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Flight and seizure motor patterns in *Drosophila* mutants: Simultaneous acoustic and electrophysiological recordings of wing beats and flight muscle activity

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Abstract

Tethered flies allow studies of biomechanics and electrophysiology of flight control. We performed microelectrode recordings of spikes in an indirect flight muscle (the dorsal longitudinal muscle, DLMa) coupled with acoustic analysis of wing beat frequency (WBF) via microphone signals. Simultaneous electrophysiological recording of direct and indirect flight muscles has been technically challenging; however, the WBF is thought to reflect in a one-to-one relationship with spiking in a subset of direct flight muscles, including muscle m1b. Therefore, our approach enables systematic mutational analysis for changes in temporal features of electrical activity of motor neurons innervating subsets of direct and indirect flight muscles. Here we report the consequences of specific ion channel disruptions on the spiking activity of myogenic DLMs (firing at ~5 Hz) and the corresponding wing beat frequency (~200 Hz). We examined mutants of: 1) voltage-gated Ca²⁺ channels (*cacophony*, *cac*), 2) Ca²⁺-activated K⁺ channels (*slowpoke*, *slo*), and 3) voltage-gated K⁺ channels (*Shaker*, *Sh*) and their auxiliary subunits (*Hyperkinetic*, *Hk* and *quiver*, *qvr*). We found flight initiation in response to an air puff was severely disrupted in both *cac* and *slo* mutants. However, once initiated, *slo* flight was largely unaltered, whereas *cac* displayed disrupted DLM firing rates and WBF. *Sh*, *Hk*, and *qvr* mutants were able to maintain normal DLM firing rates, despite increased WBF. Notably, defects in the auxiliary subunits *Hk* and *qvr* could lead to distinct consequences, i.e. disrupted DLM firing rhythmicity, not observed in *Sh*. Our mutant analysis of direct and indirect flight muscle activities indicates that the two motor activity patterns may be independently modified by specific ion channel mutations, and that this approach can be extended to other dipteran species and additional motor programs, such as electroconvulsive stimulation-induced seizures.

Keywords

microphone; wing beat frequency; dorsal longitudinal muscle; flight initiation; high speed videography; potassium channel; *Shaker*, *Hyperkinetic*, *quiver/sleepless*, *slowpoke*, *cacophony*, calcium channel; BK channel; bang-sensitive mutants; electroconvulsive seizure

Introduction

Dipteran tethered fly preparations have been used to study a wide variety of neural processes involved in flight control (e.g. Götz, 1968; Heisenberg & Wolf, 1984; Lehmann & Dickinson, 1997; Balint & Dickinson, 2001). *Drosophila*, like other dipteran flies, are equipped with both direct and indirect flight muscles (Miller, 1950). The indirect flight muscles, consisting of the dorsal longitudinal muscles (DLMs, the primary wing depressors) and dorsal ventral muscles (DVMs, wing elevators), are the largest muscles in the adult fly that undergo isometric contractions powering flight. In DLMs and DVMs, tension oscillations are stretch-activated, at different phases with respect to the thorax case resonance and thus the muscle contractions are synchronous with the wing beat (Chan & Dickinson, 1996). In contrast, a set of smaller direct flight muscles are involved in controlling wing positions during flight maneuvers (Dickinson & Tu, 1997). However, due to their small size, electrical recordings from direct flight muscles are relatively sparse in the literature (Nachtigall & Wilson, 1967; Heide & Götz, 1996; Balint & Dickinson, 2001). However, DLMs have been extensively studied for their electrical activity (Salkoff & Wyman, 1983; Koenig & Ikeda, 1983; Elkins, et al., 1986) and contractile mechanisms (Pringle, 1949; Irving & Maughan, 2000; Vigoreaux, 2001; Dickinson, et al., 2005). To date, correlations between the electrical activity of the direct and indirect flight muscles have not been fully established.

A diverse range of spiking characteristics has been documented during flight for different sets of flight muscles. While isometric contractions in the DLMs are triggered by stretching occurring with each wing beat cycle (~200 Hz), their firing rate is much lower (~5 Hz), reflecting the spiking rate of their innervating motor neurons (Levine, 1973; Harcombe & Wyman, 1977; Koenig & Ikeda, 1983). A relatively low firing rate in indirect flight muscles is sufficient to maintain the Ca^{2+} influx required for the tension oscillations (Pringle, 1978; Gordon & Dickinson, 2006). Furthermore, visual cue-triggered transient changes in the firing rate precisely modulate changes in power output (Dickinson et al., 1998; Lehmann et al., 2013). In contrast, several direct flight muscles fire at the wing beat frequency. These muscles fire in phase with the wing beat, but the firing patterns vary, presumably associated with flight maneuvers. Some fire in intermittent bursts (e.g. basilar b2 muscle), while others (e.g. the basilar b1 muscle) fire continuously at one-spike to one-wing beat during flight (Heide & Götz, 1996; Balint & Dickinson, 2001).

In this report, we simultaneously record electrical activity from the DLM and acoustic signals (collected from a high-gain microphone) of wing beats during flight. Although the WBF alone does not fully describe wing kinematics or enable inferences on power output during flight (both of which depend on measurements of the stroke amplitude which may not be as easily ascertained from acoustic data), our WBF observations may serve as a first order proxy for spiking in motor neurons driving a subset of direct flight muscles that fire in a one-to-one relationship with wing beats (Nachtigall & Wilson, 1967; Heide & Götz, 1996; Balint & Dickinson, 2001). Thus, potential genetic factors preferentially affecting central pattern generation driving spiking in either direct or indirect flight muscle motor neurons can may be drawn via analysis of WBF and DLM spiking in collections of wild-type (WT) and mutant flies.

Using this approach, we examined flight in a collection of *Drosophila* ion channel mutants to determine whether disruption of individual channel types differentially alters indirect or direct flight muscle activities. We focused on K⁺ and Ca²⁺ channel mutants with identified motor coordination defects, starting with *slowpoke* Ca²⁺ activated K⁺ BK channel mutants, known for their flight defects (Elkins, et al., 1986). We also examined flight in mutants of *cacophony*, a Ca²⁺ channel gene, which was named for disrupted courtship songs (von Schilcher, 1976). We compared another set of locomotion mutants, ether-induced leg-shaking mutants of the Shaker K⁺ channel subunit genes *Shaker*, *Hyperkinetic*, and *quiver* (Kaplan & Trout, 1969; Ganetzky & Wu, 1986; Wang, et al., 2000). We extended this approach to characterize additional *Drosophila* species, and another well-established pattern of muscle spike activity, electroconvulsively induced seizures that trigger highly stereotypic sequences of wing buzzing and DLM activity (Lee & Wu, 2002).

Materials and Methods

Drosophila Stocks

Flies were reared on standard cornmeal media at room temperature (~22 °C), and were between 5 and 11 days old when studied. The *D. melanogaster* wild-type (WT) strain was *Canton-S* (CS). Male flies were used for all flight recordings, except for Supplemental Figure 2. The *Drosophila robusta* strains used (identified as ‘SL’ that were derived from a single female fly caught at Saylorville Lake, Iowa, USA and ‘IR’ from a single female fly caught on the Iowa River in Iowa City, Iowa, USA) were a gift from Prof. B. McAllister of the University of Iowa.

Ion channel mutants examined in this study include alleles of *slowpoke* (*slo: slo¹, slo⁹⁸*) encoding a BK Ca²⁺-activated K⁺ channel (Elkins, et al., 1986; Atkinson, et al., 1991), and *cacophony* (*cac: cac^S, cac^{NT27}*), encoding an N-type Ca_v channel (von Schilcher, 1976; Rieckhof, et al., 2003). Alleles of other K⁺ channel genes include: *Shaker*, (*Shr: Shr^M, Shr¹²⁰, Shr¹³³, Shr⁵*), encoding the pore-forming alpha subunit of the transient A-type channel K_v1 (Kaplan & Trout, 1969; Jan, et al., 1977; Ganetzky & Wu, 1982; Ganetzky & Wu, 1983; Haugland & Wu, 1990; Wu & Ganetzky, 1992); *Hyperkinetic* (*Hk: Hk¹, Hk^{IE18}*), encoding an intracellular auxiliary subunit of the Shaker channel K_vβ (Kaplan & Trout, 1969; Chouinard, et al., 1995; Wang & Wu, 1996); and *quiver/sleepless* (*qvr: qvr¹, qvr^{EY04063}*, henceforth referred to as *qvr^{EY}*), encoding an extracellular modulatory subunit of the Shaker channel (Wang, et al., 2000; Koh, et al., 2008; Wang & Wu, 2010; Dean, et al., 2011).

The bang-sensitive flies used include mutants of the genes *slamdance* (*sda^{iso7.8}*), which encodes an aminopeptidase (Zhang, et al., 2002), and *easily shocked* (*eas¹*) encoding an ethanolamine kinase (Pavlidis, et al., 1994).

Tethered Preparation

Flies were briefly anesthetized on ice and glued with cyanoacrylate glue (Aron Alpha Type-203TX, Toagosei America, Inc., W. Jefferson, OH, USA) to a tungsten pin using a modified procedure described previously (Gorczyca & Hall, 1984; Lee & Wu, 2002). All

experiments were carried out at room temperature (21 - 23 °C). Tethered flies were allowed to rest for at least 30 min prior to experimentation.

In the tethered preparation, most flies would spontaneously start flying during the “rest” period, during handling, or after positioning and electrode insertion into the DLM. In cases where spontaneous flight initiations were not observed, air puffs were used to trigger flight (see below). “Sustained flight” data were collected during a period at least 30 s after flight initiation and recordings lasted at least 60 s, frequently up to 240 s. Afterwards, the fly was allowed to rest for approximately 3 min prior to the trials of air puff-triggered flight initiations. The fly was subjected to a series of 500-ms air puffs spaced 5 s apart until a flight initiation was observed. (Non-fliers were defined as those that failed to initiate flight within five air puffs.) Importantly, this protocol requires a total of no more than 45 min to ascertain the relevant parameters for electrical and acoustic characterization from a single fly. This approach enabled us to sample enough flies for each line for a neurogenetic analysis across many genotypes.

Flights could be reliably triggered by a gentle air puff delivered through a 4 mm diameter aluminum tube placed ~ 4 mm away from the fly. Air puffs were generated by an aquarium air pump (Whisper 10-30, Tetra, Blacksburg, VA, USA), switched by a 3-way solenoid valve (ASCO scientific, P/N AL4312, Florham Park, NJ, USA) controlled via an USB 6210 DAQ card (National Instruments, Austin, TX USA) in conjunction with a custom-written LabVIEW script (LabVIEW 8.6, National Instruments).

Acoustic and high speed video analysis of flight

Acoustic signals were acquired via a high-gain microphone (PA3-IL, Supercircuits Inc., Austin, TX) placed approximately 4 mm below a tethered fly, sampled by a sound card (8 bit depth, 22050 Hz sampling rate, Creative Sound Blaster X-Fi Go! Pro, Creative Technology Ltd., Singapore) connected to a standard PC. A LabVIEW script was used to control data acquisition of acoustic signals, filtered offline via a 16th order zero-phase Butterworth filter (3 dB bandwidth 60 – 600 Hz) implemented in a custom-written MATLAB script (MATLAB r2012a, Mathworks, Natick, MA, USA).

Wing beat frequency was determined from the filtered acoustic signal by computing the short-time FFT at 22-kHz sampling rate with 500-ms running windows and a 75% overlap between adjacent windows (via the ‘*spectrogram*’ function, MATLAB). Flight was confirmed when the 500-ms windows exhibited a peak power greater than -50 dB V² and displayed a highly skewed frequency-power distribution (skewness > 20, Sokal & Rohlf, 1969).

For experiments correlating video and audio data of flight, a high-speed video camera (MotionScope 8000S, Redlake Imaging Corp., Morgan Hill, CA, USA) fitted with a macro lens was placed above the tethered fly. Images were acquired at 2000 frames per second. A homemade transistor switching circuit synchronized audio and video data acquisition, and video data was digitized and analyzed in MATLAB.

DLM recordings during flight and seizure activity

Recordings were performed from modified protocols described previously (Engel & Wu, 1992; Lee & Wu, 2006). An electrolytically sharpened tungsten electrode was inserted into the indirect flight muscle DLM, with a reference electrode inserted into the dorsal abdomen. The left or right dorsal-most DLMa was used throughout the study, as indicated by its attachment site (Levine & Hughes, 1973). Signals were amplified by an AC amplifier (bandwidth of 10 – 20,000 Hz, AM Systems Model 1800, Carlsborg, WA, USA) and were digitized by an USB 6210 DAQ card at a sampling rate of 20 kHz. A custom-written LabVIEW script was used to control both acoustic and electrophysiological data acquisition.

Electroconvulsive Stimulation

Electroconvulsive stimulation (ECS), used to induce seizures, was delivered across the brain via sharpened tungsten electrodes inserted into each eye (cf. Lee & Wu, 2002). A train of 0.1 ms 80 V pulses delivered at 200 Hz for 0.5 or 2 s (as specified) reliably triggered seizure activity. Stimuli were generated by an isolated pulse stimulator (Model 2100, AM Systems).

Statistical Analysis

Kruskal-Wallis tests were used for ANOVA analysis, with the Rank Sum test (Bonferroni correction applied) used for post hoc analysis.

Results

Acoustic signals produced during tethered flight in *Drosophila*

Spike patterns from DLMs as well as other indirect flight muscle fibers during tethered flight have been well described (Levine, 1973; Harcombe & Wyman, 1977; Engel & Wu, 1992; Lehmann & Dickinson, 1997) but the relationships between DLM spiking and wing beats are less well understood. We sought to facilitate the correlation of DLM electrical activity to mechanical signals produced by wing movement by using a simple, commercially available, transducer. As shown in Figure 1A, we employed a high-gain microphone directly under a tethered fly and picked up a rhythmic tone during flights (Fig. 1B-C). Our acoustic signal was similar in frequencies and waveforms to previously described force-transducer signals with direct coupling to the thorax of a tethered fruit fly (Zanker & Götz, 1990). To examine how the acoustic waveform acquired corresponded to the phases of the wing beat cycle, we placed a high-speed video camera (2000 frames per second) above the fly. The microphone signal during a wing beat cycle is expanded and shown in Figure 1C, along with the corresponding high-speed video frames in Figure 1D. Representative spectrogram samples (30-s) are shown in Figure 1E from two different flies, with their integrated power spectra displayed on the right panels. The microphone signal clearly contained a single dominant frequency, which corresponded to the wing beat frequency (WBF). The expected harmonics at higher frequencies were also observed in the microphone signal (outside the plotted range, see Supplemental Figure 1).

We first focused on ‘sustained flight’ and analyzed bouts of flight lasting longer than 30 s in WT male *D. melanogaster*. Consistent with previous reports of WBF in *Drosophila* (e.g. Zanker & Götz, 1990; Lehmann & Dickinson, 1997; Fry, et al., 2005), we found that the

range of WBFs in male *D. melanogaster* fell between 170 and 220 Hz, with a median of 191.7 Hz (Fig. 1F, female flies showed a similar distribution, Supplemental Fig. 2A). Interestingly, each fly maintained its own characteristic WBFs with relatively small variability during trials up to 240 s ($\sigma_{\text{ind}}^2 = 23.6 \text{ Hz}^2$, $n_{\text{bin}} > 160/\text{fly}$). However, consistent with previous reports (Curtsinger & Laurie-Ahlberg, 1981), there was a substantially greater variability across the population examined in WBF than what would be expected due to WBF variability within-individuals ($\sigma_{\text{pop}}^2 = 172.0 \text{ Hz}^2$, $n = 21$ flies, $\sigma_{\text{means}}^2 / \sigma_{\text{ind}}^2 = 7.30 \gg F_{0.001}(23, \infty) = 2.135$). We examined if differences in age could explain this variability, and found that there was no significant correlation between WBF and age in our population ($R^2 = 0.028$, $p = 0.44$ Supplemental Figure 3A). Despite this variability in WT *D. melanogaster*, several mutants displayed WBF modifications beyond this range in the following analysis.

Simultaneous monitoring of DLM firing and wing movements in *Drosophila* species: *melanogaster* and *robusta*

—It is well established that individual action potentials of the indirect flight muscles, including DLMs, do not correspond with wing beats in a one-to-one manner during flight, but the WBF does reflect the spiking activity of several identified direct flight muscles (Pringle, 1949; Dickinson & Tu, 1997). We obtained the DLM firing frequency data together with recordings of wing beat frequency during the sustained flight bouts analyzed in Figure 1 for male WT *D. melanogaster* (Fig. 2A), and compared their flight parameters to those of two *D. robusta* lines (designated SL and IR, see methods), demonstrating the applicability of our approach to other dipteran species (Fig 2A).

Presumably due to *robusta*'s substantially larger sizes (Stalker & Carson, 1947), both *D. robusta* lines showed substantial reductions in median WBF as compared to WT *D. melanogaster*, (Fig 2B, 121.4 and 155.2 Hz for SL and IR respectively versus 191.7 Hz for WT *melanogaster*, $p < 0.001$). Notably, the sustained-flight WBF values in the two *robusta* lines were not overlapping, despite the absence of obvious differences in body size, suggesting that a large variation of the WBF during sustained flight can occur among wild populations of the same species. We found that the sustained-flight DLM firing frequency was not significantly different across the two populations of *robusta* (Fig 2D, 5.80 and 6.93 Hz for SL and IR, respectively, $p = 0.17$). However, the results unexpectedly revealed that even though *D. robusta* had a lower WBF, their DLM firing rate was actually higher when compared to *D. melanogaster* (Fig 2C, median WT = 4.53 Hz, $p < 0.05$).

Our recordings also demonstrated a wide variability in DLM firing rates among individuals within the same WT line of *D. melanogaster*. For male flies, it ranged from 2.61 to 7.05 Hz (Fig 2C). Female *D. melanogaster* showed a similar variability in DLM firing frequency, ranging from 2.22 to 5.98 Hz with a median of 4.57 Hz (Supplemental Figure 2B). Interestingly, we found that for both *D. melanogaster* and *D. robusta* males, the DLM firing rate was not correlated with WBF across individuals ($R^2 = 0.0185$ and 0.130 , $p = 0.526$, and $p = 0.250$, respectively). Furthermore, the observed firing rate variability was not correlated with age ($R^2 = 0.0049$, $p = 0.75$ Supplemental Figure 3B). Our data also provides a convenient entry to the analysis of the intrinsic regularity of DLM firing. We found that there were no significant differences between *D. robusta* vs. *D. melanogaster* in terms of the

coefficient of variation (CV) of the DLM inter-spike-intervals (Fig 2D, median CV = 0.14 and 0.13 for SL and IR, respectively, vs. 0.15 for WT *melanogaster*, $p = 0.23$).

Flight initiation: Relationships between DLM Firing and Wing Beat Frequency

In tethered flight studies, gentle air puffs are often used to trigger flight in *Drosophila*. We found that a 500-ms puff reliably triggered flight in WT flies. Figure 3A displays the characteristic transient features in DLM firing and WBF during and immediately following flight initiation. In majority WT initiations, the DLM firing frequency during the air puff itself is much higher than the ensuing flight, with a mean spiking rate of 19.65 ± 2.9 Hz compared to 4.25 ± 0.9 Hz for sustained flight bouts (Fig 3B). Immediately after the air puff delivery, there was a sharp decline in the DLM firing, approaching the final firing rate within seconds (90 % decay averaged 2.1 s). The WBF showed a similar pattern (Fig 3C), with a higher initial value (mean of 212.8 ± 2.0 Hz) and a slower decline to the final WBF, (90 % decline averaged 6.0 s). Despite individual variability in WBF and DLM firing, the above characteristics of flight initiation could serve important and sensitive indicators in mutant analysis of how different genes affect the motor pattern generators for indirect and direct flight muscle activity.

Motor outputs during flight in slowpoke Ca^{2+} -activated K^+ and *cac* Ca^{2+} channel mutants

Disruption of ion channel function leads to a wide variety of motor coordination deficits in *Drosophila*. For example, mutants of *slo* (encoding a BK Ca^{2+} activating K^+ channel) exhibit poor flight ability (Elkins, et al., 1986) and altered synaptic transmission due to prolonged Ca^{2+} entry at the nerve terminal (Lee, et al., 2014). We asked how different flight characteristics could be altered in *slo* mutant flies. We examined flight initiation in two alleles: *slo^l* and *slo⁹⁸*, and found that both alleles showed dramatically decreased success rates in air puff-triggered flights (0/6 in *slo^l*, 1/10 in *slo⁹⁸*, Fig 3E). Interestingly, *slo⁹⁸*, but not *slo^l*, flies displayed spontaneous sustained flight (observed in 7 out of 12 *slo⁹⁸* flies, Fig 4A), with apparently normal wing beat frequency (Fig 4B), DLM firing rate (Fig 4C), and CV of DLM inter-spike intervals (Fig 4D). The fact that *slo⁹⁸* flies can fly normally indicates that flight initiation mechanisms could be preferentially affected by *slo* mutations.

Besides *slo* mutants, flight defects have been described in several other mutations that disrupt intracellular Ca^{2+} regulation, e.g. *inositol triphosphate receptor* (*itpr*, Banerjee, et al., 2004). However, it is not known how flight is affected by mutations of the primary presynaptic voltage-gated Ca^{2+} channel, encoded by *cacophony* (*cac*, also known as *Dmca1A*, Kawasaki, et al., 2000; Lee, et al., 2014). Interestingly, the first *cac* mutants were isolated on the basis of their altered courtship songs (von Schilcher, 1976); presumably reflecting altered motor programs driving certain subsets of direct flight muscles. We asked whether the courtship song defects of *cac* are indicative of disruptions in flight motor patterning (Fig 4A). We examined two *cac* alleles, *cac^S*, and *cac^{NT27}*. Similar to the *slo* mutants, both *cac* alleles performed poorly in the air puff-triggered flight initiations (0/11 in *cac^{NT27}*, and 1/8 for *cac^S*, Fig 3E). However, similar to *slo⁹⁸*, but unlike *slo^l*, most *cac* flies displayed spontaneous flights (7/10 in *cac^S*, and 9/10 in *cac^{NT27}*, Fig 4).

Nevertheless, unlike *slo*⁹⁸, both *cac* alleles showed significantly increased WBFs (medians of 208.7 and 216.6 Hz, respectively, vs. 191.7 Hz for WT, $p < 0.05$ for both alleles, Fig 4B) coupled with strikingly higher DLM firing frequencies (medians of 10.3 and 11.2 Hz, respectively, vs. 4.53 for WT, $p < 0.001$, Fig 4C), well beyond the maximum firing rate observed among WT flies during sustained flight (7.1 Hz). To examine the intrinsic regularity of the DLM firing, we measured the inter-spike-interval CV in *cac* mutants. We found only a subtle, albeit statistically significant, increase in the CV for *cac*^S mutants (Fig 4D).

It should be noted that previous studies have shown an important functional role of DLM firing in regulating mechanical power output during tethered flight; therefore, the relationship between DLM firing frequency and WBF in normal flight varies depending on visual cues in the flight arena (Gordon & Dickinson, 2006; Lehmann et al., 2013). Our findings were more pertinent to the basic parameters under simplified conditions, with which the separable effects of mutations of distinct genes on DLM firing and WBF can be clearly indicated. This is further supported by analysis of additional sets of ion channel mutants.

A diversity of flight phenotypes in mutants of Sh channel and its auxiliary subunits Hk and qvr

We further analyzed how alterations in voltage-gated K⁺ channels could differ from Ca²⁺-activated K⁺ channels in exerting influences on flight behavior (Fig 5A). The quantitative parameters enabled us to detect subtle changes in either direct or indirect flight muscle activities. We focused on several well-characterized alleles of the *Sh* gene, encoding the pore-forming α subunit (K_V1) for a voltage-gated K⁺ channel that mediates transient, fast-inactivating A-type currents. We chose to study 1) a null allele *Sh*^M, affecting both nerve and muscle, 2) a hypomorphic allele, *Sh*^{I20}, which affects largely neuronal current while leaving muscle currents relatively intact, and 3) a neomorphic allele *Sh*⁵ that alters the voltage dependence of the A-current (Haugland & Wu, 1990). Consistent with previous studies (Engel & Wu, 1992), individuals of each allele examined were capable of sustained flights (7/10, 9/10, and 10/10, respectively, showed spontaneous flights longer than 10 s). Flight patterns in *Sh*^M, as well as *Sh*^{I20}, were distinct from WT. Both of these *Sh* alleles showed increased WBF (Fig 5B, median values of 204.0 and 207.6 vs. 191.7 Hz for WT), but displayed largely normal DLM spiking activity, including average firing rate and CV of the inter-spike interval (Fig 5 C-D). These observations are a contrast to *cac* mutations, which affect both WBF and DLM firing rate. Unexpectedly, the neomorphic allele *Sh*⁵ displayed largely normal flight parameters, with WBF, DLM firing rate, and inter-spike-interval CV not significantly different from WT.

A hallmark of mutations that affect A-type K⁺ currents is the ether-induced leg-shaking phenotype. This is true also for the mutants of Shaker channel auxiliary subunits: *Hk* encodes an intracellular beta subunit (K_Vβ, Chouinard, et al., 1995) while *qvr* encodes an extracellular, GPI-anchored subunit (Koh, et al., 2008). We asked how *Hk* and *qvr* mutations modify flight characteristics in comparison to *Sh* mutations.

Of the two *Hk* mutants examined, the null allele *Hk^{IE18}* showed an increased WBF (median 214.0 Hz), similar to *Sh^M* and *Sh^{I20}*, but also a subtle, yet statistically significant, decrease in DLM firing rate, unlike *Sh* mutations (Fig 5C). *Hk^I* did not appear to alter either WBF or DLM firing rate. However, both *Hk^I* and *Hk^{IE18}* affected the regularity in DLM spiking as measured by a significant increase in the DLM inter-spike interval CV (Fig 5D).

The examined *qvr* mutants, *qvr^I* and *qvr^{EY}*, showed distinct, significant alterations in flight patterns. Similar to *Sh^M*, *Sh^{I20}*, and *Hk^{IE18}*, individual *qvr^I* flies displayed significantly increased WBF with a relatively normal DLM firing rate. Interestingly, the null allele *qvr^{EY}* displayed normal WBF (Fig 5B). Further, the average DLM firing rate in this allele was apparently normal during flight (Fig 5C). However, the DLM spiking pattern of *qvr^{EY}* flies strikingly lacked regularity (Fig 5D), as measured by the CV of inter-spike intervals close to what would be expected from random firing (i.e. from an exponential distribution).

Temporal dissociation of the DLM motor neuron activity from wing movements during electroconvulsive seizures in bang-sensitive mutants

In a number of bang-sensitive mutants, “wing buzzing” is a striking aberrant motor activity associated with mechanical shock-induced seizure episodes (Wu & Ganetzky, 1982; Burg & Wu, 2012). In electrophysiological experiments, high-frequency (200 Hz) electroconvulsive stimulation (ECS) across the brain also triggers a stereotypic DLM seizure discharge (Pavlidis, et al., 1994; Pavlidis & Tanouye, 1995) that could be correlated with wing buzzing behaviors in tethered WT and mutant flies (Fig 6A, Lee & Wu, 2002; Lee & Wu, 2006). The DLM firing rates are known to be radically altered in several bang-sensitive mutants, but the activity pattern in other muscle groups, including the direct flight muscles, during seizures is not known. We took advantage of the readily accessible WBF acoustic signal to examine the temporal relationships between DLM activity and wing buzzing during the ECS-induced seizures.

Using simultaneous DLM and microphone recordings, we analyzed these wing buzzing events in both WT and two representative mutants of the bang-sensitive genes, *easily shocked* (*eas*, Pavlidis, et al., 1994), encoding an ethanolamine kinase, and *slamdance* (*sda*, Zhang, et al., 2002) encoding an aminopeptidase. In WT flies, we could acquire a tone that accompanied the ECS-triggered DLM spike discharge that was triggered immediately following the ECS, with gradual decline in frequency, and abruptly terminated at the same time as cessation of DLM spiking (Fig 6B). Interestingly the WBF during the seizure discharge was within the normal range of sustained flight (compare to Fig 1E).

Surprisingly, in the bang-sensitive mutants, *sda* and *eas*, we found a decoupling of the temporal relationship between DLM firing and wing buzzing (Fig 6B). In both mutants, a drastic increase in DLM firing frequency was observed following ECS with a pattern characteristic to the individual genotypes (Fig 6B and data not shown), consistent with previous reports (Pavlidis & Tanouye, 1995; Zhang, et al., 2002; Lee & Wu, 2002). The temporal characteristics of the ECS-induced wing buzzing showed a gross discrepancy with the DLM firing pattern. Unlike the WT pattern, the WBF in these mutants did not appear to be temporally coupled with the DLM firing episode. Instead, an abrupt termination of wing beats occurred during DLM firing, followed by quiescence prior to a second bout of wing

beats when DLM firing was terminated. Among the half-dozen flies for each genotype, these general patterns of DLM and direct flight muscle activities have been consistently observed. Our results demonstrate that some bang-sensitive mutations can affect spike patterning in indirect and direct flight muscles more extremely than the channel mutants described above. Furthermore, this approach can facilitate studies of alterations of motor program outputs associated with epileptiform seizures and other excitability disorders.

Discussion

Simultaneous assessment of direct and indirect flight muscle activity

Flight tones have been used for over a century to analyze dipteran flight (Landois, 1866; cited in Williams & Galambos, 1950). Off-the-shelf microphones could be readily added to monitor flight tones in the tethered preparations previously used to monitor spiking activity in DLMs to analyze properties of the giant-fiber escape reflex (Engel & Wu, 1992; Engel & Wu, 1996) as well as seizure discharges (Lee & Wu, 2002; Lee & Wu, 2006) in *Drosophila*. Because the fundamental frequency of the flight tone corresponds to the wing beat frequency (Fig 1B-C, Williams & Galambos, 1950), our approach enables direct comparisons between indirect muscle firing with wing beats in the same fly (Fig 2). More importantly, analysis of different categories of mutants reveals that mutations in particular genes may affect either WBF (e.g. *Sh*) or DLM firing (*qvr*) only, or both (e.g. *cac*), suggesting that the firing of direct and indirect flight muscles is driven by distinct motor pattern generators that are genetically separable.

Among the populations sampled in this study, we observed a span of variability in wing beat frequency (range of 170 - 220 Hz) and DLM firing (range of 2.22 - 5.98 Hz) in WT and various mutants. While significance of such variability is unclear, previous analyses of WBF across several thousand flies from several isogenized lines of *D. melanogaster* males report similarly large variability with relatively small contributions due to differences age, ambient temperature or flight duration alone (Curtsinger & Laurie-Ahlberg, 1981). However, it should be noted that WBF and DLM spiking did not co-vary across individuals, i.e. for any particular fly, higher WBF did not necessarily couple with higher DLM firing rates during sustained flight (Fig 2). Our observations suggest that this holds true in other *Drosophila* species (Fig 2).

Significantly, the performance of tethered flies does not reflect all aspects of free-flight behavior. For example, the WBF has been observed to be systematically higher in free flight. It has been suggested that such differences may be introduced by the unnatural posture of horizontally positioned tethered fly, in contrast to a more vertical orientation during free flight resulting in sensory cues not usually experienced by the fly (Fry, et al., 2005). It is also known that flight power control depends on both wing stroke frequency and amplitude, with a lower stroke amplitude associated with higher stroke frequency, and vice versa (Lehmann and Dickinson, 1997). It will be desirable in future studies to include both WBF and amplitude measurements. Further, muscle mechanical power output also depends on temperature (Gilmour & Ellington, 1993; Lehmann 1999), stretch activation (Pringle 1978), intramuscular Ca^{2+} levels (partially from influx during muscle firing, Gordon & Dickinson, 2006; Lehmann et al., 2013), and conceivably mechano-sensory feedback (Dickinson, 1999;

Sherman & Dickinson, 2003). It would be interesting to compare in further studies how the changes in the parameters observed in our study translate to modified free-flight parameters in the various mutants.

Ion channels and flight performance

Ca²⁺-dependent K⁺ channels—The ion-channel mutants we examined all have previously documented motor coordination deficits. The *slo* mutants with defective K_{Ca} BK channels were first identified on the basis of their uncoordinated, sluggish behavior at high temperature (38°C, Elkins, et al., 1986; Elkins & Ganetzky, 1988). Mutant adults display “poor flight” as measured by a drop-and-flight Flight-Tester assay (Elkins, et al., 1986; Babcock & Ganetzky, 2014), and larvae also show abnormal central pattern generation responsible for peristaltic contractions (McKiernan, 2013). The altered BK currents in the *slo* alleles studied here have been described in several tissues, including the DLM (Elkins, et al., 1986), larval muscles (Singh & Wu, 1989), presynaptic terminals (Lee, et al., 2014), and cultured embryonic neurons (Saito & Wu, 1991; Peng & Wu, 2007). These studies demonstrate the striking consequences of altered BK channels resulting in prolonging Ca²⁺ influx through Ca²⁺ channels, broadening action potentials, promoting repetitive firing, and altering synaptic transmission.

Our observations highlight aspects of tethered flight that depend on the functioning of the *slo* BK channel. Air puff-triggered flight initiation was severely affected, with no success in *slo^l* and severe reductions in *slo⁹⁸* in a large number of trials. However, several *slo⁹⁸* individuals were able to initiate spontaneous flight, which showed relatively normal WBF and DLM firing patterns (Figs 3, 4). These results suggest several possibilities, including: 1) the *slo* mutations preferentially affect putative command components of the circuit for flight initiation, 2) potential defects in relevant sensory contributions required for flight, and 3) homeostatic adjustments in neural circuits since *slo* is known to initiate compensatory up-regulation of *Sh* channels in cell bodies and presynaptic terminals of neurons (Peng & Wu, 2007; Lee, et al., 2014).

Voltage-dependent K⁺ channels—The impact of disrupted *Sh* channels on locomotion has been well studied in a variety of experimental paradigms (Fox, et al., 2005). The *Sh* channel complex is composed of the α subunit (K_V1) encoded by *Sh*, the auxiliary β subunit (K_V β 1) encoded by *Hk*, and the extracellular modulatory subunit encoded by *qvr*. Mutations of these genes display the hallmark ether-induced leg-shaking behavior (Kaplan & Trout, 1969; Wang, et al., 2000) plus locomotive defects, including larval crawling (Wang, et al., 1997) and adult walking (Iyengar, et al., 2012). Additionally, the roles of *Sh* and *Hk* in the giant fiber-mediated escape reflex and its habituation have been documented (Engel & Wu, 1998). Previous voltage-clamp experiments have demonstrated the altered biophysical parameters of *Sh* channels caused by *Sh*, *Hk*, and *qvr* mutant alleles (Haugland & Wu, 1990; Wang & Wu, 1996; Wang, et al., 2000).

Our results reveal previously unnoticed features and relationships among these alleles. *Sh^M*, a null allele, and *Sh^{l20}*, displaying mild effects on muscle currents but severe neurotransmission defects (Haugland & Wu, 1990), show similar defects in WBF, among the

three parameters analyzed (WBF, DLM firing frequency, CV of DLM inter-spike intervals). However, *Sh*⁵, a neomorphic allele, produced WT-like flight in all three parameters, even though it is known to have a unique wing-scissoring phenotype in addition to leg-shaking, coupled with a unique I-V curve, requiring stronger depolarization for activation (Haugland & Wu, 1990). These observations suggest a potential link between the allele-dependent phenotypic differences and the known extensive post-transcriptional mRNA splice-forms of the *Sh* gene (Kamb, et al., 1988; Pongs et al., 1988; Schwarz, et al., 1988).

In comparison with *Sh* mutants, the null allele, *Hk*^{IE18}, produced phenotypes consistent with *Sh*^M in terms of WBF, whereas *Hk*^I displays nearly normal WBF. However, a higher DLM inter-spike-interval CV is observed in both *Hk* alleles, indicating less regular firing due to this auxiliary β subunit. In contrast, mutations in the *qvr* subunit can lead to more extreme consequences. Flight in *qvr*^I flies with disrupted mRNA splicing (Koh, et al., 2008) displayed WBF changes, similar to *Sh*^M. However, the null allele, *qvr*^{EY}, was clearly more extreme than *Sh*^M. In fact, *qvr*^{EY}'s increased DLM inter-spike interval CV was greatest among all mutants examined in this study. This result indicates the possibility that *qvr* also modulates, in addition to Sh channels, other molecular components regulating membrane excitability. Recently, *qvr* was found to modify nicotinic acetylcholine receptor channel function, an abundant excitatory neurotransmitter receptor in the *Drosophila* central nervous system (Wu, et al., 2014).

Voltage-dependent Ca²⁺ channels—It should be noted that among mutants of the Sh channel complex, the average DLM firing rate was largely preserved across mutant genotypes, similar to the consequences of Slo BK mutations. In contrast to K⁺ channel mutations, severe DLM firing rate modifications were found in *cac* Ca²⁺ channel mutations. The gene *cac* encodes an N-type Ca²⁺ channel homologous to vertebrate Ca_v2 channels (Kawasaki, et al., 2000). These channels are present in motor neuron soma (Ryglewski, et al., 2012) and are also localized in the nerve terminal to mediate Ca²⁺ influx required for neurotransmitter release (Kawasaki, et al., 2004). Both *cac* alleles we examined displayed consistent increases in WBF and DLM firing rates. Furthermore, because WBF serves as a proxy for direct flight muscle activity, our results demonstrate that *cac* mutations disrupt spike pattern generation during flight in the motor neurons driving direct and indirect flight muscle groups. This contrasts with the K⁺ channel mutations in which WBF increases, but DLM firing rates remain largely unaltered. Importantly, previous studies indicate that *cac* currents are not prominent in muscles (Lee, et al., 2014), and thus the observed alterations in both WBF and DLM spiking rate are unlikely to be due to changes in muscle properties, but rather due to changes to the motor neuron and its input.

Acoustic monitoring of wing movements during seizures and other motor activities

Wing movements are known to be involved during a number of behaviors, such as male courtship song production, which has been predominantly studied acoustically. An immediate question with interesting implications is if there is any relationship between wing beats during courtship song and flight. A number of well-studied courtship mutants are available and correlational analysis may produce insight. One such mutant, *cac*, studied here, has well-characterized song defects (von Schilcher, 1976) and our study demonstrates its

severe consequences on key parameters involved in flight control. Motor programs involved in these two activities may be correlated in the same fly, using previously devised methods to induce courtship songs in tethered males (Ewing, 1977; Ewing, 1979).

Another well known, striking behavioral phenotype involving patterned wing beats is seizure, observed in certain bang-sensitive mutants following mechanical shock (Ganetzky & Wu, 1982; Burg & Wu, 2012) or in other genotypes induced by ECS (Lee & Wu, 2002). While DLM spiking during ECS-triggered seizures is well described, the activity in the direct flight muscles has not been monitored during these events. We have demonstrated that the wing buzzing patterns characteristic to individual genotypes during seizures may also be monitored acoustically (Fig 6), enabling correlation between DLM and direct flight muscle activities during another stereotypical behavioral repertoire.

In such, our findings may provide an opportunity to extend the scope of analysis across motor programs underlying different categories of behaviors. With the wealth of behavioral mutant collections, it is conceivable that future *Drosophila* studies will elucidate the common and distinct features of discrete programs driving different muscle groups during behaviors, such as flight, seizure, and other stereotypic motor activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Monitoring *Drosophila* wing beat frequency during flight via acoustic recording.

(A) Photograph of the tethered preparation. A fly is glued at the dorsal thorax-head junction to a tungsten pin. A high-gain microphone is placed directly below the fly and an air puff tube (used to initiate flight) is placed below and in front of the fly. (B) A sample microphone waveform trace during sustained flight. (C) Expanded trace of a single wing beat for simultaneous analysis. Numbered points correspond to high speed video stills in (D). (D) The sequence of high speed video stills corresponding to the points of the wave form in (C). (E) Spectrograms (left) and overall power spectrum densities (right) of sustained flight in two individuals (fn: 647, and fn: 751, see Materials and Methods for details on calculation). (F) Distribution of wing beat frequency for 21 flies sorted from lowest average frequency (167.0 Hz) to highest (214.4 Hz). Error bars represent the root mean square error of the WBF over the duration of the flight. Arrows indicate the WBF properties from the flies in (E).

Figure 2.

Wing beat frequency and indirect flight muscle spiking in two *Drosophila* species: *melanogaster* and *robusta*.

(A) Example trains of DLM spiking and corresponding microphone spectrograms during sustained flight in *D. melanogaster* and *D. robusta*. (B) Comparison of wing beat frequency (WBF) of sustained flight in the *melanogaster* CS line and two *robusta* lines (IR and SL), sorted from lowest to highest individual average WBF. Both *robusta* lines displayed decreased WBF as compared to *melanogaster*, and the SL line was significantly lower than the IR line. (C) Plots of the average DLM firing frequency from individuals of each line. Points are sorted in the same order as they are in (B). Both *robusta* lines showed increased DLM firing frequency relative to the *melanogaster* line. (D) Plot of the average coefficient of variation (CV) of the inter spike interval of DLM spikes during flight. Individual points are sorted in the same order as they are in panel (B). Higher CV values indicate less rhythmic spiking, while lower values indicate higher spiking rhythmicity. (***) $p < 0.001$, Kruskal Wallis test, rank-sum post hoc analysis).

Figure 3.

Air puff-triggered flight initiation in WT and mutant flies.

(A) Example air puff-induced flight initiation. Upper: trace indicating the 500 ms air puff, Middle: DLM spikes, Lower: corresponding microphone spectrogram. (B) Average time course of DLM spiking upon air-puff triggered flight (n=14 individuals, 37 air-puff triggered events). (C) Corresponding average time course of WBF upon air puff initiation. (D) Example of an abortive flight initiation in *slo*¹ fly, where sustained flight was not reached (DLM spikes above, spectrogram below). (E) Bar graph of the proportion of flies for the *cac* and *slo* alleles examined that displayed air puff-triggered flights greater than 10 s. Number of flies are indicated above each bar.

Figure 4.

Altered sustained flight DLM and wing beat frequency in *cac* and *slo* mutants.

(A) Example DLM spikes and corresponding microphone spectrogram from WT, Ca²⁺ channel mutant *cac*^{NT27}, and the BK K⁺ mutant *slo*⁹⁸ during sustained flight. (B)

Distribution of wing beat frequency (WBF) for two *cac* alleles *S* and *NT27*, as well as an allele of *slo* (*slo*⁹⁸) plotted from lowest to highest, in a similar manner to Fig. 2B. Both alleles of *cac* displayed significantly higher WBF compared to WT, while *slo*⁹⁸ was not significantly different from WT. (C) Distribution of average DLM firing frequencies for the corresponding individuals in (B). Both *cac*^S and *cac*^{NT27} showed highly increased DLM

firing rates during flight as compared to WT, while *slo*⁹⁸ did not. (D) Distribution of the CV of DLM spikes during flight for the respective individuals plotted in a manner similar to Fig. 2C. (*p < 0.05, ***p < 0.001, Kruskal Wallis test, rank-sum post hoc analysis).

Figure 5.

Diverse range of flight phenotypes observed in Shaker channel mutants.

(A) Representative DLM spikes and corresponding microphone spectrograms for WT, as well as the null alleles *Sh^M*, *Hk^{IE18}*, and *qvr^{EY}*. (B-D) Distribution of wing beat frequency (B), sorted from lowest individual WBF to highest, average DLM firing frequency (C), sorted in the same order as (B), and DLM firing CV (D), sorted in the same order as (B) for the *Sh*, *Hk*, and *qvr* alleles examined. *Sh^M* and *Sh¹²⁰* showed increased median WBF compared to WT, as did *Hk^{IE18}* and *qvr¹*. Only *Hk^{IE18}* showed a lower DLM firing rate. The auxiliary subunit mutants *Hk¹*, *Hk^{IE18}*, and *qvr^{EY}*, showed significant increases in the CV of DLM firing. (*p < 0.05, ** p < 0.01, ***p < 0.001, Kruskal Wallis test, rank-sum post hoc analysis).

Figure 6.

Electroconvulsive stimulation-induced wing buzzing dissociates wing beats from DLM Firing.

(A) High frequency electroconvulsive stimulation across the brain triggers a stereotypic pattern of wing buzzing corresponding to a seizure discharge (panel adapted from Lee & Wu, 2002). (B) DLM activity and microphone activity during electroconvulsively triggered seizure discharges in WT (2.0 s ECS stimulation) as well as in the bang-sensitive mutants *sda* and *eas* (2.0 and 0.5 s ECS stimulation respectively). During WT discharges, wing buzzing was also evident in the microphone spectrograms monitored over the same time period. However, in *sda* and *eas* mutants DLM spikes were observed after wing buzzing initially stopped, and a second buzz was observed that was unaccompanied by DLM spikes.