Mutation of orthologous prickle genes causes a similar epilepsy syndrome in flies and humans

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Abstract

Objective: Genetically tractable fruit flies have been used for decades to study seizure disorders. However, there is a paucity of data specifically correlating fly and human seizure phenotypes. We have previously shown that mutation of orthologous PRICKLE genes from flies to humans produce seizures. This study aimed to determine whether the prickle-mediated seizure phenotypes in flies closely parallel the epilepsy syndrome found in PRICKLE patients.

Methods: Virtually all fly seizure studies have relied upon characterizing seizures that are evoked. We have developed two novel approaches to more precisely characterize seizure-related phenotypes in their native state in prickle mutant flies. First, we used high-resolution videography to document spontaneous, unprovoked seizure events. Second, we developed a locomotion coordination assay to assess whether the prickle mutant flies were ataxic. Third, we treated the mutant flies with levetiracetam to determine whether the behavioral phenotypes could be suppressed by a common antiepileptic drug.

Results: We find that the prickle mutant flies exhibit myoclonic-like spontaneous seizure events and are severely ataxic. Both these phenotypes are found in human patients with PRICKLE mutations, and can be suppressed by levetiracetam, providing evidence that the phenotypes are due to neurological dysfunction. These results document for the first time spontaneous, unprovoked seizure events at high resolution in a fly human seizure disorder model, capturing seizures in their native state.

Interpretation: Collectively, these data underscore the striking similarities between the fly and human PRICKLE-mediated epilepsy syndromes, and provide a genetically tractable model for dissecting the underlying causes of the human syndromic phenotypes.

Introduction

The fruit fly Drosophila melanogaster has been used to study seizure disorders for well over two decades.1–3 Flies are genetically tractable, with a rapid generation time, and the fly community has at its disposal a variety of tools to facilitate seizure research. First, hypomorphic and loss-of-function mutations exist for greater than 2/3 of all fly genes,4 and the relatively straightforward genetics of flies makes it possible to combine seizure-promoting mutations with other potential suppressor or enhancer mutations in order to study genetic pathway interactions. Additionally, a deficiency kit exists which contains large numbers of deletion mutants covering virtually the entire genome, and this kit can also be used to screen for genomic regions showing modifier effects to the seizure phenotype.5 Second, RNAi lines which can be expressed in particular structures at particular times using the...
UAS-Gal4 system, exist for approximately 90% of all genes, making it possible to perform gene knockdowns in only the CNS, for example, with less worry regarding lethal effects to the organism.

Early on, a class of mutants was identified that showed seizure-like behaviors after a brief mechanical stimulation (usually via vortex); this assay was referred to as the bang sensitivity behavioral assay, or “bang test,” and the mutants were referred to as bang-sensitive paralytic mutants. A range of phenotypes was observed after mechanical stimulation, including initial seizure-like behaviors such as leg shaking and wing flapping oftentimes followed by a paralysis phase before additional rounds of seizure-like activity were observed. The rate of seizure recovery followed by subsequent climbing behavior of the flies in the vial was then used to “quantify” fly seizures. Paired with these behavioral studies, electrophysiology methods were developed to evoke seizure-like activity in adult flies by direct stimulation of the brain in combination with flight muscle recordings. These experiments led to the discovery that bang-sensitive flies had reduced seizure susceptibility at the electrophysiological level. The first paralytic (para) mutant (para$^{ps}$) was identified as a conditional temperature-sensitive paralytic mutant, while para$^{ps}$ and para$^{ps2}$ alleles were shown to render flies seizure-resistant.9,10 The identification of para (the single sodium channel-encoding gene in Drosophila with multitudes of isoforms due to alternative splicing9,11) was particularly exciting given that many human epilepsies (such as Dravet syndrome and GEFS+ (Genetic Epilepsy with Febrile Seizures plus)) are caused by mutations in sodium channel genes (including SCN1A), suggesting that Drosophila might be a good proxy for studying human seizure disorders. Along these same lines, other fly ion channel gene mutants were identified which caused hyperexcitability phenotypes.13–15

Although these initial studies were critical in identifying the first class of seizure-prone mutants in flies, they were somewhat restricted by both the limited number of available behavioral mutants to screen, as well as limitations of the bang test assay in identifying different aspects of seizure-like activity. Recently, in an attempt to better model human seizure disorders in flies, two para mutants were generated in the laboratory which specifically altered amino acid residues previously shown to cause either Dravet syndrome or GEFS+. Strikingly, both fly mutants exhibited seizure phenotypes which were most severe at elevated temperatures, suggesting that very specific missense mutations causing epilepsy in humans could be engineered into flies to produce similar phenotypes. These studies provided a springboard for the current work where we have begun to explore the behavior of fly seizure disorders at an even higher level of resolution.

Mutations in prickle orthologs from flies to humans cause seizure disorders. The fly prickle (pk) gene encodes two postembryonic protein isoforms, Prickle-prickle (Pk$^{pk}$) and Prickle-spiny-legs (Pk$^{ple}$). Recently, we showed that the Pk isoforms influence microtubule (MT) growth dynamics and polarity, in addition to modulating vesicle transport, in larval neurons, and that reducing the levels of Pk$^{ple}$ (but not Pk$^{pk}$) predisposed the flies to seizures. We also showed that enhanced anterograde transport was the cause of the seizure phenotype, which could be suppressed by reducing the levels of either of two kinesin anterograde motor proteins. This study was the first to show that mutations in an ortholog of a known human epilepsy gene produce seizures through enhanced anterograde vesicle transport. Although at present it is unclear how such altered transport is promoting a seizure phenotype, investigations are currently underway to address whether the seizure phenotype is due to differential activities of inhibitory versus excitatory neurons, altered distributions of neuronal components (i.e., increases in the concentrations of synaptic components), or whether altered transport is affecting connectivity of particular neuronal circuits. Kinesin inhibitors developed as potential anticancer medications are also being tested to determine whether they might suppress the seizure phenotype.

The first human PRICKLE mutation (in PRICKLE1) was identified in three families with myoclonus epilepsy-ataxia syndrome, which is characterized by early onset (~4 year) ataxia followed by later onset myoclonic seizures (5–10 year), both worsening with age. Subsequently, missense mutations were identified for both PRICKLE1 and PRICKLE2 in patients with myoclonus. Aside from the seizure phenotypes, ataxia (otherwise described as uncoordinated gait) was also noted. Here, we show that flies with $p^{ple}$ mutations not only exhibit unprovoked, spontaneous myoclonus-like seizure activity, but are also ataxic when compared to control flies of the same genetic background. Additionally, we show that reduction in an anterograde vesicle motor protein in the context of the $p^{ple}$ flies can suppress the ataxia, similar to its suppression of the seizure phenotype which we previously reported. Finally, treatment with levetiracetam (LEV), a human antiepileptic drug, can suppress both the spontaneous seizure as well as ataxia phenotypes in the $p^{ple}$ flies. Collectively, these data demonstrate that the PRICKLE-associated syndrome in humans is comparable to phenotypes observed in the fly, revealing a conserved role for Prickle in controlling multiple aspects of motor control.
Methods

Fly stocks

Stocks used were Oregon-R (OR, obtained from the Chun-Fang Wu laboratory, University of Iowa); easily shocked (eas^{2}) and hang senseless (para^{bar}) Outcrossed pk^{ple} and pk^{pk} flies were generated as previously described.21

Fly flip assay

Seven-day-old flies were transferred to an empty vial and the vial flug (fly plug; www.Flystuff.com) was pushed downward until a gap of 2 inches (from vial bottom to flug) was created, thus discouraging flies from climbing as well as preventing them from moving out of the camera frame so that individual flies could be tracked. Flies were then mechanically stimulated with a vortex mixer (Fisher Scientific Vortex Mixer; maximal setting of 10) for 5 sec. After vortexing, flies were digitally recorded for 1 min (from the start of the vortex); only flies that flipped on their back and remained at that position for at least one second were considered to be exhibiting muscle-jerk seizure activity.

Video-tracking locomotor and spontaneous seizure analysis

Newly enclosed flies were collected and aged for 7 days. Flies were individually placed into standard mating chambers using a manual aspirator and allowed to acclimate for 5 min. Fly behavior was recorded using a web camera for lower resolution (Logicool Quickcam IM, Logitech, Fremont, CA), or a Canon High Definition Vixia HF M31 Camcorder with zoom (resolution 1920 x 1080) for higher resolution. Images of individual flies were captured at up to 30 frames/sec for 10 min. We used pySolo 23 to track fly locomotion and computed x, y coordinates during every frame for a total of 9000 frames. The percentage of time a fly spent inside an inner circle (70% diameter of the chamber) during a 5 min observation period was calculated from its x, y coordinates. Turning angle was determined from the x, y coordinate data for three consecutive frames and presented in degrees.

The same videos recorded for the locomotor assay were analyzed for the spontaneous seizure events, while others were generated with higher resolution videography. We assessed the flies inside the mating chambers for abrupt involuntary movements for a total of 5 min. These involuntary movements usually manifest as rapid repositioning of the flies within the chamber. For each genotype, we calculated the total number of mutant flies that exhibited spontaneous seizure events and compared them to control flies using a Fisher’s exact test. For the seizing events per fly, we calculated the number of seizure events exhibited per fly compared to control using a student’s t-test.

Tripod synchrony assay

Males and females of each genotype were aged for 7 days after pupation and recorded in separate Petri dishes according to gender. Nonvortexed controls (OR) and homozygous mutant flies (pk^{ple}/pk^{ple}, pk^{pk}/pk^{pk}) were placed directly into 35 x 10 mm style Petri dishes containing nonscented clay formed into approximately 2.5 by 2 cm rectangles to contain the flies during recording. The flies were recorded for 1–3 min using a Sony 10.2 mega pixel Handycam. The videos were converted from 1920 x 1080 .MTS (the raw video format) to 640 x 360 .MOV in Quicktime and loaded in DIAS 3.4.1 to step through the movies and score individual leg movements.24 The trimmed videos contain segments where flies are walking linearly at a steady velocity, typically less than a second.

DIAS allows the trimmed segments to be viewed in 24 frames per second; on average the trimmed videos were 30 frames in duration. Once in DIAS, each frame was analyzed in Excel by assigning each leg to a column, and each leg was assigned an identifier (L1, L2, L3, R1, R2, R3), later organized into T1 (L1 R2 L3) and T2 (R1 L2 R3). Raised legs were assigned a “1” (out of contact with the Petri dish), while legs in contact with the Petri dish were assigned a “0”. Starting from frame one, each frame was analyzed and compared to the previous frame to determine position changes for each leg in T1 and T2. To aid in analysis, consecutive images were toggled back and forth from one frame to the next to determine foot position relative to the surface.

The Excel data were then converted into five graphs; front leg, middle leg, and back leg pairings, as well as left, right, left coordination (T1), and right, left, right coordination (T2). From these graphs, the number of coordinated versus uncoordinated frames was counted and recorded in a separate Excel file. Graphs of T1 were counted for coordination and separate graphs of T2 were also counted for coordination. Each graph was counted as a separate event and the two events were added together to obtain the total number of frames. The percentage of coordinated frames out of the total number of frames was assessed, and mutant flies were then compared to the controls using a Mann–Whitney test. Sixty videos in total were collected (10 males and 10 females from each genotype).

The same protocol as above was repeated for two sets of 60 flies (20 each of pk^{pk}/+, pk^{ple}/+, and the OR control), one analyzed without mechanical stimulation and a
second analyzed after 30 sec of vortexing to match the mechanical stimulation used in the bang sensitivity assays of previous experiments. Finally, a set of 60 \( pk^{sple}/Khc, pk^{sple}/+, Khc/+ \) flies (20 each) was vortexed for 30 sec and analyzed as indicated above.

**Drug treatment**

One gram of levetiracetam (Sigma-Aldrich, Saint Louis, MO; catalog number PHR1447-1G) was directly dissolved into 200 mL of cornmeal molasses fly food which had been liquefied by heating, for a final concentration of 5 mg/mL. The food was cooled so that it could be manually tolerated prior to adding of the drug, and the drug/food mixture was vigorously stirred to ensure uniform dispersion. The food was then pipetted into standard glass *Drosophila* vials (~10 mL) or bottles (~40 mL) and allowed to cool prior to use. Flies were kept on standard fly food for 4 days after eclosion, starved for 4 h in empty humidified vials, and then placed on the drug food for an additional 3 days, after which the flies were assessed for seizure activity or ataxia.

**Results**

\( pk^{sple} \) flies exhibit seizure-induced flipping behavior

We previously reported that both \( pk^{sple} \) homozygous and heterozygous mutant flies were seizure-prone using mechanical stimulation\(^{18,21} \) whereby flies are vortexed for a fixed time and then allowed to climb the sides of the vial; seizure-prone flies are delayed from beginning their climbing behavior since they must first recover from the seizure activity. We also noted that the \( pk^{sple} \) homozygotes were particularly hyperexcitable, oftentimes exhibiting what appeared to be spontaneous seizure activity in their vials. However, given that the \( pk^{sple} \) homozygotes exhibit planar cell polarity (PCP) defects on the legs, we sought to develop an alternative seizure assay for the homozygotes that could still capture muscle-jerk activity in these flies. We thus developed the "fly flip assay" which further modifies the mechanical stimulation assay to discourage the flies from climbing\(^{26} \) while assessing "flipping" behavior caused by muscle-jerk activity in the legs. Using this assay, we found that only the seizure-prone \( pk^{sple} \) homozygotes showed a significant increase in the percent of flipping flies per vial (\( P < 0.0001 \); Fig. 1A), demonstrating that this assay is robust in assessing muscle-jerk seizure activity in a known seizure-prone mutant.

\( pk^{sple} \) flies have increased seizure-associated spiking activities

In order to determine whether the \( pk^{sple} \) homozygotes possess electrophysiological abnormalities characteristic of other fly mutants used to study seizure disorders,\(^{10,27–29} \) we used the electroconvulsive stimulation (ECS) paradigm\(^{7,21,27,30} \) whereby an electrical shock is administered across the fly’s brain followed by recording of spike activity from

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Fly flip assay and electroconvulsive seizure stimulation paradigm demonstrate that \( pk^{sple} \) homozygotes are seizure-prone. (A) Percent of seizing flies per vial: \( pk^{sple}/pk^{sple} \) (\( sple/sple \)) flies show a significant increase in the percent of seizing flies, compared to the Oregon-R controls (\(+/+\)) or nonseizure-prone \( pk^{pk}/pk^{pk} \) (\( pk/pk \)) flies. Ten vials were used per genotype, each containing five males and five females. (B) Representative spiking activity traces indicating that both \( pk^{sple} \) heterozygotes and homozygotes exhibit strong, but similar, spiking activity compared to control flies. (C) Plots of spike counts triggered by ECS (60 V). Median spike counts: \(+/+ = 32; pk^{sple}/+ = 66; pk^{sple}/pk^{sple} = 62\). \(*P < 0.05, ****P < 0.0001, \) using Mann–Whitney U-test. ECS, electroconvulsive stimulation.
the dorsal longitudinal flight muscle (DLM). Both \(pk^{ple}\) heterozygotes, as previously reported,\(^{21}\) as well as \(pk^{ple}\) homozygotes, showed a significant but similar increase \((P < 0.05)\) in median spike counts (66 and 62 respectively) compared to OR control flies (32) (Fig. 1B and C), suggesting that once a seizure threshold is reached, the resultant seizure activity is comparable.

**\(pk^{ple}\) mutants exhibit abnormal locomotor activity and spontaneous, unprovoked seizures**

Next, we tested whether the \(pk^{ple}\) homozygous mutant flies showed abnormal locomotor activity by tracking the movement of individual flies using video-tracking locomotor analysis software.\(^{23,31–33}\) Thus, single flies were placed in circular chambers and movement was tracked for 5 min using videography (Video S1). The percentage of time a fly spends in the center of the chamber, as well as the average speed and turning angles of the fly, were then assessed. Wild-type flies have been shown to walk along the edges of the chamber, with very little deviation in movement.\(^{31}\) The tendency to avoid open spaces is not limited to invertebrates, as it has also been seen in rodents.\(^{31,34–37}\) Such center avoidance behavior in the fly is thought to be controlled by the mushroom body (a central region of the brain partially responsible for locomotor activity).\(^{31–33}\) Mutant flies exhibiting locomotor defects, on the other hand, have increased turning angles, tend to spend more time in the center, and show overall reduced activity, presumably due to the added effort to walk.\(^{31,38,39}\)

Only male and female \(pk^{ple}\), but not \(pk^{pk}\), homozygotes showed a significant increase in the percent of time an individual fly spent in the center of the chamber compared to controls (Fig. 2A, \(P < 0.001\) and < 0.01 for female and male, respectively). Additionally, only the \(pk^{ple}\) homozygotes showed a significant decrease in average activity (Fig. 2B, \(P < 0.05\) and < 0.01 for female and male, respectively) as well as increase in turning angle (>90°) compared to controls (Fig. 2C, \(P < 0.01\) for both female and male). These results demonstrate that the \(pk^{ple}\) mutation results in abnormal locomotor activity consistent with what would be observed in a fly with altered gait. However, since the increased turning angle could also indicate slight involuntary leg movements that reposition the fly, we thus reviewed the videos by eye and were intrigued by the observation that a significant proportion of the \(pk^{ple}\) homozygotes exhibited pronounced spontaneous muscle-jerk seizures (Video S1; top panel represents OR controls, bottom panel represents \(pk^{ple}\) homozygotes). A more extreme example of seizure activity can be seen in the \(pk^{ple}\) mating chamber 13 (Video S1), with the fly experiencing multiple seizures over the time imaged (a frame by frame depiction of a portion of this seizure sequence is portrayed in Fig. 3A and B), compared to OR controls and nonseizure-prone \(pk^{pk}\) homozygotes. Additionally, flies in several other mating chambers (including 4, 7, 8, and 9) showed mild to moderate myoclonic-like seizures. We thus quantified this behavior in both the \(pk^{pk}\) and \(pk^{ple}\) homozygotes and found that only the \(pk^{ple}\) homozygotes showed a significantly increased percentage of flies exhibiting spontaneous seizure activity compared to controls (Fig. 3C). Intriguingly, we also found that \(pk^{ple}\) homozygotes had a significant increase in discrete seizure events per seizing fly when compared to controls (Fig. 3D). Thus, \(pk^{ple}\) flies

![Figure 2](https://example.com/figure2.png)

**Figure 2.** \(pk^{ple}\) homozygous flies exhibit abnormal locomotor activities. (A) Both female and male \(pk^{ple}\) homozygous mutant flies show a significant increase in the time spent in the center of the arena compared to control flies. (B) Both female and male \(pk^{ple}\) homozygous mutant flies show a significant decrease in activity, which results in a reduction of total distance traveled. (C) The \(pk^{ple}\) homozygous mutant flies (both male and female) show a significant increase in turning angle >90°, which may be due to a sudden jerk or spontaneous seizure. Error bars = SEM; Mann–Whitney U-test was used to generate the \(P\) values. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\). The number of flies used in the experiment was between 14 and 56.
Analyzing Myoclonus and Ataxia Patterns in Flies

S.N. Ehaideb et al.
are not only more susceptible to initiating a seizure event, but also are likely to exhibit multiple seizure events after the first seizure. Importantly, unlike the fly flip assay where the seizures are evoked by mechanical stimulation, this analysis quantifies unprovoked, spontaneous fly seizure events that more closely mimic the spontaneous nature of human seizures.

In order to better assess the nature of the spontaneous seizure events we observed, we used high-resolution slow-motion videography of individual \(pk^{ple}\) homozygotes. We were able to identify two main classes of commonly observed seizure behaviors in the flies: Firstly, we observed what we refer to as the “rhythmic jerking” phenotype, whereby one or two legs of a fly (usually on the same side) are seen contracting and extending multiple times (see Video S2, 1st and 2nd clips). On occasion, a leg contraction may displace a second leg on the side of the contraction, thereby causing the fly to flip on its back (see Video S2, 3rd clip). Secondly, we observed what we refer to as the “severe seizure” phenotype whereby multiple legs are seen contracting and extending over a longer period of time (see Video S2, 4th clip). This class of seizure makes up approximately 5% of the observed seizures. Most of the observed phenotypes involve myoclonus (repetitive jerks of isolated muscle groups), and thus these seizures are similar in appearance to the myoclonic epilepsy phenotype observed in humans with \(PRICKLE\) mutations.$^{18,22,40}$

**easily shocked mutants exhibit spontaneous, unprovoked seizures**

In order to determine whether other previously identified seizure-prone fly mutants were susceptible to spontaneous, unprovoked seizures, we performed the mating chamber spontaneous seizure assay in \(eas^2\) and \(bang\) \(para^{bat}\) mutant flies and quantified seizure events.$^{1,2,41,42}$ As can be seen in Figure S1, the \(eas^2\) flies were indeed susceptible to spontaneous, unprovoked seizures, quantifiably similar to the \(pk^{ple}\) homozygous mutant flies. In contrast, the \(para^{bat}\) flies showed no evidence of unprovoked seizure activity compared to controls.

**pk\(^{ple}\) flies exhibit ataxia-like behavior**

Given the defects we uncovered by the locomotor assay, we developed an additional method to specifically assess coordination of locomotor movement in the \(pk^{ple}\) mutant flies to determine whether they were indeed ataxic. We placed flies into small Petri dishes positioned perpendicular to the ground and imaged them walking up the side of the dish closest to the camera. These data were then analyzed to monitor fly movement frame by frame. As has been observed by others, we noted that wild-type flies coordinate their locomotor activity such that, in general, the front right (R1), using conventional nomenclature), rear right (R3), and left middle (L2) legs move in unison, with the front left (L1), rear left (L3), and right middle (R2) legs moving in unison and in opposition to the others.$^{13,44}$ This type of movement creates two stable tripod configurations, and the walking behavior is referred to as tripod gait. We used frame by frame analysis to determine when the individual legs were in contact with the surface as well as time points when the legs were raised above the surface and calculated how often the legs for a particular Tripod configuration were similarly positioned (all three down, or all three up), allowing us to calculate the degree of synchronous movement. Only the \(pk^{ple}\) homozygotes showed reductions in synchronous movement compared to the other genotypes, (Fig. 4A,B; compare Movies 3, 4 and 5), demonstrating that these flies were indeed ataxic.

Next, we assessed whether the heterozygous \(pk^{ak}\) and \(pk^{ple}\) flies, which only have one functional copy of \(pk^{ak}\) or \(pk^{ple}\), respectively, were ataxic compared to controls. Upon administering of a brief mechanical vortex stimulation followed by synchronized walking analysis, only the \(pk^{ple}\) heterozygotes showed a significant reduction of coordinated locomotion (Fig. 4C and D; \(P < 0.01\) and 0.05 for female and male, respectively). Since a mechanical vortex is only required to elicit the ataxia in heterozygotes but not homozygotes, these data suggest that gene dosage plays a significant role in promoting the ataxia phenotype; namely, eliminating \(pk^{ple}\) produces a strongly penetrant phenotype, whereas reducing the amount of \(pk^{ple}\) by 50% requires an additional stressor (in this case, mechanical stimulation) to reveal the phenotype. These data are similar in pattern to what we have observed using the bang sensitivity behavioral assay whereby \(pk^{ple}\) heterozygotes are less seizure-prone than homozygotes.$^{18}$ although it is important to note that we only assay postvortexed flies that are actively walking and not seizing during the video monitoring.

![Image](https://example.com/image.png)

**Figure 3.** \(pk^{ple}\) homozygotes exhibit spontaneous, unprovoked seizures. (A) Still images showing normal circular walking for a representative OR (+/+) and \(pk^{ak}\) homozygous mutant (pk/pk) fly, as well as images for a spontaneously seizing \(pk^{ple}\) homozygous mutant (sple/sple) fly. (B) Cartoon illustration of the merged pictures in panel A. (C) Quantification of spontaneous seizures demonstrate that both male and female \(pk^{ple}\) homozygotes exhibit a significantly higher number of spontaneous seizures compared to control flies. (D) \(pk^{ple}\) homozygotes exhibit a significantly higher number of recurring seizure events compared to control or pk/pk flies. Eighty OR, \(pk^{ple}\), and \(pk^{ak}\) flies were used for analysis. Error bars = SEM. Fisher’s exact test and student’s t-test were used to generate the \(P\) values for (C) and (D), respectively. $^{**^{**}p < 0.0001$.
To determine whether we could suppress the ataxia by reducing the dosage of Kinesin heavy chain (Khc) antero-grade motor protein in the context of the sple heterozygotes, we created sple/Khc transheterozygotes which were subjected to a brief mechanical vortex stimulation followed by synchronized walking analysis. Notably, reducing the dosage of Khc in the context of the sple heterozygous mutation could fully suppress the ataxia (Fig. 4C and D), once again similar to the suppression of the seizure activity previously observed after mechanical stimulation.21

Although the pkple homozygotes were severely ataxic based on both the synchrony assay and the video evidence, we were concerned that at least a portion of this phenotype might be due to the planar cell polarity defects on the legs of the mutants. We thus treated adult pkple homozygotes with the human antiepileptic drug LEV, followed by testing the flies in the tripod synchrony assay. Importantly, the drug treatment was able to fully suppress the ataxia of the flies (Fig. 5A; compare Video S5 with Video S6). Similarly, LEV was able to suppress the unprovoked, spontaneous seizures we observe in this mutant, both at the level of reducing the percentage of seizing flies as well as the number of independent seizing events per seizing fly (Fig. 5C and D). Moreover, an increase in
overall activity levels was seen in females ($P < 0.01$), although males trended toward increased activity (Fig. 5B). These data suggest that, due to the antiseizure/ataxia properties of the drug, the flies were better able to initiate and carry out movements and provide compelling evidence that both the spontaneous seizure activities and ataxia are due to neurological dysfunction which can be pharmacologically corrected with a drug known to be effective on seizure disorders.

**Discussion**

Epilepsy is a generalized term to describe a variety of different syndromes and phenotypes, which may or may not include overt convulsive seizures. With regard to convulsive seizures, myoclonic seizures differ from clonic seizures in that the former oftentimes manifest as sporadic jerks which may or may not involve both sides of the body, whereas the latter tend to be repetitive and rhythmic in nature and are oftentimes more severe, involving both sides of the body (as seen in the classic “tonic-clonic”, or “grand mal”, seizure). Although great headway has been made in modeling human seizure activity in flies, there has been a paucity of experiments that attempt to correlate fly seizure behaviors with human seizure phenotypes. Using high-resolution slow-motion videography of the $pks$ mutant flies, we specifically observed phenotypes consistent with sporadic muscle-jerk, or myoclonic, seizures that were spontaneous and unprovoked, which closely parallel those exhibited by epileptic patients with PRICKLE mutations and which can be suppressed by a common antiepileptic drug, levetiracetam. Levetiracetam binds to synaptic vesicle glycoprotein 2A (SV2A), potentially modifying its function in vesicle exocytosis.45 This is particularly intriguing since we have previously shown that PRICKLE1 can directly interact with SYNAPSIN I, a protein that works with SV2A during synaptic vesicle exocytosis at synapses.46 Therefore, levetiracetam may be particularly effective in suppressing Prickle-associated seizures by targeting a direct link in the Prickle pathway.

We observed that $pks$ mutant flies, after exhibiting an initial seizure, were prone to a significant number of subsequent independent seizure events. Spontaneous seizure events could be observed in a second seizure-prone $eas^{2}$ fly mutant but not in $par^{bxx}$ mutants.41,42 The observation that seizures in $pks$ and $eas$ fly lines are unprovoked and do not require mechanical stimulation provides a model for seizures that more closely mimics the “natural state” of epileptic seizure types seen in humans. Future studies are planned to utilize this spontaneous seizure assay to screen for novel chemical compounds with anticonvulsant activities.

We also assayed locomotion in the $pks$ mutant flies, and found a significant reduction in coordinated movement resulting in ataxia, the second key aspect of the syndrome found in humans with PRICKLE1 mutations. Flies have diverged from humans around 500–600 million years ago, so it is striking that flies with prickle mutations exhibit both key features of the human disorder. Such data suggest a conserved prickle pathway as it relates to

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neuronal function, and this is further supported by the observation that Prickle mutations or knockdowns in mice and zebrafish also predispose these animals to seizures.\textsuperscript{18,47} We have recently provided additional evidence for a conservation of a seizure-associated prickle pathway by identifying another key gene whose interaction with prickle is conserved from fly to mouse (the deubiquitinase encoded by USP9X in mouse and human, and its ortholog fat facets, or \textit{fas}, in flies).\textsuperscript{26} Additionally, the antiepileptic drug, valproic acid, which has been effective in treating epilepsy patients harboring \textit{PRICKLE} mutations,\textsuperscript{48} suppresses seizure activity in \textit{pk}^{ple} mutant flies.\textsuperscript{18}

To examine ataxia, we developed the tripod synchrony assay to determine whether the tripod gait used by flies was coordinated in the \textit{prickle} mutant flies. We found that the homozygous (or mechanically stimulated heterozygous) seizure-prone \textit{pk}^{ple} mutant flies, but not the \textit{pk}^{ple} mutant flies, showed uncoordinated gait compared to controls. The \textit{pk}^{ple} homoyogotes had difficulty walking in a straight line (see Video S5), often dragging legs and moving only one or two legs at a time. Although these flies manifested the aforementioned PCP defects on their legs, the suppression of the ataxia phenotype by levetiracetam (Video S6) clearly demonstrates that these morphological defects were not the cause of the ataxia and lessens the possibility that the ataxia is due to a major developmental miswiring defect of neuronal circuits. It is thus more likely that dysfunction at the level of neuronal physiology, or wiring defects involving very specific classes of neurons, is the primary cause of the ataxia. Consistent with this idea, we only rarely observe miswiring of neurons in larval filet preps which we routinely prepare in order to assess bouton morphology. Additionally, our demonstration that enhanced antero-grade transport is likely the cause of both the seizure and ataxia phenotypes, both of which can be suppressed by reducing the dosage of genes encoding anterograde motor protein subunits,\textsuperscript{21} further drives the point that perturbations in neuronal physiology contribute to the observed motor defects.

How similar are fly seizure disorders to human seizure disorders, and do they involve the same sets of genes? Many orthologous genes or gene pathways have already been linked to both fly and human-associated seizures, including genes that encode calcium channel subunits,\textsuperscript{13,15,50} potassium channel subunits,\textsuperscript{13,15,50} the mTOR signaling pathway,\textsuperscript{51} and the synaptic vesicle recycling pathway.\textsuperscript{52–54} Of the three mutants assayed here for spontaneous seizure activity, two involve genes whose orthologs are known to be mutated in human epilepsies (\textit{prickle}, with human epilepsy-associated mutations in \textit{PRICKLE1} and \textit{parabss1}; \textit{para} \textit{bou}, with human epilepsy-associated mutations in \textit{SCN1A} \textsuperscript{55,56}). The third, \textit{easily shocked}, encodes the ethanolamine kinase enzyme which is involved in phospholipid metabolism. Although mutations in human orthologs of this gene have not yet been identified in epilepsy patients, exome sequencing efforts of large epilepsy cohorts have identified \textit{de novo} nonsynonymous mutations in several genes involved in phospholipid metabolism or biosynthesis, including \textit{AGPAT3}, \textit{PLA1A}, and \textit{TPTE2}.\textsuperscript{57,58} Additionally, \textit{CLN8} mutations, which have been shown to alter phospholipid levels, were identified in patients suffering from the epilepsy disorder ceroid-lipofuscinosis.\textsuperscript{59} Given that epilepsy is now regarded as a multigenic disorder likely involving hundreds of genes, the overlap between genes associated with fly and human seizure disorders will only continue to expand.

By bringing together both clinicians and basic scientists in this study, we have begun to explore the behavioral similarities between fly and human seizure disorders. Unprovoked, spontaneous seizure behavior has not been routinely assessed for fly seizure mutants; in fact, we are unaware of published studies that document and quantify such seizures at this level of resolution. Most studies focus on mechanical or electrical stimulation to evoke seizure activities,\textsuperscript{2} neither of which allows one to observe a seizure in its native state. Although we recognize that many human disorders could never be recapitulated in a fly model, a deeper understanding of fly seizures at the level of motor activity in the context of the various seizure-associated mutations may allow additional connections to be established between the human and fly seizure disorders; in cases where a correlation is made, the power of the \textit{Drosophila} genetic toolkit can be leveraged to tease apart the relevant genetic pathways, as we have begun to do for \textit{prickle} and the genes encoding Prickle interactors conserved in flies and vertebrates.\textsuperscript{18,26,46} It is important to note that, comparable to humans, the fly utilizes a complex network of excitatory and inhibitory neurons which are interconnected into circuits in order to control precise and accurate motor movements, using evolutionarily conserved neurotransmitters and their associated receptors. The ability to move in a coordinated, efficient fashion is no less important to the survival of a fly than it is to the survival of a human. Thus, it is perhaps not so surprising that mutation of orthologous motor control genes in “hard wired” evolutionarily divergent species yield similar neuropathological phenotypes.

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Author Contribution

S.N.E., T.K., and J.R.M. conceived and designed the experiments. S.N.E. performed and analyzed the fly flip locomotion parameter assays. S.N.E. and A.J.L. performed and analyzed the spontaneous seizure assay for the OR, pk+pk, and pkpk flies. E.A.W. performed and analyzed the tripod synchrony assay. J.K. performed the spontaneous seizure assay for the cas and para flies, which was analyzed by S.N.E. W.H.E., H.L.K., and J.R.M. performed the drug treatment experiment which was analyzed by H.L.K. and S.N.E. A.I. performed and analyzed the electroconvulsive seizure stimulation experiment. A.G.B. analyzed the high magnification seizure videos. S.N.E., E.A.W., J.K., W.H.E., A.I., H.L.K., A.J.L., A.G.B., T.K., and J.R.M. reviewed and interpreted the data. S.N.E. and J.R.M. wrote the paper.

Conflict of Interest

Dr. Iyengar reports grants from NIH, during the conduct of the study.

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Analyzing Myoclonus and Ataxia Patterns in Flies

S.N. Ehaideb et al.

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Individual control (OR; top) and \( pk^{ple} \) homozygous mutant flies (bottom) were assayed for locomotor activity in mating chambers. For the majority of the recorded time, the OR control flies walk along the edge of each chamber, whereas, \( pk^{ple} \) mutant flies are less active and spend a significant amount of time in the center of the chamber, both consistent with locomotor defects. In addition, the \( pk^{ple} \) mutants exhibit spontaneous, unprovoked seizure events, several of which are indicated with flashing red arrows. The seizure phenotypes varied from “weak” to “severe”; the fly in chamber 13 represents a severe seizure phenotype.

**Video S2.** \( pk^{ple} \) mutant flies exhibit myoclonus-like seizures. \( pk^{ple} \) homozygous mutant flies exhibit either a myoclonus-like rhythmic jerking phenotype involving only one or two legs (clips 1–3), or a more severe seizure phenotype, where multiple legs are observed jerking (clip 4); this latter event starts with jerking of a single leg, followed by involvement of other legs. All original video clips have been slowed down 10× to reveal leg movement patterns.

**Video S3.** OR control flies exhibit coordinated tripod gate. OR wild-type flies walk in a coordinated fashion using tripod gate, where the left front, middle right, and left back legs move in opposition to the right front, middle left, and right back legs.

**Video S4.** \( pk^{pk} \) mutant flies exhibit coordinated tripod gate. \( pk^{pk} \) homozygous mutant flies walk in a coordinated fashion using tripod gate, similar to OR controls.

**Video S5.** \( pk^{ple} \) mutant flies exhibit severe locomotor defects. \( pk^{ple} \) homozygous mutant flies have poorly coordinated leg movements, with inappropriate combinations of legs moving at the same time. In addition, the rear legs are oftentimes dragged while walking.

**Video S6.** The antiepileptic drug levetiracetam fully suppresses the ataxia in \( pk^{ple} \) mutant flies. \( pk^{ple} \) homozygous mutant flies treated with levetiracetam for 3 days exhibit coordinated tripod gate, similar to control flies.