Distinctions among electroconvulsion- and proconvulsant-induced seizure discharges and native motor patterns during flight and grooming: quantitative spike pattern analysis in *Drosophila* flight muscles

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ABSTRACT

In *Drosophila*, high-frequency electrical stimulation across the brain triggers a highly stereotypic repertoire of spasms. These electroconvulsive seizures (ECS) manifest as distinctive spiking discharges across the nervous system and can be stably assessed throughout the seizure repertoire in the large indirect flight muscles dorsal longitudinal muscles (DLMs) to characterize modifications in seizure-prone mutants. However, the relationships between ECS-spike patterns and native motor programs, including flight and grooming, are not known and their similarities and distinctions remain to be characterized. We employed quantitative spike pattern analyses for the three motor patterns including: (1) overall firing frequency, (2) spike timing between contralateral fibers, and (3) short-term variability in spike interval regularity (CV) and instantaneous firing frequency (ISI⁻¹). This base-line information from wild-type (WT) flies facilitated quantitative characterization of mutational effects of major neurotransmitter systems: excitatory cholinergic (Cha), inhibitory GABAergic (Rdl) and electrical (ShakB) synaptic transmission. The results provide an initial glimpse on the vulnerability of individual motor programs to different perturbations. We found marked alterations of ECS discharge spike patterns in terms of either seizure threshold, spike frequency or spiking regularity. In contrast, no gross alterations during grooming and a small but noticeable reduction of firing frequency during *Rdl* mutant flight were found, suggesting a role for GABAergic modulation of flight motor programs. Picrotoxin (PTX), a known pro-convulsant that inhibits GABA_A receptors, induced DLM spike patterns that displayed some features, e.g. left-right coordination and ISI⁻¹ range, that could be found in flight or grooming, but distinct from ECS discharges. These quantitative techniques may be employed to reveal overlooked relationships among aberrant motor patterns as well as their links to native motor programs.

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Introduction

For nearly half a century, *Drosophila* mutants have opened the avenue to the mechanistic elucidation of how genetic variations contribute to the physiological basis of epileptiform behaviors and related seizure disorders (Benzer, 1971; Judd, Shen, & Kaufman, 1972; Kaplan & Trout, 1969). Early mutants were isolated on the basis of seizures triggered by stressors such as high temperature (e.g. *shibire*, Grigliatti, Hall, Rosenbluth, & Suzuki, 1973; seizure, Jackson, Wilson, Strichartz, & Hall, 1984) or mechanical shock (e.g. *bang-senseless*, easily shocked, knockdown and *bang-sensitive*, Ganetzky & Wu, 1982), while other mutants displayed spontaneous spasms (e.g. *Shudderer*, Williamson, 1982). Mutational studies have led to identifications of individual genes associated with seizure disorders, including those encoding for or regulating ion channels (Kawasaki, Felling, & Ordway, 2000; Loughney, Kreber, & Ganetzky, 1989; Marley & Baines, 2011; Titus, Warmke, & Ganetzky, 1997), ionic transport and distribution (Hekmat-Scafe, Lundy, Ranga, & Tanouye, 2006; Melom & Littleton, 2013; Palladino, Bower, Kreber, & Ganetzky, 2003), and enzymes related to energy metabolism (Fergestad, Bostwick, & Ganetzky, 2006; Royden, Pirrotta, & Jan, 1987; Trotta, Rodesch, Fergestad, & Broadie, 2004; Zhang et al., 1999).

Different adult preparations (Lee & Wu, 2002; 2006; Pavlidis & Tanouye, 1995; Salkoff & Kelly, 1978) have been employed to monitor seizure activity in the nervous system. In particular, the indirect flight muscles, dorsal longitudinal muscles (DLMs), have been most widely utilized as readout of aberrant motor activities, especially during seizure discharges. High-frequency stimulation across the brain can trigger an electroconvulsive seizure (ECS, Pavlidis & Tanouye, 1995) with a highly stereotypic repertoire consisting of an initial spike discharge (ID), a period of paralysis, and a second delayed spike discharge (DD, Lee & Wu, 2006).
A number of activity patterns driven by the innervating DLM motor neuron (DLMn) have been characterized in detail, including flight, grooming, courtship and giant-fiber mediated jump-and-flight escape. During flight, the DLM fibers (six on each side) undergo myogenic, stretch-activated, isometric contractions to generate tension in phase with the oscillation of thorax case to power wing depression during the wing beat cycles (~200 Hz, Dickinson & Tu, 1997; Iyengar & Wu, 2014). DLM action potentials, evoked by DLMn spikes, fire continuously and rhythmically at approximately 5 Hz (Harcombe & Wyman, 1977; Levine & Wyman, 1973) solely to facilitate Ca\(^{2+}\) influx required for continued oscillation in muscle tension (Gordon & Dickinson, 2006; Lehman, Skandalis, & Berthe, 2013). In contrast, discrete individual spikes are associated with giant-fiber pathway activation (Card & Dickinson, 2008; Engel & Wu, 1992, 1996; Tanouye & Wyman, 1980; Trimarchi & Schneiderman, 1995), while distinct spike bursting activities are associated with grooming (Engel & Wu, 1992) and courtship song production (Ewing, 1977).

In this report, we quantitatively describe the firing patterns of the DLM during electroconvulsion-induced seizure discharges versus the native spiking patterns during flight and grooming. In addition to ensemble statistics, we analyzed bilateral coordination of motor activities, as well as the local temporal features of spike trains, including instantaneous firing rate, short-term firing regularity and timing relationships among adjacent spikes.

We further examined how activity patterns characteristic of each motor programs were affected upon genetic or pharmacological manipulations. We studied well-characterized mutants of major neurotransmission systems known to have clear alterations in physiology and behavioral expression: (1) picrotoxin (PTX), a non-competitive antagonist of the GABAA receptor (Takeuchi & Takeuchi, 1969); (2) a related receptor mutant, Resistant to dieldrin (Rdl) that encodes the GABAA receptor α subunit (Ffrench-Constant, Mortlock, Shaffer, MacIntyre, & Roush, 1991); (3) Choline acetyltransferase (Cha) mutants with reduced acetylcholine (ACh) synthesis (Gorczyca & Hall, 1984; Greenspan, Finn, & Hall, 1980); and (4) mutants of ShakB, encoding an innexin gap junction protein required for transmission at electrical synapses (Thomas & Wyman, 1984). These treatments revealed clear quantitative distinctions between the seizure discharge patterns evoked by ECS and that induced by GABA receptor blockade via PTX. Thus, the quantitative approaches undertaken here may be applicable to the study of additional aberrant DLM IDs to explore their potential association with or distinction from the endogenous spike patterns during normal motor activities.

**Materials and methods**

**Fly strains**

*Drosophila melanogaster* strains were kept in standard glass vials containing cornmeal medium (Frankel & Brosseau, 1968) and reared at room temperature (23 °C). Adult flies used for the experiments were 3–12 d old. Wild-type (WT) flies were of the strain Canton S (CS). Synaptic transmission mutants examined include those alleles defective in Cha encoding a Choline Acetyltransferase (Cha\(^{12}\), Greenspan et al., 1980), Rdl encoding the GABA\(_A\) receptor a subunit (Rdl\(^{MD-RR}\), Ffrench-Constant et al., 1991), and ShakB encoding the structural subunit of an innexin (Shak\(^{b2}\), Honyk & Grigliatti, 1983; ShakB\(^{D2}\), Thomas & Wyman, 1984, also known as ShakB\(^{g}\), Lindsley & Zimm, 1992). Data from male and female individuals were pooled, as previous results have indicated that DLM firing properties are not generally different between sexes during flight or seizure activity (Iyengar & Wu, 2014; Lee & Wu, 2002, 2006).

**Tethered preparation and DLM recording**

The fly tethering procedure used for DLM recordings has been described in detail previously (Iyengar & Wu, 2014; Lee & Wu, 2002; 2006). Flies were briefly cold- or ether-anesthetized and glued to a tungsten pin using cyanoacrylate or nitrocellulose based glue. Flies were allowed to recover from anesthesia for at least 30 min prior to recording. All recordings were done at room temperature (~23 °C).

Electrolytically sharpened tungsten electrodes were used to access DLM spikes, with an electrode inserted into the dorsal-most fiber (DLM\(_a\), 45a, c.f. Miller A., 1950), and a similarly constructed electrode inserted into the abdomen for reference. Signals were amplified by an AC amplifier either DAM-5A (World Precision Instruments, New Haven, CT) or AM Systems Model 1800 (Carlsborg, WA) and digitized via a data acquisition card at a sampling rate >10 kHz (DIGIDATA 1200, Axon Instruments, San Jose, CA, or USB 6210, National Instruments, Austin, TX). Spike detection and analysis were done in PCclamp 6, LabVIEW 8.6 and in MATLAB r2017b using custom-written scripts.

**Electroconvulsive stimulation**

Electroconvulsive stimulation was delivered across the brain via tungsten electrodes inserted into each eye and consisted of a train of 0.1-ms pulses delivered at 200 Hz at a specified voltage (30–100 V) and train duration (0.5–4 s). Seizure susceptibility of individual flies was examined for each genotype (Figure 6) using five different intensity levels 1–5 (stimulus voltage, train duration): 1. (50 V, 0.5 s); 2. (50 V, 1.0 s); 3. (50 V, 2.0 s); 4. (100 V, 2.0 s); and 5. (100 V 4.0 s). Each level corresponds to a progressive doubling of stimulation intensity (Lee & Wu, 2006). When appropriate, after the electroconvulsive stimulation, test pulses sufficient to directly activate the giant-fiber neuron (24 V or higher, 0.1 ms duration, delivered at 1 Hz) were applied to examine synaptic transmission along giant-fiber pathway.
Pharmacology

PTX was used to block ionotropic GABAergic signaling (Lee & Wu, 2002). Two methods were employed to administer PTX systemically in intact, tethered flies: (1) small droplet applied on an eye incision (‘eye drop’), and (2) a fixed volume injection into the dorsal vessel (‘DV injection’). For eye drop application, a stimulation electrode was advanced to penetrate the basement membrane of the retina and then retracted. A small drop of 1 mM PTX solution dissolved in 0.6% NaCl solution (or Control NaCl solution without PTX) was applied to the incision and was allowed to diffuse to the hemolymph. The DV injection procedure was adapted from Howlett and Tanouye (2013). A filamneted glass electrode (1.0 mm OD, 0.58 mm ID, A-M Systems #601500) was pulled in a Brown-Flaming electrode puller (P87, Sutter Instruments, Novato, CA). After loading the distal end of the electrode with PTX solution, capillary action consistently drew 0.33 μl of solution to the tip. To inject the loaded solution, the electrode tip was broken and was inserted into the dorsal vessel (Miller A., 1950) of the tethered fly. The solution was then injected using air pressure delivered manually through a syringe.

Spike train analysis

Firing rate was analyzed using two measures: instantaneous firing frequency and overall spiking rate over a specified time window of interest. The instantaneous firing frequency was defined as the reciprocal of the inter-spike interval (ISI) measured in seconds, between successive spikes (Knight, 1972; Lansky, Rodriguez, & Sacerdote, 2004), referred to as ISI\(^{-1}\) with units of Hz. For sequential ISI\(^{-1}\)s in a spike train, the occurrence of the first spike is used to mark the temporal location of the particular ISI. The overall spike rate was defined as the total spike count during the specified time window divided by its duration. Poincaré trajectories (Figures 4, 7, and 9) were constructed by plotting the time series of ISI\(^{-1}\) within a spike train, with each ISI\(^{-1}\) against the ISI\(^{-1}\) of the subsequent interval, i.e. ISI\(^{-1}\) vs. ISI\(^{-1}\)+1. Therefore, for a spike train of n spikes, there will be n-1 data points in the Poincaré trajectory.

To quantify the regularity of ISIs within a spike train, the coefficient of variation (CV), defined as the standard deviation of ISIs divided by the mean ISI, has been extensively used (e.g. Werner & Mountcastle, 1963). However, to accurately estimate variability, the average firing frequency of the spike train is usually assumed to be stationary over a sufficiently long period of firing for which the CV is computed. To assess variability in spike trains with varying firing frequency over relatively short time periods, Holt and associates developed CV\(_2\) which estimates variability between adjacent spike intervals. The CV\(_2\) is computed for each spike sequentially along the spike train from each pair of two adjacent intervals ISI, and ISI\(_{i+1}\) as:

\[
CV_{2i} = 2 \frac{|ISI^{-1}_{i} - ISI^{-1}_{i+1}|}{(ISI^{-1}_{i} + ISI^{-1}_{i+1})}
\]

For cases of spike trains with stationary average firing rates over time, estimates of variability derived from CV and CV\(_2\) are in close agreement (Holt, Soffky, Koch, & Douglas, 1996).

For each spike train, the scatterplot of the ISI\(^{-1}\) vs. CV\(_2\) documents the local firing frequency (or ISI\(^{-1}\)), and firing frequency variability (CV\(_2\)) around individual spikes (represented as points in the 2-D plot). After connecting temporally adjacent spike-points, the trajectory thus traces the history of the spike-interval variability along with the sequential instantaneous spike firing frequencies. Note that the plot of the ISI\(^{-1}\) vs. CV\(_2\), may be derived from the Poincaré trajectory, which registers sequentially the ISI\(^{-1}\) before and after each individual spike throughout the same spike train.

For assessment of the general trends and for clarity in presentation, we applied a running average filter to smoothen the raw ISI\(^{-1}\) vs. CV\(_2\) plots. We verified that filter window sizes ranging from 3 to 9 pts provided qualitatively similar results without severely distorting major features of the trajectory for the three motor programs examined here. A 6-spike window was applied in ISI\(^{-1}\) vs. CV\(_2\) plots because it appropriately captures the characteristics for both sustained flight and short grooming bouts (Supplemental Figure 1).

The filtered ISI\(^{-1}\) vs. CV\(_2\) plots also enabled us to obtain smoothened ‘overall’ trajectories for the different motor patterns based on the ensemble of spike trains from recorded individuals, even though each train could differ somewhat in numbers of spikes. Briefly, durations of individual spike trains were normalized, such that ‘0’ represented the initial spike and ‘1’ the last spike, with the relative temporal locations for the rest of spikes reassigned proportionally along the normalized unitary duration. Thus, an overall average trajectory of a specific motor pattern could be constructed based on the collective temporal information from an ensemble of spike trains, i.e. to determine the averaged ISI\(^{-1}\) vs. CV\(_2\) values, or (ISI\(^{-1}\), CV\(_2\)), along the unitary duration. For a spike train in the ensemble, each rescaled ISI segment still carries the same (ISI\(^{-1}\), CV\(_2\)), associated with the ith spike. The averaging process for the ensemble (ISI\(^{-1}\), CV\(_2\)) was carried out with an increment of 0.01 (bin size) along the normalized duration. The resulting variation of (ISI\(^{-1}\), CV\(_2\)) along the normalized time base, or unitary duration, was re-plotted in the 2-D graph with ISI\(^{-1}\) vs. CV\(_2\) coordinates. The range of number of ISIs for this treatment was between ~60 and ~300 for DD, ~400 and ~1200 for flight, and 20 and ~500 for grooming spike trains. The outlier spike trains were eliminated from the calculation (mostly occurred in grooming).

The bilateral phase (θ) relation between individual spikes from the left and right DLM units was calculated as the proportion of the time point within the contralateral ISI that the ipsilateral unit generated a spike, i.e. θ ranges from 0 to 1 (Koenig & Ikeda, 1980; Levine & Wyman, 1973).

Results

Three identified motor programs driving DLM activity: electroconvulsive seizure discharges, flight and grooming

High frequency stimulation across the brain triggers a previously described ECS repertoire in WT and different mutants (Lee & Wu, 2002; Pavlidis & Tanouye, 1995), which consists
of an initial discharge (ID) of DLM spikes corresponding to an initial spasm, a period of paralysis where conduction fails along the giant fiber (GF) jump-and-flight escape pathway, followed by a delayed discharge (DD) of DLM spikes, and finally recovery of GF pathway transmission and voluntary motor behaviors. This study focuses on DD spike patterns since quantitative aspects of DLM spiking during the ID have been characterized (Fergestad et al., 2006; Iyengar & Wu, 2014) whereas spike patterning during the DD has received less attention (Lee & Wu, 2006). We noted that flies often display a ‘wings-up’ posture during DD (Figure 1(A); Supplemental Video 1, cf. Lee & Wu, 2002). During flight, the wings beat at approximately 200 Hz (Curtsinger & Laurie-Ahlberg, 1981; Iyengar & Wu, 2014; Lehmann & Dickinson, 1997), and during grooming, wing depression facilitates leg sweeping across the wing (Figure 1(A), Engel & Wu, 1992; Phillis et al., 1993; Seeds et al., 2014).

We observed characteristic patterns of DLM spiking corresponding with these behaviors (Figure 1(B)), each with distinct profiles of instantaneous firing frequency (defined as the reciprocal of each ISI, ISI$^{-1}$, with units Hz, Figure 1(C)). Electroconvulsion-induced DDs consisted of DLM activity of varying firing frequencies, which quickly accelerates, peaks (~20 Hz), and gradually decelerates (Figure 1(C)). During sustained flight, the DLM firing displayed highly regular spike intervals (hereafter described as ‘rhythmic’), whereas grooming spike patterns differed markedly, with short bursts of high-frequency spikes (ISI$^{-1}$$>$100 Hz in contrast to 5–10 Hz for flight) coincident with wing depression events.

Figure 2(A) depicts histograms of the ISI$^{-1}$ during the respective motor programs from a representative fly. It is evident that the three motor programs had clearly distinct frequency profiles. DD seizure spiking displayed a right-skewed ISI$^{-1}$ distribution with most spiking occurring between 1 and 10 Hz, with a clear ‘tail’ of vanishing activity and a brief session of fast-firing spikes, beyond 25 Hz, corresponding with the early peak of DD firing. During flight, the distribution of ISI$^{-1}$s was markedly less variable, reflecting a rhythmic firing. During grooming, DLM spiking displayed a highly-variable ISI$^{-1}$ distribution, with some short spike bursts exceeding 100 Hz – well above the maximum rate observed during either DD or flight.

With an ensemble of tens of flies, we examined firing rate regularity across the population for each motor pattern, by comparing the fractional distribution of the average ISI$^{-1}$ along the spike train against that of the overall firing rate (defined as the total spike count divided by recording duration, typically 60–120 s). In general, rhythmic firing produces similar results from the two measures and disparity between the two measures suggests highly arrhythmic firing. As Figure 2(B) illustrates, rhythmic DLM firing during flight produced highly similar distributions of firing rate based on the two measures (average ISI$^{-1}$ = 6.70 Hz, overall firing rate = 4.90 Hz), whereas grooming behavior was associated with irregular short bursts of a few spikes during, leading to drastically different rate distributions derived from the total spike count or from the instantaneous firing frequency (average ISI$^{-1}$ vs. overall firing rate = 16.62 vs. 0.55 Hz). The discrepancy between the two measures of firing rate during delayed seizure discharges (DDs) fell in between (average ISI$^{-1}$ vs. overall firing rate = 7.46 vs. 5.08 Hz).

**Interactions between bilateral DLM motor units during delayed seizure discharges, flight and grooming**

Analysis of spike timing relationships between left and right DLM pairs can reveal potential interactions between the
bilaterally symmetrical pairs of motor neurons that innervate them and can yield important clues to distinctive mechanisms of spike pattern generation among different motor programs. We recorded from top-most pair of DLMs (#45a, Miller A., 1950), each of which is innervated by a contralateral MN5 motor neuron in the mesothoracic ganglion (Coggshall, 1978). Of the three motor patterns examined here, relationships between bilateral DLM pairs during flight have been best characterized (Harcombe & Wyman, 1977; Koenig & Ikeda, 1980; Levine & Wyman, 1973), while the relationships during grooming and DDs have yet to be documented. Within individual flies, we found that for flight and DD, the firing frequency profiles of the left and right DLM units were not identical, but similar to each other (Figure 3(A), top two panels, data from a representative individual). In contrast, striking discrepancies in firing frequency between left-right DLM pairs were observed during grooming (Figure 3(A), bottom panel). In behavioral context, the left-right pairs perform coordinately during flight and DD, but can be decoupled during grooming.

We further quantified bilateral phase relationship of DLM firing in the three motor programs by using the protocol described by Koenig and Ikeda (1980). Briefly, the fraction of an ISI that elapsed prior to the contralateral muscle firing was measured to define the left to right (or right-to-left) phase from zero to one for individual spikes along the left (or right) DLM spike train (Figure 3(B), data from a representative individual, see also Methods). Histograms of the bilateral phase relationships between contralateral units appeared relatively unremarkable during DD, compared to the other two motor programs (Figure 3(C), derived from Figure 3(B)). An ensemble distribution created from DD spike trains across different flies did not indicate significant deviation from a uniform distribution (Figure 3(D), p>.05, χ²-test). However, during flight, the bilateral phase relations of DLM units displayed a characteristic spike exclusion, as indicated by the observation that immediately after a spike, the contralateral unit rarely spiked (±10% of ISI period, approximately 40 ms; arrows in Figure 3(D)). This ‘exclusion band’ feature was previously described by Levine and Wyman (1973), and may serve to differentiate flight-associated spiking from DD seizures within individual DLMs. In contrast to the relatively uniform distributions associated with DD and bilateral exclusion during flight, grooming spike activity was characterized by phase-correlated firing in the left-right DLM pairs, with most firing occurring within 10% of the ISI period on the contralateral side. Thus, our analysis of the phase relationship provides a quantitative indicator for degrees of independence, exclusion or coupling between bilateral pairs of DLMs during the respective motor programs.

**Poincaré and ISI⁻¹ vs. CV² plots: distinct signatures of rate and rhythmicity among DLM spike patterns**

In addition to the ensemble ISI⁻¹ distributions in a somewhat overlapping ranges (Figure 2), DD, flight and grooming spike patterns may be qualitatively distinguished based on differences in variation of ISI⁻¹ between adjacent spikes (Figures 1 and 3(A)). The temporal structure of sequential spike intervals may be distinct among these firing patterns, but are not retained in the firing frequency distributions and
left-right phase relationships (Figures 2 and 3(B,C)). Poincaré plots (also known as return maps, c.f. Longtin, 1993; Szucs, 1998) have been used to visualize spike interval dynamics, which may contain both deterministic and stochastic components. We plotted sequentially the ISI$_{n}$ each of spike interval against the subsequent interval (ISI$_{n}$ vs. ISI$_{n+1}$, hereafter designated as Poincaré trajectory or PT), to quantify sequential changes along the progression of a spike train (Figure 4(A)). When plotted in this manner, irregular spike trains will have data points deviating from the lower-left to upper-right diagonal to different degrees, depending on how abruptly the sequential ISI$_{n}$ changes. The degree of deviation of individual data points will depend on the magnitude of change between successive ISI$_{n}$'s. A spike train with gradual frequency-modulation with relatively small changes in successive ISIs will have data points wandering around the diagonal of the plot. A constant-frequency spiking train will have successive points in the PT converging to a single location on the diagonal. Within individual flies, grooming spike patterns displayed much higher variability in successive spike intervals in their PT, compared to both DD and flight patterns that showed lower ISI$_{n}$ to ISI$_{n+1}$ variability. Thus, the PT for individual grooming spike trains traversed abruptly in broad strides across the diagonal, whereas successive data points in DD clustered closer to the diagonal and flight to almost a point (Figure 4(A)), uncovering the special morphological features of the spike trains hidden in the above population-level firing frequency distributions (Figure 2(B)).

The above Poincaré trajectories clearly illustrated distinctions among the three motor patterns. However, multiple PTs cannot be readily treated to produce an ensemble trajectory based on larger sample sizes to highlight the global dynamic differences between motor patterns. We employed a transformation of the PTs to enable overlays of individual trajectories for construction of an 'average' trajectory across a number of individual trajectories (Figure 4(B)). It is known that the distributions of both instantaneous firing frequency and variability between successive spike intervals can be extracted directly from the Poincaré plot (Brennan, Palaniswami, & Kamen, 2001; Holt et al., 1996). Since deviations of the PT from the diagonal are related to variability of spike train intervals, one established treatment is to quantify the deviation from the diagonal along the local firing frequency adjacent to each spike in the sequence by adopting a measure for the instantaneous CV$_2$, defined as (2 |ISI$_{n}$ - ISI$_{n+1}$|)/(ISI$_{n}$ + ISI$_{n+1}$) (Holt et al., 1996, see also Methods). The transformed plot of the ISI$_{n}$ vs. CV$_2$, using the same sequential ISI$_{n}$ information, is essentially a re-scaling of the sequential deviations from the diagonal in the PT plotted against the ISI$_{n}$. After this transformation, high CV$_2$ values correspond with abrupt,
large changes in adjacent spike intervals in either direction, increasing or decreasing.

This treatment generates $ISI^{-1}$ vs. $CV_2$ plots that display clear distinctions among the three motor programs with an advantage of generating ensemble statistics for repeated trials (see Methods for averaging details). During DDs, average trajectory of $ISI^{-1}$ vs. $CV_2$ (Figure 4B, left panel, temporally color coded, average trajectory superimposed on individual trials in grey lines) illustrates a stereotypic trend of an initial acceleration ($ISI^{-1}$ from $\sim 7$ to $\sim 15$ Hz, blue segments) accompanied by increasing regularity, lower $CV_2$ ($<0.1$), which then gradually decreases in firing frequency as well as regularity with increasing $CV_2$ values (Figure 4B, left panel, cyan-green segment, climbing toward the upper-left corner, cf. Figure 1(C), left panel). For sustained flight, DLM spiking appears as a tight trajectory with relatively little drift within a confined region (Figure 4B, middle panel, $ISI^{-1} \sim 5$ Hz, $CV_2 \sim 0.03$, cf. Figure 1(C) middle panel). Notably, the initial phase of the DD trajectory and the flight trajectories occupy adjacent regions of low $CV_2$ values; although for DD (thick grey average trajectory re-plotted in Figure 4B for comparison), instantaneous firing frequencies are somewhat higher ($\sim 10$–$15$ Hz vs. $3$–$10$ Hz) across the population sampled. In contrast, for the case of grooming, DLM spiking trajectories occupy a region separate from either DD or flight, displaying significantly higher $CV_2$ values, usually above 0.5, consistent with the qualitative observations of arrhythmic firing punctuated with abrupt changes in $ISI^{-1}$ (Figure 4B, right panel cf. Figure 1(C) right panel). Thus, the temporal evolution of the spike patterns of the three motor programs is intrinsically distinct since the above quantitative treatment generates averaged trajectories that occupy three separable regions in the $ISI^{-1}$ vs. $CV_2$ plot.

**Differential vulnerability of three motor patterns to genetic perturbations of synaptic transmission systems**

To provide a first glimpse of the general vulnerability of spike patterns of the three motor programs to perturbations of the major synaptic transmission systems, we examined
mutants of three genes that have been well-established to regulate inhibitory, excitatory and electrical synapses in the CNS: resistant to dieldrin (Rdl, encoding the inhibitory GABA<sub>A</sub> receptor subunit; FFrench-Constant et al., 1991), choline acetyltransferase (Cha, acetylcholine synthesis enzyme; Greenspan et al., 1980) and ShakingB (ShakB, encoding innexin gap junction subunits; Crompton, Griffin, Davies, & Miklos, 1992; Homyk, Szidonya, & Suzuki, 1980; Phelan et al., 1998). These mutants have been well-characterized for their effects on the giant-fiber pathway, from the brain afferents to DLM output (Gorczyca & Hall, 1984; Lee & Wu, 2002; Thomas & Wyman, 1984). We specifically examined the Rdl<sup>MD-RR</sup> allele, a natural variant of Rdl that has developed resistance to the insecticide dieldrin, which also displays enhanced GABAergic inhibition (FFrench-Constant, Williamson, Davies, & Bass, 2016; Wang, Zhang, Rocheleau, FFrench-Constant, & Jackson, 1999; Zhang, FFrench-Constant, & Jackson, 1994). The Cha allele examined, Cha<sup>12</sup> displays defects in cholinergic transmission at room temperature, even though its enzymatic activity is more severely disrupted at elevated temperatures (Gorczyca & Hall, 1984; Greenspan et al., 1980). Both ShakB alleles studied here (ShakB<sup>2</sup> and ShakB<sup>pas</sup>) are known to severely compromise signal transmission along the GF pathway that involves identified electrical synapses (Phelan et al., 1996; Thomas & Wyman, 1984).

We first examined instantaneous firing frequency of the three motor patterns (cf. Figure 1). Notably, DD discharges in the respective mutants showed distinct defects (Figure 5(A)). A substantial reduction was observed in both the number of spikes and the peak instantaneous firing frequency in Rdl mutants with enhanced GABA<sub>A</sub> receptor function (mean peak ISI<sup>−1</sup> of 6.4 vs. 13.0 Hz for WT, Figure 5(Aii)). Interestingly, disruption of excitatory cholinergic transmission in Cha mutants also led to a reduction in instantaneous firing frequency (ISI<sup>−1</sup>, 6.3 Hz), comparable to Rdl mutants. In contrast, altered electrical transmission in ShakB mutants did not significantly alter the peak ISI<sup>−1</sup> in the DD (Figure 5(Aii)). However, the qualitative temporal profile was more resistant to these genetic manipulations, since across the three mutants, the rate of rising to the peak firing frequency remained unaltered, despite the clear modifications in their firing frequencies (Time to Peak, Figure 5(Aiii)).

Compared to the clear effects on DD spike trains, the flight and grooming pattern generators showed much higher resilience to the same perturbations of the above transmission systems. During flight, only Rdl mutants displayed a modest reduction in firing frequency compared to WT counterparts (mean ISI<sup>−1</sup> of 5.9 Hz vs. 8.2 Hz, respectively, Figure 5(B)). For grooming spike patterns, which is intrinsically highly variable, we did not detect significant differences among the mutants tested (Figure 5(C)), with average mean ISI<sup>−1</sup> ranging from 0.39 to 1.22 Hz across genotypes, and maximum ISI<sup>−1</sup> observed between 92 and 120 Hz.

Given the clear alterations in ISI<sup>−1</sup> profiles of DD spike trains compared to the other motor patterns, we further characterized the impacts of Rdl, Cha and ShakB mutations on other parameters of ECS discharge repertoire (Figure 6(A), cf. Lee & Wu, 2006). Specifically, we examined the stimulation threshold to trigger DD, the onset timing of DD, its duration, and the duration of transmission failure along the GF pathway following electroconvulsive stimulation. While WT and Rdl displayed similar thresholds and DD durations (Figure 6(B,C)), Cha and ShakB were apparently hypoexcitable as indicated by significantly higher ECS thresholds, and the reduced DD duration (mean duration of 10.0 and 9.4 s, respectively) than WT and Rdl (13.3 and 11.8 s, Figure 6(C)). Notably, only Cha affected the onset time of DD (22.7 vs. 18.3 s for WT, Figure 6(D)), which has been seen only in a subset of hyperexcitability mutants (Lee & Wu, 2006). In contrast, all three mutants examined displayed prolonged periods of GF failure upon electroconvulsive stimulation, as determined by DLM failure to respond to 1-Hz brain stimuli (Figure 6(A), cf. Lee & Wu, 2002). Modest increases in DLM failure was observed in Rdl and Cha (36.8 and 36.0 vs. 26.0 s for WT) and extreme lengthening in ShakB (>80 s, Figure 6(E)), a powerful demonstration for the important role of electric synapses in the transmission along the GF pathway that mediate the escape reflex (Engel & Wu, 1992; Phelan et al., 1996; Thomas & Wyman, 1984; Trimarchi & Schneiderman, 1995).

Poincaré plots and CV<sub>2-ISI</sub> maps as distinct signatures for electroconvulsive seizure spike patterning in synaptic transmission mutations

The diverse range of alterations to DDs evident in the above analyses (Figures 5 and 6) is corroborated by characteristic differences further revealed through Poincaré plots and CV<sub>2-ISI</sub> trajectories of DD spike trains (cf. Figure 4) in neurotransmission system mutants (Figure 7). We found the reduction in peak firing rate of DD in Rdl mutants (Figure 6(A)) was accompanied by an increase in irregular firing, indicated by relatively large deviations from the lower-left to upper-right diagonal of Poincaré plot (Figure 7(A)). The same spike trains plotted on ISI<sup>−1</sup> – CV<sub>2</sub> phase trajectory (cf. Figure 3(B)) also illustrate more variable trajectories, lacking a general characteristic trajectory compared to WT (Figure 7(B)). In contrast, Cha mutants showed somewhat less irregular firing and ISI<sup>−1</sup> vs. CV<sub>2</sub> trajectories. However, both Rdl and Cha DD trajectories appeared to be confined to region with a modest ‘left-shift’ compared to WT, consistent with decreased ISI<sup>−1</sup> values described above (cf. Figure 5(A)). Although ShakB displayed a similar peak ISI<sup>−1</sup> value compared to WT flies (Figure 5(A)), the Poincaré plot indicates more erratic variability in firing frequency within DDs (Figure 7(A)), which is also illustrated in the ISI<sup>−1</sup> vs. CV<sub>2</sub> trajectories, with many trajectories scattered over a wide range (Figure 7(B)). However, the average trajectory suggests similar firing frequency range even though ShakB displayed shorter trajectories compared to WT (Figure 7(B)).

Picrotoxin blockade of inhibitory GABA<sub>A</sub> receptors: evolving seizure activity patterns from flight-like firing to bursting discharges

The enhanced GABA<sub>A</sub> receptor function by the Rdl<sup>MD-RR</sup> mutation reduced firing rate and altered DLM spike patterning
without detectable changes in ECS threshold (Figures 5 and 6). We examined the effects of suppressing the GABAergic system on motor patterns, including seizure-like behaviors. Ingestion of PTX, a non-competitive antagonist of ionotropic GABAA receptors (Chen, Durkin, & Casida, 2006) results in spontaneous spasms that are accompanied by DLM IDs (Lee & Wu, 2002). We extended this previous observation to carry out quantitative comparisons between PTX- and ECS-induced discharges, DD. We found that application of a small drop of PTX solution (1 mM, ~0.1 μl) to an incision on the compound eye resulted in abnormal DLM spiking, starting within 10 min of application (Figure 8(A)). Initially, DLM firing appeared rhythmic, with spiking frequencies somewhat higher than those observed during tethered flight (mean ISI<sup>−1</sup> ~10–20 Hz, Figure 8(B), cf. Figure 1(B)). Over the course of 30 min, short bursts of spikes emerged (Figure 8(A))

**Figure 5.** Modifications of DLM firing characteristics during DD, flight and grooming activity in WT, and the mutants of GABAA receptor, RdI, choline acetyltransferase, Cha and electrical synapse, ShakB. Instantaneous firing frequency (left) and sample population statistics (right) in indicated genotypes. (Ai) Representative instantaneous firing frequency plots during DD. (Aii) Box plots of peak instantaneous firing frequency. (Aiii) Time to peak frequency in the respective mutants. (Bi) Sample plots of instantaneous firing frequency during flight in the respective mutants. (Bii) Box plots of mean instantaneous firing frequency. (Biii) Minimum frequency for the respective synaptic transmission mutants. (Ci) Sample plot of instantaneous firing frequency during grooming in the respective mutants. (Cii) Mean and (Ciii) Maximum instantaneous firing frequencies during grooming. Boxes represent the 25th, 50th and 75th percentiles while whiskers indicate 5th and 95th percentiles of the distribution. Arrowheads indicate distribution mean. Sample sizes as indicated. (**p<.01, ***p<.001, Mutant vs. WT, Students t-test).
consistent with the previous report on PTX-fed flies (Lee & Wu, 2002). The ISI$^{-1}$ of spikes within these bursts sometimes exceeded 100 Hz (Figure 8(B)), and the ISIs were highly variable. These two modes of PTX-induced spiking activity are referred to as ‘flight-like’ and ‘bursting’ activity, respectively, below. Importantly, the sequence of PTX-induced DLM spiking was gradual and continuous. Furthermore, distinct from ECS-evoked seizure pattern, a period of paralysis followed by a stereotypic discharge pattern was missing in PTX-induced motor activity, suggesting two different modes of seizure activity.

An analysis of the phase relations between bilateral pairs of DLM units revealed striking distinctions between the flight-like and bursting spike patterns induced by PTX (Figure 8(C,D)). Flight-like firing displayed a relatively uniform phase distribution, with a clear spike-exclusion band approximately ±10% of contralateral spike (Figure 8(D)), resembling the observations during sustained flight (Figure 3(D)). Bursting spiking activity, in contrast, displayed a phase-relation histogram with most firing occurring in-phase, resembling the bilateral relationships observed during grooming (Figure 8(D), cf. Figure 3(D)).
In addition to the application of PTX via an incision in the compound eye, we adopted a second procedure, dorsal vessel injection (DV injection, Howlett & Tanouye, 2013) to investigate whether a global, systemic application of PTX could induce additional spiking patterns. The dorsal vessel serves as a major pulsatile organ which circulates hemolymph (Miller T., 1985), and injected solutions circulate throughout the fly within a few seconds. We injected a fixed volume of solution marked with blue #1 dye (0.33 µl) in the tethered fly (Figure 9(A); Supplemental Video 2) to ensure the systemic spread of PTX throughout the abdomen thorax and head. We found the two approaches induced the same sequence of spike patterns even though DV injection triggered these events on an accelerated timescale. As Figure 9(A) shows, PTX injection led to a stereotypic repertoire of a brief wing buzz followed by a ‘wings-up’ posture (see also Supplemental Video 2). Consistent with eye application, PTX injection between 50 and 100 µM induced flight-like DLM spike patterns which evolved into bursting activities within seconds to minutes (Figure 9(B)). At concentrations of 200 µM or above, we found the flight-like firing pattern was skipped and progression of burst discharge patterns was observed within seconds of injection (Figure 9(B)).

The two categories of PTX-induced spike patterns are clearly contrasted by their trajectories in Poincaré plots (Figure 9(C)). The flight-like spike patterns displayed PTs which were relatively compact, with occasionally sharp deviations, corresponding to spike doublets or brief gaps in firing, which were notably absent during sustained flight in WT flies (compare to Figure 4(A)). Bursting spike patterns displayed strikingly different PTs, characterized by stereotypic recurrent sequences of successive ISI$^{-1}$ values,
Figure 8. DLM spiking patterns evoked by picrotoxin application. (A) Representative traces of DLM spikes triggered by PTX applied to a punctured eye (see Methods for details) at 10 min intervals over 40 min. Note the transition from rhythmic ‘flight-like’ pattern to repetitive burst discharges. (B) Plots of $\text{ISI}^{-1}$ during flight-like and bursting periods from a single individual. Spiking in the left and right DLMs is indicated by open and closed circles respectively. (C) Corresponding phase relations between left and right DLM spiking during the flight-like and bursting states shown in B. (D) Histogram of bilateral phase relations in (C). Note the similarity between the PTX-evoked flight-like and burst firing with respectively the phase relations during flight and grooming shown in Figure 3(C,D).

Figure 9. Rapid effects from systemic dorsal vessel (DV) injection of picrotoxin. (A) Video frames illustrating behavior and posture from a fly before and after 100 μM PTX DV injection. A transient wing ‘buzz’ was often observed, and flies displayed a ‘wings-up’ posture for several hours before death. (B) Representative spiking traces from three individual flies injected with either 50, 100 or 200 μM PTX, respectively. Traces illustrate the sequential events, evolving from flight-like to bursting patterns, at different times after injection as indicated. At 50 μM, discrete inter-burst gaps did not fully develop, and at 200 μM, the initial flight-like state was not captured and spike activity (7 s) rapidly evolved into bursting activity. (C) Poincaré plots of DLM spiking displaying flight-like or bursting spike patterns (~5 s) from two representative individuals (100 μM PTX inject). (D) $\text{ISI}^{-1}$ vs. $\text{CV}^2$ trajectories from ~ 5 s portions of the spike trains shown in C during the flight-like and bursting DLM spiking phases.
corresponding to recursive bursts. Plots of the ISI$$^{-1}$$ vs. CV$_2$ served to further contrast these features (Figure 9(D)). PTX-induced flight-like firing displaying a more stable phase trajectory with CV$_2$$\sim$0.1, and ISI$$^{-1}$$ higher than flight ($\sim$10–20 Hz), whereas bursting appeared as a looping trajectory. Each burst cycle started with a relatively lower ISI$$^{-1}$$ and high CV$_2$, then accelerated to high ISI$$^{-1}$$ and lower CV$_2$, corresponding to the long inter-burst interval followed by the fast peaking and slow decay of the firing frequency within the burst.

**Discussion**

Due to the ease of electrical monitoring, coupled with the tractable genetic manipulations in *Drosophila*, the indirect flight muscles, DLMs, have been frequently used for studying consequences of mutations of identified genes on membrane excitability and synaptic transmission (for early examples, see Elkins, Ganetzky, & Wu, 1986; Salkoff & Kelly, 1978; Salkoff & Wyman, 1981; Siddiqi & Benzer, 1976). Mutational analyses of a number of motor programs that drive DLM activity have also been carried out. DLM responses have enabled characterization of the GF pathway-mediated jump-and-flight escape reflex triggered by visual or vibrational stimulation (Engel, Xie, Sokolowski, & Wu, 2000; Engel & Wu, 1992; 1996; 1998; Gorczyca & Hall, 1984; Kadas, Tzortzopoulos, Skoulakis, & Consuolas, 2012; Mu, Bacon, Ito, & Strausfeld, 2014; Phelan et al., 1996) which leads to escape-related wing depression (Trimarchi & Schneiderman, 1995; von Reyn et al., 2014). Additionally, DLM recording has enabled detailed genetic analyses of the pattern generators that drive flight (Brembs, Christiansen, Pfluger, & Duch, 2007; Elkins & Ganetzky, 1988; Iyengar & Wu, 2014), grooming (Engel & Wu, 1992; Melzig et al., 1996; Seeds et al., 2014), and electroconvulsively induced seizure discharges, including the ID, and DD have also been carried out (Kuebler et al., 2001; Kuebler & Tanouye, 2000; Lee & Wu, 2002; 2006; Parker, Padilla, Du, Dong, & Tanouye, 2011; Pavlidis & Tanouye, 1995).

In this study, direct comparisons of spike patterning aim at an initial qualitative and quantitative delineations among three distinct modes of DLM spiking associated with DD, flight and grooming motor programs.

**DLM-Activity signatures associated with seizures, flight and grooming motor patterns**

The spiking activity of the six DLMs on each side of the fly thorax is driven by five motor neurons in the mesothoracic ganglion, with the lower four muscles (DLMc-f) each innervated by a single motor neuron (MN1 – 4) on the ipsilateral side, while the top two muscles (DLMa and DLMb) are innervated by a single contralateral motor neuron, MN5 (Coggshall, 1978; Ikeda & Koenig, 1988; King & Wyman, 1980). The MN5 is particularly well characterized, in terms of neurite outgrowth (Consuolas, Restifo, & Levine, 2002; Fernandes & VijayRaghavan, 1993; Hebbar & Fernandes, 2004) as well as excitability (Ryglewski, Kilo, & Duch, 2014). Individual Ca$$^{2+}$$ (Ryglewski, Lance, Levine, & Duch, 2012) and K$$^+$$ (Ryglewski & Duch, 2009) currents within the MN5 soma have been characterized, and their contributions to action potential generation computationally modeled (Herrera-Valdez et al., 2013). Notably, the MN5 and the DLMa,b fibers are capable of following direct stimulation one-to-one well beyond 100 Hz (Elkins et al., 1986; Engel & Wu, 1992). Thus, recording from DLMa or DLMb faithfully registers the MN5 activity, facilitating the analyses of a variety of motor patterns spanning a wide range of firing frequencies.

To quantitatively differentiate DLM spiking patterns associated seizure discharges, flight and grooming activities (Figure 1), we characterized several parameters to capture salient features of spike firing frequency (Figure 2), spike timing relationships between contralateral units (Figure 3), and the evolving trajectory of varying firing regularity and frequency during episodes of spiking activity (Figure 4). The instantaneous firing frequency, ISI$$^{-1}$$ has been extensively utilized to quantify DLM spiking frequency (e.g. Koenig & Ikeda, 1980; Wyman, 1965), and our analysis revealed distinctive ISI$$^{-1}$$ distributions during the three respective motor programs. Further, the spike timing relationships between the left and right units (Figure 3), as well as temporal characteristics revealed by Poincaré plots and ISI$$^{-1}$$-CV$_2$ trajectories clearly separate these firing patterns into distinct categories (Figure 4).

Consistent with previous reports (Harcombe & Wyman, 1977; Koenig & Ikeda, 1980), we noted that the left-right phase distribution of flight spike trains displayed ‘exclusion band’ in which synchronous firing between the bilateral DLM pair is very rare. In contrast, during DD spiking no discernable phase relation in timing between the left and right units was observed, while grooming spikes tended to be phase-correlated (Figure 3). These findings suggest that the exclusion band is specific to flight motor patterns, although the precise cellular and molecular mechanisms remain to be uncovered. Indeed, electrical coupling between motor neurons (Koenig & Ikeda, 1980), or inhibitory glutamate channels (gluCl, Liu & Wilson, 2013) has been proposed to mediate this mutual feedback inhibition.

Among DD, flight and grooming, a readily apparent qualitative distinction is the ISI variation in their spike patterns, with low variability associated with flight, high variability with grooming, and DD in between (Figure 1). To contrast the spike interval variability associated with the three motor patterns, we employed two related approaches to analyze spike trains, Poincaré plots (Figure 4(A)) and of the ISI$$^{-1}$$ vs. CV$_2$ plots (Figure 4(B)). Poincaré plots have been frequently utilized in characterizing variability in heartbeats (e.g. Goldberger, Rigney, & West, 1990; Huikuri et al., 2000) and in neuronal action potentials (e.g. Avila-Akerberg & Charcon, 2011). We found that Poincaré plots succinctly display the differences in spike interval variability among the three types of spike patterns. Because the Poincaré trajectories from individual spike trains cannot be readily combined to produce an ‘average’ ensemble trajectory, we utilized the instantaneous CV$_2$ (Holt et al., 1996, Figure 3(B)) to compile the merged, ensemble spike interval variability across a
Neurotransmitter systems in shaping seizure discharges and native motor patterns

Despite their obvious importance, a more comprehensive characterization remains to be accomplished to reveal the involvement of individual neurotransmitter systems in generating flight, grooming, and ECS-related activity in Drosophila. We aimed to provide an initial glimpse of the general vulnerability of the respective motor patterns to perturbations of excitatory, inhibitory and electrical transmission systems. The results also provide a context for the modulatory role of biogenic amines on these motor programs that have been examined to some depth (Brembs et al., 2007; Sadaf, Reddy, Sane, & Hasan, 2015; Yellman, Tao, He, & Hirsch, 1997). We selected mutant alleles with well-described perturbations of neurotransmission. Specifically the Cha and ShakB flies displayed clear disruptions of cholinergic and electrical transmission respectively along GF pathway (Gorczyca & Hall, 1984; Thomas & Wyman, 1984), a robust neuronal circuit critical for escape behaviors (Card & Dickinson, 2008; Engel & Wu, 1996; Tanouye & Wyman, 1980; Trimarchi & Schneiderman, 1995; von Reyn et al., 2014). For the Rdl mutant, the insecticide resistance phenotype has led to identification of the GABA<sub>A</sub> receptor gene and this particular allele that has been extensively studied (Ffrench-Constant et al., 2016). Studies in cultured neurons have demonstrated altered sensitivity to GABA<sub>A</sub> blockade (Lee, Su, & O’Dowd, 2003; Zhang et al., 1994).

Given the importance of these synaptic transmission systems on activity in the nervous system, it was somewhat unexpected that relatively minor modifications to flight and grooming patterning were uncovered (Figure 5(B,C)). Indeed, we observed that these mutant flies were capable of flight and grooming. Due to the intrinsically high variability across grooming spike trains, it is difficult to conclusively quantify any minor differences caused by the mutations. During flight, however, it was clear that only Rdl mutants displayed a reduction in DLM firing frequency, supporting the hypothesis of direct feedback regulation among DLM motor neurons underlying flight patterning (Harcombe & Wyman, 1977; Koenig & Ikeda, 1980). Conceivably, such feedback interactions may be mediated via GABA<sub>A</sub>ergic interneurons.

ECS discharges, in contrast to flight and grooming motor activities, appeared to be quite sensitive to disruption of neurotransmission systems. Interestingly, perturbations to GABA<sub>A</sub>ergic, cholinergic and electric synaptic systems each led to alterations in overlapping but not identical subsets of DD parameters (Figures 5–7). We found that enhancing GABA<sub>A</sub>ergic effectiveness in the Rdl allele and suppressing cholinergic transmission in Cha mutations severely reduced the peak firing frequency of DDs (Figure 5(A)), while Cha and the gap junction mutant ShakB displayed increases in the threshold to induce a DD seizure with a corresponding shortened DD duration. Consistent with their integral role of transmission along the GF pathway, mutations in these transmission systems also prolonged the period of circuit failure following the high-frequency electroconvulsion stimulation. In particular, none of the ShakB mutants recovered over the >80s recording period (Figure 6). Notably, our analysis of DD in ShakB corroborates findings of a previous report of increased seizure thresholds in the same mutant alleles (Song & Tanouye, 2006). The alterations in spike patterning during DD were also evident in that Rdl and ShakB displayed more variable Poincaré plots and distinct ISI<sup>−1</sup> vs.

Figure 10. ISI<sup>−1</sup> vs. CV<sub>2</sub> plots reveal distinctions in DLM spike patterns during flight, grooming and seizure discharges. Summary of averaged ISI<sup>−1</sup> vs. CV<sub>2</sub> trajectories of DLM spiking during different motor patterns. Insets: Representative traces illustrating different spiking activities associated with the distinct trajectories occupying different regions of the ISI<sup>−1</sup>-CV<sub>2</sub> plot. (A) Trajectories of ‘native’ spike patterns during flight and grooming. (B) Trajectories of electroconvulsive stimulation- and PTX-evoked seizure spike patterns. Arrow heads indicated trajectory direction during seizure discharges.
CV2 trajectories compared to WT and Cha counterparts (Figure 7).

**Distinct modes of seizure discharges induced by electroconvulsive stimulation and GABAergic blockade**

Electroconvulsive stimulation and proconvulsant administration are two means for seizure induction commonly used in both vertebrates and invertebrates (Löschler & Schmidt, 1988). Several studies have utilized the proconvulsant PTX, a non-competitive GABA_A antagonist, to induce seizure activity in *Drosophila* (Giachello & Baines, 2015; Lee & Wu, 2002; Stilwell, Saraswati, Littleton, & Chouinard, 2006). However, the resulting seizure discharge pattern was reported to be qualitatively distinct from the ECS discharge repertoire (Lee & Wu, 2002). We found that PTX application evoked a stereotypic, evolving DLM activity patterns (Figures 5 and 6). Unlike DD which occurred over a discrete period (~15 s), PTX-evoked activity continued throughout the recording period (>1 h), invoking the impression of the release or run-away of certain native, patterned activity. Initially, the DLM displayed a spontaneous, rhythmic spiking pattern reminiscent of flight activity in terms of firing frequency and the phase relation between left and right DLM spiking (compare Figure 4 ‘flight’ and Figure 5 ‘flight-like’). This mode of activity was succeeded by bursts of spikes with instantaneous firing frequencies exceeding 100 Hz during which spiking was generally synchronized between the left and right sides. Loss of central inhibition is known to release a number of motor programs in insects (Harris, Pfeiffer, Rubin, & Truman, 2015), with a notable example of decapitation-triggered courtship motor programs in preying mantids (Roeder, 1935). Therefore, it is tempting to speculate that automatisms observed in this study during ECS- and PTX-induced seizures may represent two separate forms of synchronized activities ‘released’ by specific treatments (Geier et al., 1976; Jasper, 1964; Noahtar & Peters, 2009).

As summarized in Figure 10(B), the ISI^−1 vs. CV2 plot of the spike trains elucidates distinctions between DD and PTX-evoked activity indicating separate neurophysiological dysfunctions. The distinction between electrically and pharmacologically triggered discharges has also been noted in vertebrate seizure models. Indeed, the expression of seizure-like behavioral sequence evoked by pentylentetrazole (a GABA_A receptor antagonist) is distinct from seizure discharges evoked by maximal electroconvulsive stimulation in rats (Luttjohann, Fabene, & van Luijtelaar, 2009; Racine, 1972).

These results serve to illustrate the importance to provide precise descriptions of seizure induction methods to avoid confusion in the literature when studying the molecular and cellular mechanisms in generating motor phenotypes. Furthermore, in future neurogenetic studies of ‘seizure’ behavior in *Drosophila*, a combination of pharmacological and electrical seizure induction methods can enhance the latitude and depth of analysis for the roles of individual genes participating in distinct mechanisms underpinning seizure activity.

Our study also demonstrates that in conjunction with spike firing rate and frequency distribution analyses, Poincaré plots adequately characterize the ISI variation along individual spike trains. The stochastic nature of spike trains, with ISI sequences varying from trial to trial within the same fly and between individuals, some inherent features characteristic of a particular motor pattern may be masked or overlooked. Nevertheless, the ability to construct CV2 for individual spike trains, which allows to derive an averaged ensemble trajectory pooled from a population flies, may serve as signatures in the ISI^−1 vs. CV2 plot for the various motor patterns and to delineate how they are modified by genetic or pharmacological manipulations.

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