 3.13 | 3.99 | 6.51 | 18.40 |
| | **EKI in MN1-Ib** | | | | |
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 2.78 | 4.98 | 8.66 | 12.64 | 28.81 |
| cycle duration | 6.89 | 9.24 | 15.55 | 19.51 | 31.72 |
| duty cycle | 0.33 | 0.57 | 0.66 | 0.73 | 0.91 |
| quiescence interval | 2.26 | 3.16 | 4.59 | 6.06 | 18.82 |

Sample size was 4 larvae. Units for burst duration, cycle duration, and quiescence interval are in seconds. Duty cycle is calculated by dividing burst duration by cycle duration, and is thus unitless on a scale of 0-1. Abbreviations: min=minimum; max=maximum; $Q_1$, $Q_2$, and $Q_3$ represent 1st, 2nd (median), and 3rd quartiles, respectively.

Since recordings in this study were made from muscles as a proxy for neural activity, it is possible compensation at the NMJ played a role in maintaining normal motor function despite EKI expression in MNs. To the author's knowledge, analysis of miniature EPSPs (mEPSPs) to test for changes in quantal content, or immunostainings of the NMJ to test for changes in postsynaptic glutamate receptor expression, have not been performed in larvae expressing EKI. However, excitability at the NMJ in larvae expressing the *Shaker* dominant-negative (SDN) transgene, a component of EKI, has been studied [22]. mEPSP frequency is higher in larvae panneuronally expressing SDN, relative to controls. Evoked EPSPs are similar in amplitude but longer in duration in SDN versus control larvae. Therefore, changes in excitability produced by neuronal expression of SDN are not fully compensated at the NMJ. Interestingly, despite these changes in excitability, intact larvae with panneuronal expression of SDN show normal crawling speeds [22]. The author is not aware of similar experiments in animals expressing only the *eag* dominant-negative (eagDN) transgene. Synapse number at the NMJ increases when both SDN and eagDN are expressed in larval MNs [58], suggesting a hyperexcitable phenotype as observed in *eag*/*Shaker* double mutants [59]. However, the strength of individual NMJ synapses has not yet been reported in animals expressing EKI.

The presence of the eagDN transgene in the EKI recombinant could be important for compensation. Eag channels can activate $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) [60], a kinase shown to regulate neuronal excitability and motor behavior in *Drosophila* [61,62], as well as other animals [63,64]. Eag channels can regulate intracellular signaling pathways that induce cell proliferation - an effect not observed in response to Shaker channel transfection [65]. Expression of an activity-dependent spliced form of the eag protein alters the structure of cultured cells [66]. In mice, *eag*-related gene (ERG) expression is regulated in response to changes in neural activity [67]. These studies suggest *eag* could act as a 'sensor of excitability' [68], inducing compensation in EKI-expressing larvae through a variety of possible downstream mechanisms. Recording from mosaic larvae expressing eagDN or SDN separately could help tease apart the role(s) of each gene.



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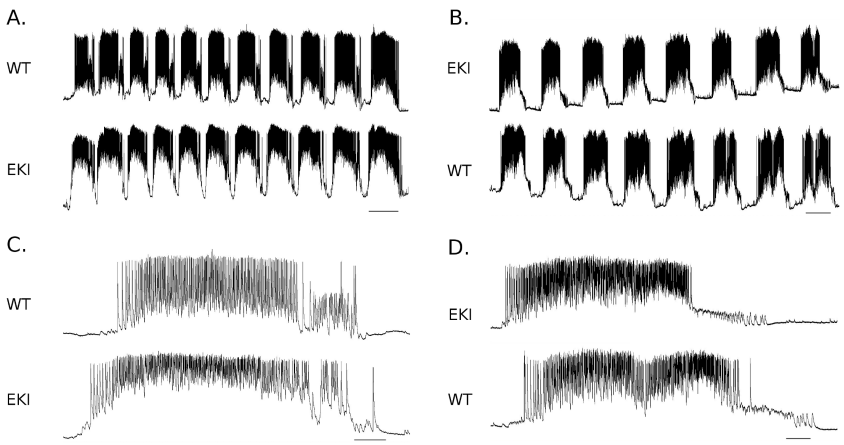


**Figure 4. Simultaneous intracellular recordings from muscles innervated by WT MNs or MNISN-Is expressing EKI.** A: Recording from one larva. M1 in segment A5 was innervated by MNISN-Is expressing EKI (bottom trace), while M1 in A4 was innervated by WT MNs (top trace). Scale bar is 10s. B: Recording from second larva. M1 in A6 (top) was innervated by MNISN-Is expressing EKI, while M1 in A5 was innervated by WT MNs (bottom). Scale bar is 10s. C,D: Single bursts from recordings in A and B, respectively. Scale bar is 1s in both.

## Possible role of sensory feedback in compensation

Sensory feedback is important for regulating the timing of motor behaviors [1, 2, 69–73]. In *Drosophila*, input from multi-dendritic (MD) sensory neurons found in the larval body wall supports wave progression from one segment to the next [42, 74, 75]. MD neurons appear to project into the same area of the neuropile where MN dendrites are located, indicating that MNs may receive direct sensory input [76]. If so, increased MN firing induced by EKI expression, and thus changes in target muscle contractions, could be detected by MD cells and relayed to MNs. Since in this study EKI was expressed throughout development, it is possible sensory feedback during embryonic stages, when peristaltic contractions first begin [77], allows the system to recalibrate and produce normal motor output. Alternatively, it may be sufficient to have cycle-by-cycle sensory feedback during the larval stages to compensate for altered MN excitability. Acute expression of EKI during the third larval instar, for example using temperature-sensitive GAL80 in concert with the UAS/GAL4 system [78], would help determine whether expression during earlier developmental stages is necessary for compensation to occur.

## What can mosaic transgenics tell us about motor behavior?

Is using mosaics to study locomotion in larval *Drosophila* the best approach? The original idea was to compare control and experimental muscle activity in the same animal to determine the effects of manipulating MN excitability. However, since this locomotor activity consists of peristaltic waves that progress from one segment to the next, it is questionable that normal activity could be seen in one segment and altered activity in an adjacent one. Sensory feedback contributes to termination of bursting in one segment and initiation of bursting in the next [42, 75]. If activity in a posterior segment is altered due to innervation by a manipulated MN, activity in the next segment, although innervated by control MNs, could in turn be altered. Even if the control muscle is posterior to the manipulated muscle, activity could still be altered as the wave slows or fails to complete and initiate a new cycle. Alternatively, sensory



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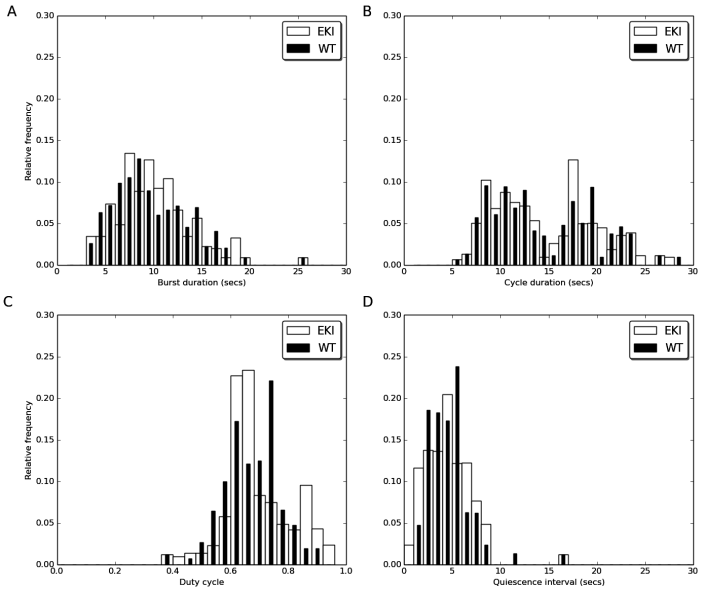


**Figure 5. Quantification of motor activity.** Histograms of burst durations (A), cycle durations (B), duty cycles (C), and quiescence intervals (D) as calculated from recordings of muscles innervated by WT MNs (black) and MNISN-Is expressing EKI (white). N = 7. Bins: A,B,C = 1s; D = 0.04.

feedback from surrounding muscles could help maintain the proper bursting pattern in the manipulated muscle. In other words, either activity will be altered in multiple segments, or maintained in all.

Related to this, including only rhythmically active animals in the analysis constitutes a form of selection bias. If the dominant-negative transgenes express with variable strength across animals, it is possible larvae with stronger expression would not show rhythmic activity, leading to a different conclusion regarding the effect of EKI. For this reason, it was important to report the percentage of recordings that were excluded from each sample, which varied from 33-50%. Since some rhythmically active animals were excluded due to electrode instability, the percentage of 'non-crawlers' tended toward the low end of the range for all samples. However, to determine whether EKI expression strength is important, quantification of $K^+$ current reduction could be compared in 'crawlers' and 'non-crawlers'. These experiments may be challenging, as they would require either patching on to cells for a full pharmacological analysis after motor activity was recorded, or performing single-cell RT-PCR on animals after recording.

Mosaics could be used to study MN excitability in isolated CNS preparations in which sensory input and muscle coupling would not play a role. However, the motor pattern is irregular in such preparations [41], which would make evaluating the effects of manipulations difficult. Furthermore, a deafferented preparation would defeat attempts to link MN excitability and gene expression to ongoing motor behavior.

## Importance of MN intrinsic properties

Although this study did not find an effect of changing MN excitability on motor output, MN intrinsic properties are likely still important for motor control. Studies in invertebrates and vertebrates have shown that MNs display specific voltage-gated currents that influence responsiveness to input and firing output [15–18, 79–86]. Ion channel expression in *Drosophila* larvae and adults is important for producing different



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**Table 2. Bursting measures in M1s innervated by WT MNs or MNISN-Is expressing EKI**

| | WT | | | | |
|---|---|---|---|---|---|
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 3.55 | 6.91 | 9.10 | 12.77 | 25.47 |
| cycle duration | 6.42 | 10.48 | 13.35 | 18.99 | 28.00 |
| duty cycle | 0.36 | 0.60 | 0.68 | 0.74 | 0.91 |
| quiescence interval | 1.34 | 3.01 | 4.46 | 5.56 | 16.95 |
| | **EKI in MNISN-Is** | | | | |
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 3.68 | 7.46 | 9.49 | 12.12 | 25.47 |
| cycle duration | 6.18 | 10.44 | 13.44 | 18.83 | 27.63 |
| duty cycle | 0.37 | 0.62 | 0.66 | 0.77 | 0.96 |
| quiescence interval | 0.40 | 2.69 | 4.30 | 6.09 | 16.40 |

Sample size was 7 larvae. Units and abbreviations are the same as in Table 1

types of excitability and response properties in MNs [21,36,68,87–91,94]. Recordings from larval MN1-Ib (aCC) neurons in abdominal or thoracic segments of the ventral ganglion show differences in the size of transient and sustained $K^+$ currents, leading to different delays to first spike and firing frequencies [91]. Decreasing expression of *eag*, which contributes to transient and sustained $K^+$ currents, causes hyperexcitable responses to current injection and increases in EPSP amplitude and frequency during rhythmic oscillations recorded from MN1-Ib [68].

It is possible some effects of changes in MN excitability were not revealed in this study because of limitations inherent to the preparation. For example, the force of muscle contraction could be altered by an increase in MN firing frequency [92,93], as is observed in EKI-expressing MNs under current injection [19]. However, such an effect, if present, was below the threshold for detection due to issues with analyzing intraburst firing frequency in muscle recordings. EPSPs recorded from M1 arise from two MNs, MN1-Ib and MNSIN-Is. These MNs are recruited at different times, fire at different frequencies, and the latter may not spike during every locomotor cycle [30]. Dual recordings from M1 and M2, innervated by one common (MNISN-Is) and one non-common (MN1-Ib or MN2-Ib, respectively) MN [29], showed that unit separation during the majority of a motor burst was not possible (S2 Fig). Thus, a rigorous analysis of the firing frequency of control and transgenic MNs could not be extracted from muscle recordings. Such analysis would require patching on to individual MNs during motor behavior. However, such recordings are difficult to obtain, since spontaneous motor activity usually runs down before the time required to enzyme and access MNs for patch recordings (McKiernan, unpublished observations).

Some studies in behaving *Drosophila* have shown that altering MN channel expression can shape motor activity. Manipulating expression of the $Ca^{2+}$-dependent $K^+$ channel gene *slowpoke* in larval MNs increases the frequency of fictive locomotion [36]. In adult *Drosophila*, EKI expression in MNs increases the probability of induced flight behavior in response to a wind stimulus and the duration of flight activity [21]. The type of motor behavior and muscle coupling is different between adults and larvae, and may explain why locomotor effects of EKI expression in MNs were seen by Duch et al. (2008) [21] but not in the current study. In addition, both Duch et al., (2008) and McKiernan (2013) [36] expressed genetic manipulations in groups of MNs, rather than the select few targeted in this study. It may be that the motor system is able to compensate if the number of MNs with altered activity is low and not affecting all



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segments, but loses this ability if the number of affected MNs exceeds a 'critical mass'.

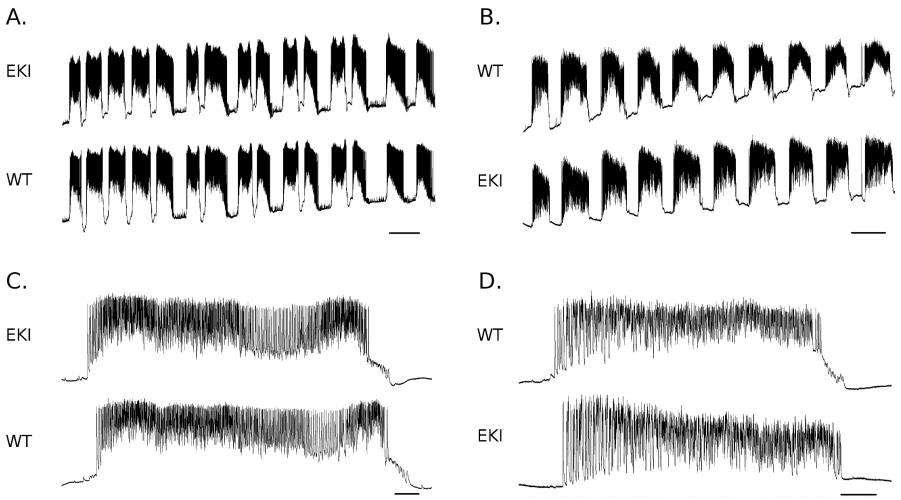


**Figure 6. Simultaneous intracellular recordings from muscles innervated by WT MNs or both MN1-Ib and MNISN-Is expressing EKI.** A: Recording from one larva. M1 in segment A5 was innervated by MN1-Ib and MNISN-Is expressing EKI (top trace), while M1 in A4 was innervated by WT MNs (bottom trace). Scale bar is 20s. B: Recording from second larva. M1 in A5 (bottom) was innervated by MN1-Ib and MNISN-Is expressing EKI, while M1 in A6 was innervated by WT MNs (top). Scale bar is 10s. C, D: Single bursts from recordings in A and B, respectively. Scale bar is 1s in both.

## MN stimulation protocols

Finally, the protocols used to stimulate genetically manipulated MNs should be considered as one possible explanation for unchanged motor activity in EKI animals. Although larval MNs expressing EKI show altered responses to square-pulse current injections, their responses to synaptic input may not be different from controls. MNs expressing EKI could be stimulated with ramps instead of square-pulse currents. The non-instantaneous change in membrane potential initiated by ramp stimulation allows for gradual activation and inactivation of ion currents and can reveal distinct neuron response properties [95–99]. Ramp currents, however, still do not adequately mimic synaptic input. Instead, MNs could be stimulated with different types of stochastic current inputs to determine their responsiveness [100–102]. An even better but more difficult approach is to record genetically manipulated MNs as they receive endogenous motor-related input, as done recently by others in WT MNs [30]. As mentioned previously, though, these recordings are challenging due to run-down of rhythmic activity.

If the response to synaptic input is not altered in EKI neurons, this would explain why motor output was unchanged and emphasize the need for realistic stimulation protocols in evaluating genetic manipulations. In this case, it would be interesting to investigate which ionic currents compensate for the change in $K^+$ channel expression. Ion channel homeostasis has previously been reported in *Drosophila* [55, 56, 103] and other animals [52, 53, 104]. If, however, the response of EKI-expressing MNs to synaptic input is altered, this suggests that the network is compensating for changes in MN excitability such that normal motor output is maintained, as seen in other systems [105]. The aCC and RP2 MNs adjust their excitability in response to changes in synaptic



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input [106]. It is possible the reverse happens, i.e. neurons in the motor network may adjust their output in response to changes in MN excitability. However, the upstream synaptic partners of larval MNs will need to be identified before this could be tested directly. Although candidate interneurons involved in *Drosophila* locomotion have been identified [107, 108], their connections with one another and with MNs have not yet been established.

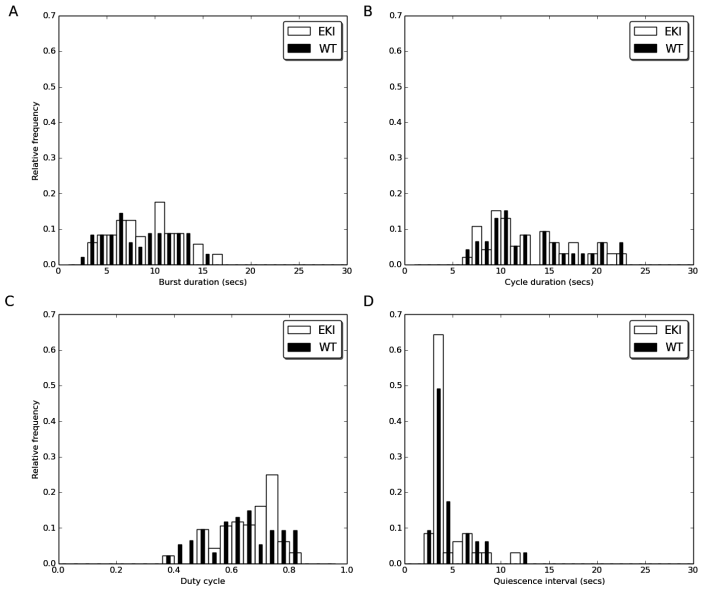


**Figure 7. Quantification of motor activity.** Histograms of burst durations (A), cycle durations (B), duty cycles (C), and quiescence intervals (D) as calculated from recordings of muscles innervated by WT MNs (black) or MN1-Ib and MNISN-Is expressing EKI (white). N=2. Bins: A,B,C = 1s; D = 0.04.

## Mathematical modeling

A mathematical modeling approach might help answer lingering questions from this study. Model MNs could be constructed in which $K^+$ channel expression is altered [109] to mimic the effects of EKI. The output of these model neurons could then be characterized under different patterns of rhythmic synaptic input or network connectivity. Considering the hypotheses outlined here, the most interesting questions have to do with the role of sensory feedback or upstream compensation. These are questions that depend highly on network organization. As long as this information is not available, modeling approaches will be limited in the answers they can provide. Future efforts should focus on determining the *Drosophila* motor network organization to strengthen the usefulness of the larval model.

# Conclusions

There is still a lot we do not understand about the interactions between MNs and other cells in the network during the generation of rhythmic motor behaviors. This study used a characterized transgenic manipulation to increase MN excitability. However, this manipulation failed to produce changes in motor output. This work raises several important questions regarding reliability and flexibility in motor networks. A number of future experiments have been outlined that could increase our understanding of the role



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**Table 3. Bursting measures in M1s innervated by WT MNs or MN1-Ib and MNISN-Is expressing EKI**

| | WT | | | | |
|---|---|---|---|---|---|
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 2.80 | 5.51 | 8.00 | 11.29 | 15.02 |
| cycle duration | 6.34 | 9.36 | 11.17 | 15.90 | 22.99 |
| duty cycle | 0.39 | 0.56 | 0.64 | 0.75 | 0.82 |
| quiescence interval | 2.49 | 3.32 | 3.89 | 4.46 | 12.90 |
| | **EKI in MN1-Ib and MNISN-Is** | | | | |
| **measure** | **min** | **$Q_1$** | **$Q_2$** | **$Q_3$** | **max** |
| burst duration | 3.28 | 6.00 | 8.26 | 11.25 | 16.15 |
| cycle duration | 6.68 | 9.40 | 11.18 | 15.89 | 22.77 |
| duty cycle | 0.36 | 0.59 | 0.68 | 0.74 | 0.81 |
| quiescence interval | 2.45 | 3.36 | 3.59 | 4.27 | 11.81 |

Sample size was 2 larvae. Units and abbreviations same as for Table 1.

of MN intrinsic properties in motor pattern generation and, more specifically, our understanding of how networks compensate for altered MN excitability to maintain proper locomotion.

## Acknowledgments

Some of the text in this article originally appeared in a series of posts on the author's blog (emckiernan.wordpress.com), and in guest posts on the sites Things We Don't Know (blog.thingswedontknow.com) and PubChase (www.pubchase.com). The author thanks Markus Dahlem, Andrew Fuglevand, Marco Herrera Valdez, Richard Levine, and Diano Marrone for feedback on earlier versions of this manuscript. The EKI line was provided by Richard Levine at the University of Arizona. The RN2-GAL4 line was provided by Matthias Landgraf at the University of Cambridge.

## Funding

ECM was funded in part by the NIH Predoctoral Training Program in Neuroscience 5 T32 AG007437 awarded to the Graduate Interdisciplinary Program in Physiological Sciences at the University of Arizona, and by NIH grant 5 R01 NS057637 03 awarded to Richard Levine at the University of Arizona. All experiments were performed at Arizona State University in the laboratory of Carsten Duch.

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# Supporting Information

## S1 Fig

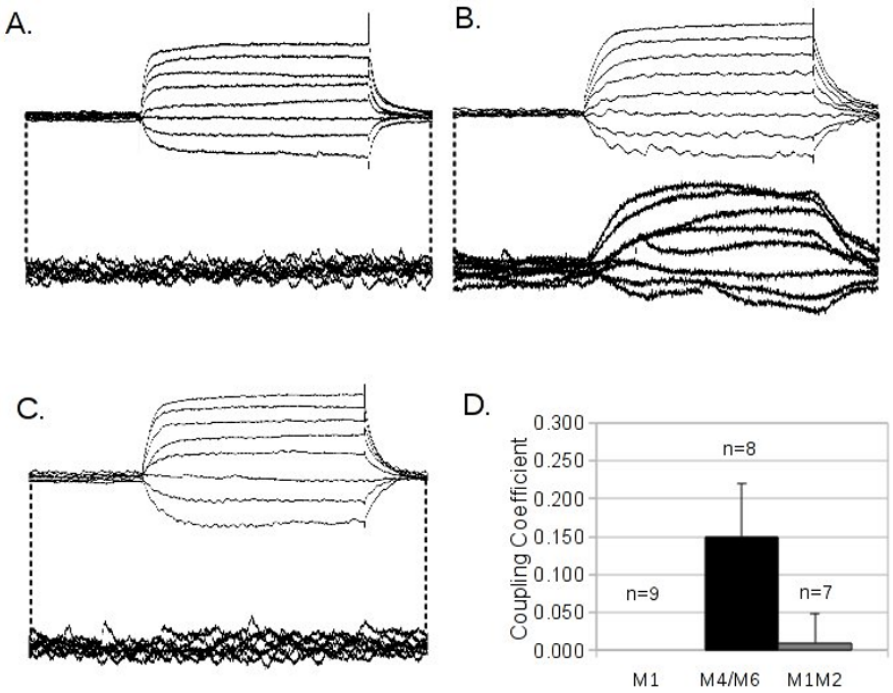


**Fig. S1** Electrical coupling of body wall muscles in WT larvae. A. Current was injected into a muscle 1 (M1) segment (top trace) and the response measured in an adjacent M1 segment (bottom trace). Dashed lines indicate that the records were made simultaneously. B. Same as in A, but for two adjacent muscle 6 (M6) segments. Note that a voltage change is measured in the adjacent segment (bottom trace) in response to current injection in the top segment, indicating electrical coupling. C. Same as in A, but for M1 and M2 in the same segment. D: Average coupling coefficient calculated for M1 pairs (n=9), M4 or M6 pairs (n=8), and M1M2 pairs (n=7). Error bars shown are standard deviations.

## S2 Fig

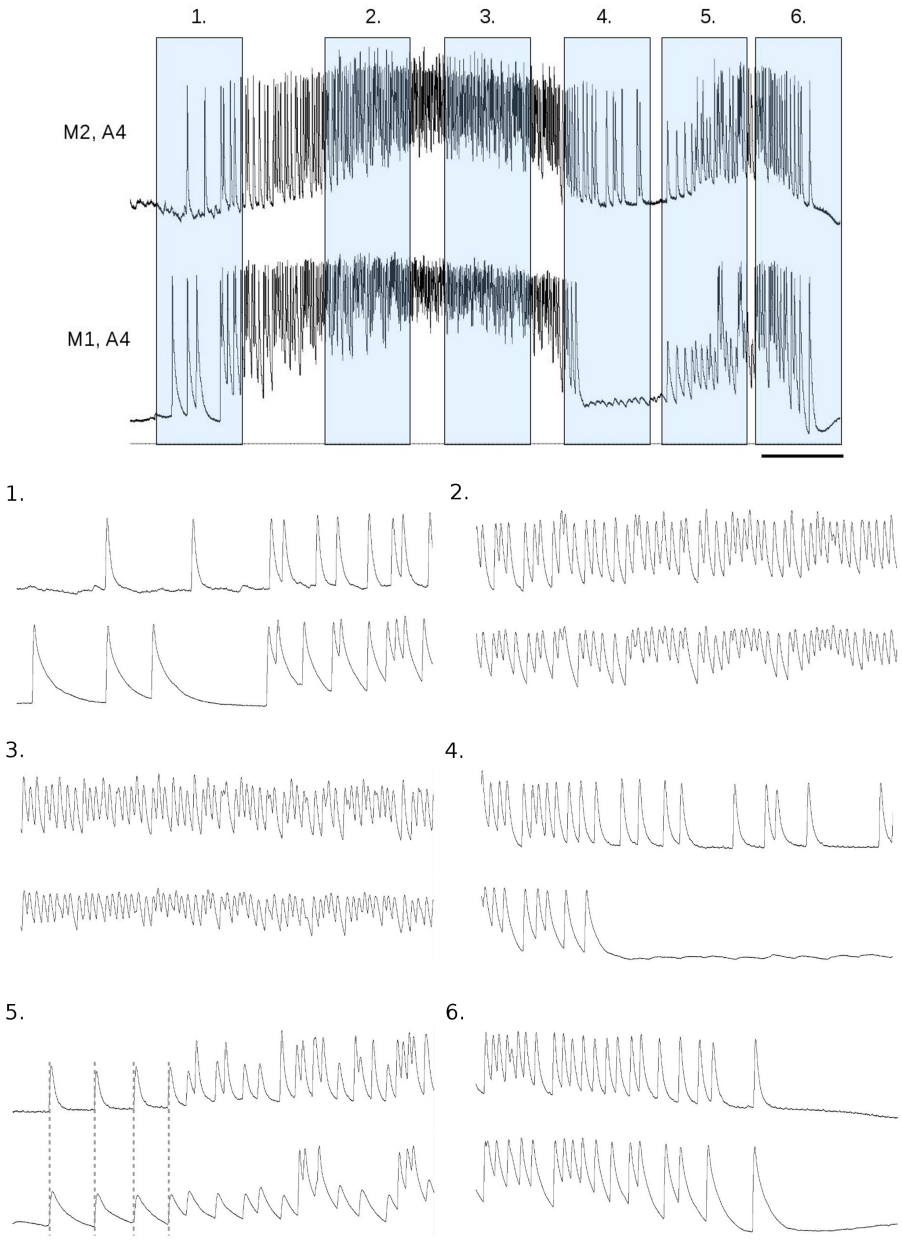


**Fig. S2** A. Simultaneous intracellular recordings from muscle 2 (top trace) and muscle 1 (bottom trace) in segment A4. Shaded rectangles 1-6 indicate regions of the burst which were examined at higher temporal resolution below. Scale bar is 1 second. Panels 1-6 are 1-second windows corresponding to the shaded and numbered regions in A. Dashed lines in panel 5 indicate coincident EPSPs. M1 and M2 are innervated by one common (MNISN-Is) and one non-common MN (MN1-Ib or MN2-Ib, respectively). It was hypothesized that by looking at coincident and non-coincident EPSPs, a unit separation could be performed. However, EPSP summation and compound events throughout the majority of the burst made this not possible.





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