Pyridox(am)ine 5′-phosphate oxidase (PNPO) catalyzes the rate-limiting step in the synthesis of pyridoxal 5′-phosphate (PLP), the active form of vitamin B6 required for the synthesis of neurotransmitters gamma-aminobutyric acid (GABA) and the monoamines. Pathogenic variants in PNPO have been increasingly identified in patients with neonatal epileptic encephalopathy and early-onset epilepsy. These patients often exhibit different types of seizures and variable comorbidities. Recently, the PNPO gene has also been implicated in epilepsy in adults. It is unclear how these phenotypic variations are linked to specific PNPO alleles and to what degree diet can modify their expression. Using CRISPR-Cas9, we generated four knock-in Drosophila alleles, hWT, hR116Q, hD33V, and hR95H, in which the endogenous Drosophila PNPO was replaced by wild-type human PNPO complementary DNA (cDNA) and three epilepsy-associated variants. We found that these knock-in flies exhibited a wide range of phenotypes, including developmental impairments, abnormal locomotor activities, spontaneous seizures, and shortened life span. These phenotypes are allele dependent, varying with the known biochemical severity of these mutations and our characterized molecular defects. We also showed that diet treatments further diversified the phenotypes among alleles, and PLP supplementation at larval and adult stages prevented developmental impairments and seizures in adult flies, respectively. Furthermore, we found that hR95H had a significant dominant-negative effect, rendering heterozygous flies susceptible to seizures and premature death. Together, these results provide biological bases for the various phenotypes resulting from multifunction of PNPO, specific molecular and/or genetic properties of each PNPO variant, and differential allele–diet interactions.

Significance

Both genetic and environmental factors contribute to epilepsy. Understanding their contributions and interactions helps disease management. However, it is often challenging to study gene–environment interaction in humans due to their heterogeneous genetic background and less controllable environmental factors. The fruit fly, Drosophila melanogaster, has been proven to be a powerful model to study human diseases, including epilepsy. We generated knock-in flies carrying different epilepsy-associated pyridox(am)ine 5′-phosphate oxidase (PNPO) alleles and studied the developmental, behavioral, electrophysiological, and fitness effects of each mutant allele under different dietary conditions. We showed that phenotypes in knock-in flies are allele and diet dependent, providing clues for timely and specific diet interventions. Our results offer biological insights into mechanisms underlying phenotypic variations and specific therapeutic strategies.

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a wide range of variation can be attributed to the differential effects of different mutations on the catalytic site, FMN binding, and/or protein folding and thermostability (17, 20, 21). While these in vitro studies show clear evidence that different PNPO mutations affect enzymatic activity to varying degrees, they do not explain the variation in seizure types, seizure onsets, and comorbidities manifested by PNPO-deficient patients carrying the same mutation (3, 17). Furthermore, PNPO variants have never been studied in vivo for their molecular or functional defects. It remains unknown whether they can also indirectly affect the gene’s function at transcriptional and/or translational levels. These molecular characterizations are crucial for understanding the phenotypic variation from a molecular perspective. Lastly, the dietary contribution to the phenotypic variations has not been systematically examined. Therefore, there is a need to develop in vivo systems to better understand individual PNPO variants, their potential contributions to phenotypic variations, and the effect of diet on the phenotype expression associated with each variant.

Drosophila melanogaster has been proven to be a valuable model system to study human diseases, including epilepsy (22–26). Our previous studies have also demonstrated that the PNPO gene is functionally conserved between humans and flies; similar to humans, PNPO deficiency in Drosophila also leads to seizures and premature death (27). Here, we generated four Drosophila knock-in (KI) strains carrying either wild-type (WT) human pyridox(am)ine 5'-phosphate oxidase (hPNPO) allele (hWT) or one of three hPNPO epilepsy-associated alleles hR116Q, hD33V, and hR95H. We examined KI flies at the molecular, circuity, behavioral, and organismal levels. In addition to the reported impaired enzymatic activity, we found that each mutant variant conferred a specific molecular effect; hD33V decreased messenger RNA (mRNA) level, hR95H reduced protein stability, and hR116Q altered the protein localization of PNPO in the brain. Furthermore, we observed a wide range of phenotypes in KI flies, including developmental impairments, behavioral hyper- or hypoactivity, spontaneous seizure discharges or abnormal firing patterns, and shortened life span. The phenotypic variation is associated with the known biochemical severity of these mutations and our characterized molecular defects. We also showed that diet treatments further diversified the phenotypes among alleles, and PLP supplementation at larval or adult stages prevented developmental impairments and seizures in adult flies, respectively. Finally, we found that hR95H had a significant dominant-negative effect, and heterozygous flies were prone to seizures upon electroconvulsive stimulation and showed increased lethality on the VB6-devoid diet.

Results

Generation of KI Drosophila Strains Carrying hPNPO Variants Identified from Epilepsy Patients. hPNPO spans ∼8 kbp in the genome and has 17 splice variants (28). The most ubiquitously expressed variant has seven exons and encodes a 261-amino acid protein (Fig. L4). In PNPO, two domains are required for the enzymatic activity; one is the oxidase domain located in the middle region, and the other one is the dimerization domain located at the C terminus. Functional PNPO is a dimer; the monomer has no enzymatic activities (14).

The Drosophila PNPO gene (sugarlath [sgll]) encodes two protein products using alternative transcriptional starts; they share the C terminus and differ at the N terminus, with one form containing nine extra amino acids (Fig. 1B and C) (10). The protein sequence of Drosophila PNPOs shares ∼45% identity and 75% similarity with hPNPO (Fig. 1C). Functionally, PNPO deficiency in flies causes seizures and premature death as it does in humans (16, 27). Both seizures and premature death can be rescued by ubiquitous expression of WT hPNPO (16, 27), suggesting that the molecules and signaling pathways underlying PNPO deficiency-induced seizures and premature death are conserved between humans and flies. Thus, Drosophila can be used as an in vivo system to study hPNPO variants identified in patients.

To date, more than 30 different hPNPO variants have been identified in neonatal epileptic encephalopathy patients and patients with early-onset epilepsy (Fig. L4). Although these mutations tend to spread across the hPNPO gene/protein, more variants are associated with oxidase and dimerization domains. Here, we generated four KI Drosophila alleles using CRISPR-Cas9, in which the Drosophila PNPO gene, sgll (10), was replaced by hPNPO cDNAs (Fig. 1B). These four KI alleles were
designated as \( h^{R116Q}, h^{D33V}, \) and \( h^{R95H} \). The three mutant alleles, \( h^{R116Q}, h^{D33V}, \) and \( h^{R95H} \), were chosen to represent different severities of PNPO deficiency based on in vitro biochemical studies of their corresponding mutant proteins (\( h^{R116Q}, h^{D33V}, \) and \( h^{R95H} \) hereafter). The residual enzymatic activities of \( h^{R116Q}, h^{D33V}, \) and \( h^{R95H} \) are \( \sim 80, 40, \) and \( 20\% \) of \( h^{WT} \), respectively (3, 18). The mutation in each allele was confirmed by Sanger sequencing (Fig. 1D). All KI alleles were initially balanced over a TM6B, Hs, Tb chromosome (TM6B hereafter) to circumvent potential homozygous lethality.

**Distinct Molecular Alterations Linked to Different hPNPO Epilepsy-Associated Variants.** Other than reducing the enzymatic activity (2, 3, 18), little is known about the molecular characteristics of hPNPO variants. Thus, after establishing the KI lines, we first studied whether these epilepsy-associated variants could affect hPNPO expression and localization using homozygous flies (SI Appendix, Figs. S1 and S2). The \( h^{R95H} \) allele was not included initially because \( h^{R95H} \) homozygotes were lethal (see below). To include all four KI alleles, we further crossed KI lines with \( w^{1183} \) to generate \(< 0.05; **P < 0.01. \) (D) Immunohistochemistry staining of hPNPO in the adult brain of \( h^{WT}, h^{R116Q}, \) and \( h^{D33V} \) homozygotes. The \( h^{R95H} \) line is not included due to the lethality of \( h^{R95H} \) homozygotes in the normal diet and the greatly reduced hPNPO protein level in their heads (Fig. 1B). A chimeric hPNPO protein could also be expressed, which contains the nine extra amino acids from sgll-RB in the front of the hPNPO protein. It is also possible that the multiple bands were due to protein modifications or proteolysis, as suggested in a previous study (14).

The reduced hPNPO protein level associated with D33V or R95H mutations could be due to changes at the transcriptional or translational level. We, therefore, examined the mRNA level of hPNPO in each genotype using qRT-PCR. Two pairs of primers that specifically targeted the N- or C-terminal regions of hPNPO cDNA were used. Results from both primer pairs showed that DI33V but not R95H led to a reduced mRNA level (Fig. 2C). Results from western blot demonstrated that the R116Q mutation did not change the hPNPO protein level, whereas D33V and R95H significantly reduced it (Fig. 2A and B and SI Appendix, Fig. S1 A and B). Interestingly, multiple bands instead of a single band appeared on the blot. The molecular nature of these bands is unclear. One possibility is that given only that the sgll-RB form was replaced in the KI allele (Fig. 1B), a chimeric hPNPO protein could also be expressed, which contains the nine extra amino acids from sgll-RB in the front of the hPNPO protein. It is also possible that the multiple bands were due to protein modifications or proteolysis, as suggested in a previous study (14).

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intrinsic to D33V. The mechanism of D33V in mediating the change is unclear.

Next, we examined the localization of hPNPO protein in the adult brain using immunohistochemistry staining with an anti-hPNPO antibody. Anti-Bruchpilot (Brp) antibody was used to visualize synapse-rich neuropils (31). We found that hWT was ubiquitously expressed in the brain with the strongest staining in the cell body rind, which comprises neuronal cell bodies and certain types of glial cells (Fig. 2D and SI Appendix, Fig. S4A) (32). The antibody is presumably specific to hPNPO as very faint staining was observed in w1118 control flies (SI Appendix, Fig. S5). There was little overlap between Brp and hPNPO staining, suggesting that hWT is not enriched in the terminal structures. Consistently, no hPNPO staining was observed in fiber bundles, such as anterior optic tract, inferior fiber system, and medial antennal lobe (AL) tract (SI Appendix, Fig. S4A). Moderate hPNPO staining was also shown in areas surrounding neuropils. One prominent region is the area surrounding the AL (Fig. 2D). The ALs are the primary centers for olfactory processing in flies, and they are organized into individual glomeruli. Each glomerulus contains synapses formed by three types of neurons: olfactory receptor neurons (ORNs), local interneurons (LNs), and projection neurons (PNs). While the cell bodies of ORNs are far away from ALs, the cell bodies of LNs and PNs surround ALs. The PNs extend their dendrites to glomeruli and receive excitatory input from ORNs to relay olfactory information to higher brain centers, such as the mushroom body calyx and the lateral horn of the protocerebrum; the LNs are interneurons, and most of them are GABAergic (33). Subcellularly, hPNPO mainly stayed in the cell bodies of surrounding neurons (SI Appendix, Fig. S4B). Furthermore, the staining of hPNPO did not overlap with DAPI, a nucleus marker, suggesting that hPNPO is mainly cytosolic. Notably, compared with the strong staining of hPNPO in cell bodies, faint hPNPO staining was also observed in the glomeruli region. However, it does not seem to overlap with the Brp staining (SI Appendix, Fig. S4B). Thus, the hPNPO staining in the glomerulus region was most likely from the dendrites of LNs or glial cells.

A similar hPNPO staining pattern was observed in hD33V brains (Fig. 2D), suggesting that D33V does not affect the protein localization in the brain. In striking contrast, strong terminal staining for hPNPO was detected in hR116Q brains (Fig. 2D and E). The increased staining appeared to occur in all terminal structures in the brain, with the most dramatic change in the AL (SI Appendix, Fig. S6). Since the hPNPO protein level in hR116Q is comparable with that in hWT (Fig. 2A and B), we speculate that the increased terminal staining is most likely caused by altered localization of hR116Q in the brain, at least in some cell types.

Taken together, molecular characterization of hPNPO variants in KI flies demonstrates that in addition to reducing the enzymatic activity as shown in previous studies (2, 3, 18, 19), hPNPO variants can also affect the gene’s function through altering the mRNA and/or protein level or the protein localization in the brain.

**Distinct Developmental Effects Associated with Different hPNPO Variants.** All balanced KI lines were viable and fertile. However, no homozygous KI adult flies were observed from hR350S/TM6B breeding bottles. To rule out the contribution of any off-target mutations, the KI line was backcrossed to w1118 for five generations. No homozygous hR350S pupae or adult flies were observed after backcross, suggesting that the lethality was most likely due to the R95H mutation in hPNPO.

To systematically study the effects of the R95H mutation as well as the two other mutations on development, we self-crossed each KI line and examined the number of homozygous KI flies in the F1 generation. The ratio of KI homozygous flies in all flies (homozygosity ratio) was further calculated. Consistent with our initial observations, no hR350S homozygotes were observed (Fig. 3A). A significantly decreased homozygosity ratio was also found from hD33V self-breeding. By contrast, the homozygosity ratio in hR116Q was comparable with that in hWT. Thus, these three mutant alleles have differential effects on development. This conclusion was corroborated by complementation tests (SI Appendix, Fig. S7).

**Allele-Dependent Diet Modifications of Life Span in KI Flies.** PLP is involved in a variety of biological processes (8), yet the potential cumulative effect of chronic PNPO deficiency has never been studied. We examined whether hR116Q and hD33V could affect the survival of adult flies on the normal diet. Survival data showed that hR116Q had a slightly shortened life span compared with hWT (median: 71 vs. 74 d for hR116Q and hWT, respectively; \( P < 0.01 \)) (Fig. 3B). In comparison, the life span of hD33V was much shorter (median: 56 d; \( P < 0.001 \)). The lethality of hR116Q and hD33V flies correlated well with the residual enzymatic activity of hR116Q and hD33V measured by in vitro studies (3, 18). Together, these studies demonstrate that even mild PNPO deficiency can have a long-term deleterious consequence, even in the presence of dietary VB6.

We have previously reported that PNPO-deficient flies (sg598) are short lived on the sugar-only diet (i.e., VB6-devoid diet) (27), suggesting that the sugar-only diet is useful for exacerbating PNPO deficiency. We thus generated homozygous (two same KI alleles), heterozygous (one hWT allele and one mutant allele), and transheterozygous (two mutant alleles) flies using four KI alleles and examined their survival on sugar. We found that the most dramatic lethal phenotype among these nine genotypes was from hD33V/hR95H flies (Fig. 3C), of which ~75% died by day 11. The
significant lethality in $h^{D33V}/h^{R95H}$ flies was not surprising since based on in vitro residual enzymatic activity studies, $h^{D33V}/h^{R95H}$ has the most severe PNPO deficiency. We also observed lethality from $h^{R116Q}/h^{R95H}$ and $h^{WT}/h^{R95H}$ flies; about 15% of them died by day 11 (Fig. 3C), suggesting that $h^{R95H}$ is indeed the most severe allele among all three mutant alleles. The fact that $h^{WT}/h^{R95H}$ showed lethality suggests that $h^{R95H}$ may cause haploinsufficiency or have a dominant-negative effect, which is unexpected because PNPO has been considered as autosomal recessive in heritance (https://omim.org). These two possibilities were further studied (see below).

Overall, these studies demonstrate that both PNPO variants and dietary conditions can affect survival of KI flies.

### Allele-Dependent Diet Modifications of Locomotor Behaviors of KI Flies

The $h^{R116Q}$ and $h^{D33V}$ homozygous flies did not exhibit noticeable behavioral deficits when reared on the normal diet (the cornmeal–yeast–molasses [CYM] media). Consistent with this observation, mutant homozygous flies traveled a similar total distance in an open-field arena, with comparable speed, percentage of active time, and speed correlation coefficient (SCC) compared with $h^{WT}$ homozygotes (SI Appendix, Fig. 8 A–E). However, when eclosed flies were reared on sugar for 4 to 6 d (Fig. 4 A), $h^{R116Q}$ homozygotes exhibited hyperactivity; they traveled farther than $h^{WT}$ and had an increased average speed (Fig. 4 B–E). The increased average speed and total traveled distance were not observed in $h^{D33V}$ homozygotes in the same breeding and testing conditions (Fig. 4 B–E). Interestingly, when flies were bred on the other standard diet, the Frankel & Brosseau’s (FB) media, in which yeast extract and nonfat dry milk were used to replace the dried yeast in the CYM media (34, 35), both $h^{R116Q}$ and $h^{D33V}$ homozygous flies exhibited hyperactivity (SI Appendix, Fig. 8 F–J). The difference was maintained when eclosed flies were further reared on sugar for 4 to 6 d (Fig. 4 F–J). Therefore, diet plays a significant role in modifying the behaviors of $h^{R116Q}$ and $h^{D33V}$ homozygous flies.

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**Fig. 4.** Behavioral analyses of KI flies in an open-field arena. (A and F) Breeding and testing conditions and representative tracks from each genotype. Flies were eclosed (E) on either the CYM or FB diet and then, transferred to the sugar-only diet (4% Suc). (B–E) Total distance traveled, percentage of active time, average speed, and SCC (Materials and Methods has calculation details), respectively, of flies from various genotypes generated and tested in A. n = 50 to 75 flies. (G–J) Total distance traveled, percentage of active time, average speed, and SCC, respectively, of flies from various genotypes generated and tested in F. n = 28 to 44 flies. The two-tailed Student’s t test with Bonferroni’s correction compared with $h^{WT}$ was used. *P < 0.05; **P < 0.01; ***P < 0.001.
Since we observed lethality in \( h^{WT}/h^{Rosh} \) and \( h^{Diss}/h^{Rosh} \) flies when they were reared on sugar (Fig. 3C), we further analyzed their behavior under different breeding and testing conditions. When bred on the CYM media, \( h^{WT}/h^{Rosh} \) flies behaved similarly to \( h^{WT} \) homozygotes in both sugar-only and CYM testing conditions (Fig. 4 B–E and SI Appendix, Fig. S8 B–E). The \( h^{Diss}/h^{Rosh} \) flies, however, were less active in both testing conditions, and some even exhibited tortuous walking paths when reared on sugar (a representative track is in Fig. 4A) and consequently, had low SCCs. The average SCC of \( h^{Diss}/h^{Rosh} \) flies is, however, comparable with that of \( h^{WT} \) (Fig. 4E). The fact that the \( h^{Diss}/h^{Rosh} \) flies exhibited behavioral deficits even on the normal diet suggests that their residual PNPO enzymatic activity is insufficient to convert dietary VB6 to PLP to maintain normal behaviors.

When bred on the FB media, \( h^{WT}/h^{Rosh} \) flies were more active than \( h^{WT} \) homozygotes in both sugar-only and FB testing conditions (Fig. 4 G–J and SI Appendix, Fig. S8 G–J), which resembles \( h^{Rosh2} \) and \( h^{Sil} \) homozygotes in the same conditions. The different behaviors of \( h^{WT}/h^{Rosh} \), \( h^{Sil} \) or \( h^{Diss} \) flies on different media are likely due to the mild PNPO deficiency in them and its interaction with different diets. Consistent with this notion, \( h^{Diss}/h^{Rosh} \) flies, which have much more severe PNPO deficiency than them, exhibited hypoactivity and tortuous walking paths on both media (Fig. 4 and Movie S1).

Taken together, behavioral analyses of KI flies on different dietary conditions demonstrate that diet can potentially interact with PNPO deficiency to modify the locomotor behaviors of KI flies, and the effect of diet–allele interactions becomes prominent when flies have mild PNPO deficiency.

**Spontaneous Seizures in KI Flies with Severe PNPO Deficiency.** The spectrum of behavioral phenotypes associated with KI lines prompted us to examine motor unit activity patterns and identify spontaneous seizure-associated spike discharges in the respective lines. We utilized a tethered fly preparation (Fig. 5A) to monitor dorsal longitudinal muscle (DLM) activity in intact, behaving flies. During flight, these muscles power the “down stroke” of the orbiting trajectories (27, 41). We found that \( h^{WT} \) homozygotes reared on sugar displayed occasional grooming-related spiking (Fig. 4E). The fact that the \( h^{Diss}/h^{Rosh} \) flies exhibited behavioral deficits even on the normal diet suggests that their residual PNPO enzymatic activity is insufficient to convert dietary VB6 to PLP to maintain normal behaviors.

Reduced GABAergic Function in KI Mutant Flies. Next, we asked what neurotransmission system(s) was responsible for the spontaneous seizures in hPNPO KI mutants. To provide an initial glance at excitatory cholinergic signaling, we examined properties of the giant fiber (GF) jump-and-flight escape pathway, a descending circuit with identified neurons as well as cholinergic, glutamatergic, and electrical synapses (44). Mutations disrupting neuronal excitability (45), cholinergic transmission (46), and gap junctions (47) lead to profound disruptions in GF pathway performance. In \( h^{WT} \) flies, direct stimulation of the GF neuron leads to a DLM spike with a stereotypic latency of 1.41 ± 0.04 ms (Fig. 5E), similar to previously reported WT and control strains (i.e., “short latency” responses (45, 48). We found that the GF latencies in \( h^{Rosh2} \) and \( h^{Diss} \) were comparable with \( h^{WT} \) (1.42 ± 0.08 and 1.46 ± 0.04 ms, respectively) (Fig. 5E). Furthermore, using a repetitive stimulation protocol across a wide range of frequencies (20 to 200 Hz), we found that \( h^{Rosh2} \) and \( h^{Diss} \) were able to follow high-frequency stimulation to a similar degree as \( h^{WT} \) (Fig. 5F). Taken together, these observations suggest that the function of both excitatory cholinergic transmission and electrical synapses is largely maintained in the KI mutants.

We further asked whether the PNPO mutations led to changes in GABAergic signaling given that PLP, the product of PNPO, is required for the GABA synthesis. In WT flies, injection of the noncompetitive GABAA receptor antagonist picrotoxin (PTX; concentration ≥50 μM) induces a stereotypic sequence of flight-like DLM spiking (~10 Hz) followed by spike bursts exceeding 100 Hz (41, 49). We reasoned that if GABAergic tone was reduced in KI flies, injection of PTX at subthreshold excitatory neurotransmission and monitored the effect on spiking activity. In flies, acetylcholine is the primary excitatory neurotransmitter in the CNS (42), while glutamate is the transmitter at the neuromuscular junction (43). We applied the nicotinic acetylcholine receptor blocker mecamylamine using a rapid systemic injection protocol (41) and found that the spike discharges in \( h^{Diss}/h^{Rosh} \) were abolished (Fig. 5C), indicating that aberrant CNS activity drove the seizure-associated motor unit discharges in these flies.

During seizure-associated discharges in \( h^{Diss}/h^{Rosh} \) mutants, bursts in \( h^{Rosh2} \), and grooming-associated activity in \( h^{WT} \) and other KI flies, the DLM spiking intervals were highly variable, with instantaneous firing rates (defined as the reciprocal of the interspike interval [ISI–1]) ranging from ~1 Hz to nearly 50 Hz. However, these activity patterns could be readily distinguished and were remarkably characteristic within mutant flies. To quantitatively delineate seizure-associated activity in KI flies from grooming-related spiking, we employed a nonlinear dynamical systems approach we had previously used to describe firing patterns in the \( slg^{105} \) mutants and other hyperexcitable flies (Fig. 5D) (27, 41). For each spike in the recording, we plotted the spike’s instantaneous firing rate (ISI–1) against the instantaneous coefficient of variation (CV2) (Materials and Methods has the definition). High CV2 values indicate irregular firing, while low values indicate rhythmic activity. Previously, we have shown that seizure-related bursting in \( slg^{105} \) mutants corresponded to a self-similar “looping” trajectory in the phase–space analysis, while grooming activity displayed a distinctive trajectory limited to high CV2 values (>0.5). Based on the ISI–1–CV2 plots (representative trajectories are shown in Fig. 5D), the orbiting trajectories of \( h^{Diss}/h^{Rosh} \) firing were qualitatively similar to spontaneous seizures in \( slg^{105} \) mutants (27), suggesting a shared neural mechanism underlying the aberrant activity.
The excitability and behavioral phenotypes seen in the tone, perhaps arising from disrupted GABA synthesis, underlies these experimental findings suggest that a reduced GABAergic abnormal DLM spiking (Fig. 5 of 30 μM) by similar looping trajectories in the ISI –1–CV plots (Fig. 5 I). The instantaneous firing frequency (ISI–1) vs. the CV 2 (a measure of firing regularity) readily distinguish grooming-related firing (hWT is shown) from self-similar bursting in hD33V and hD33V/hR95H. Plots from representative firing patterns are shown (Materials and Methods has details on construction of ISI–CV2 plots). (E) Representative DLM spikes triggered by stimulation of the GF escape circuit (gray bar and lightning bolt). Note the similar stimulus-response latency in the respective genotypes (∼1.4 ms). (F) GF circuit following ability. DLM spike response rate (% Resp) to trains of 10 GF stimuli at varying frequencies. For clarity, error bars are omitted. No significant differences between genotypes were observed. (G) DLM spiking evoked by injection of a low dose of the GABA<sub>A</sub> receptor blocker PTX (30 μM). Occasional spikes in hWT corresponded with grooming, the hR116Q individual selected showed repetitive DLM firing, and the hD33V flies displayed spike bursts. (H) Average DLM firing frequency following PTX injection (n = 18 to 39 traces from five to six flies per genotype). (I) ISI–CV2 plots of DLM firing in a representative hD33V fly following PTX injection. Kruskal–Wallis ANOVA rank-sum post hoc was used. n.s.: P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

Concentrations would trigger abnormal DLM spiking activity in hR116Q and hD33V but not hWT. Indeed, following injection of 30 μM PTX, none (zero out of five) of the hWT flies displayed abnormal DLM spiking (Fig. 5 G and H). In contrast, two of five hR116Q and five of six hD33V flies displayed continuous DLM spiking following PTX injection (Fig. 5 G and H). In fact, we observed burst firing in hR116Q and hD33V individuals reminiscent of the spontaneous seizure phenotype in hD33V/hR95H flies, as revealed by similar looping trajectories in the ISI–1–CV<sub>2</sub> plots (Fig. 5I). These experimental findings suggest that a reduced GABAergic tone, perhaps arising from disrupted GABA synthesis, underlies the excitability and behavioral phenotypes seen in the hR116Q and hD33V flies.

**PLP Is Required for Both Development and Function of Adult Brain.**

No hR95H homozygous flies were generated on the normal diet (Fig. 3A), suggesting that the PNPO activity in these homozygotes is insufficient to convert dietary VB6 to PLP to support development. Thus, PLP supplementation should rescue the developmental impairment. We further asked whether there is a basal level requirement for PLP to support development. To answer the question, we supplemented breeders with varying doses of PLP, ranging from 0 to 400 μg/mL. We found a dose-dependent rescue associated with PLP supplementation. Specifically, we observed a complete rescue with 40 or 400 μg/mL of PLP, a partial rescue with 4 μg/mL, and no rescue with doses below 4 μg/mL (Fig. 6A).

To examine whether PLP was also indispensable for adult flies, we maintained hR95H homozygous flies (developed with PLP supplementation; 400 μg/mL) on sugar with or without continued PLP supplementation (400 μg/mL). With PLP supplementation hR95H mutants survived for several weeks (Fig. 6B) (median life
spontaneous firing and seizure discharges in hR95H homozygotes are rescued by PLP supplementation. While homozygous flies on sugar-only (S) or sugar supplemented with PLP (S + P) media for 2 d (D–G) Quantification of hR95H walking over a 3-min interval for flies reared on indicated media. n = 9 to 16 flies. One-way ANOVA with Tukey’s post hoc test was used. **P < 0.01. (C) Representative traces of DLM firing from tethered hR95H flies. Note that the burst discharges present in flies reared on S media are suppressed by PLP supplementation. (G) Representative traces of ISIs1–CV2 trajectories. Note that seizure-associated bursts are suppressed after PLP supplementation, and grooming (high-CV2) associated patterns are observed.

span: ~ 18 d). In contrast, without PLP supplementation, these flies could not survive for more than 3 d, demonstrating that PLP is also required for the survival of adult flies.

Similar to hD33V/hR95H flies (Fig. 4), hR95H homozygous flies also exhibited seizure-like behaviors before their death. To characterize these abnormal behaviors and to further study the role of PLP in the behavioral output, we monitored the locomotor activity of hR95H homozygotes with or without PLP supplementation (Fig. 6C and Movie S3). Compared with hR95H homozygotes without PLP supplementation, hR95H homozygotes with PLP supplementation for 2 d showed significantly improved levels in total distance traveled, percentage of active time, average speed, and SCC (Fig. 6 D–G), demonstrating that PLP deficiency is responsible for the low behavioral performance of hR95H homozygous flies. The improvement of the behavioral performance declined with age, which correlates well with the increased lethality (Fig. 6B).

Consistent with our previous findings that PNPO deficiency leads to increased spontaneous firing and seizure discharges (27) (Fig. 5), hR95H homozygotes on sugar also exhibited clear spontaneous seizure discharges in the tethered fly preparation (Fig. 6A and Movie S4). Remarkably, these seizure-associated discharges were completely suppressed by PLP supplementation, as demonstrated by greatly reduced median firing rate (Fig. 6D) (1.9 to 0.5 Hz for flies on sugar and sugar only supplemented with PLP, respectively) and the alteration from burst-associated trajectories to grooming-associated patterns in the ISIs1–CV2 plots (Fig. 6I).

Taken together, results from stage-specific PLP supplementation demonstrate that PLP is required for both development and normal brain function in adult flies. Notably, PLP deficiency in larval stages will lead to a dose-dependent developmental impairment; the higher the level of a deficiency, the more severe the impairment.
The dominant-negative effect would presumably make hWT/hR95H flies prone to seizures. However, these flies did not show spontaneous seizures (Fig. 5A and B). To determine whether hWT/hR95H flies were prone to seizures, we stimulated them with high-frequency electrical stimulation (HFS). HFS across the brain can induce stereotyped electroconvulsive seizure (ECS) discharges in flies (Fig. 7E and Movie S5) (40, 50). Compared with control flies, seizure-prone flies usually require lower stimulation intensities to induce seizures (25, 26). Many seizure-prone mutants have thus been identified and characterized (26, 37, 38, 40, 50, 51). By applying a range from 30 to 80 V of HFS intensities to hWT/hR95H heterozygous and hWT homozygous flies, we found a substantial reduction in the ECS stimulation threshold in hWT/hR95H compared with hWT. When hWT/hR95H and hWT flies were stimulated with a low voltage (30 V), seizures were already induced in 50% of hWT/hR95H flies, while no seizures were observed in hWT (Fig. 7F). The increased sensitivity in hWT/hR95H was maintained when the stimulation intensity was gradually increased. At 80 V, essentially all hWT/hR95H flies showed ECS discharges, while only about 60% of hWT were affected. Thus, the dominant-negative effect of hR95H confers the susceptibility of hWT/hR95H flies to HFS-induced seizures.

Discussion

Studying metabolic genes presents unique opportunities to uncover the contribution of gene–environment interactions to human health conditions and can offer potential treatment options for diseases. Yet, it is challenging to study mutations of such genes in humans since they often lead to lethality during development or rare diseases due to the functional essentiality of metabolic genes. Here, we generated a series of KI Drosophila models carrying hPNPO variants and examined these epilepsy-associated variants at molecular, circuitry, behavioral, and organismal levels. This genetically tractable model system and well-controlled dietary conditions have enabled the functional and molecular characterization of three epilepsy-associated PNPO alleles with vastly different phenotypes. Our results reveal that PNPO has multiple biological functions; that different PNPO variants have distinct molecular properties; and that individual PNPO mutations, diet, and allele–diet interactions all contribute to the final phenotype expression.

Our data demonstrate how the diet’s effect on the phenotype expression depends on PNPO alleles, with more evident outcome in genotypes showing milder deficiency of PNPO activity. For example, with specified dietary breeding and testing conditions, hR116Q, hD33V, or hWT/hR95H flies exhibited readily detectable behavioral outcomes, whereas hD33V/hR95H stayed relatively invariant (Fig. 4 and SI Appendix, Fig. S8). Notably, a less variable phenotype is also known for patients carrying severe PNPO mutations. The seizure onsets in patients with severe PNPO mutations (e.g., R95H) range from hours to weeks compared with the wide variation spanning from hours to years in patients with mild mutations (e.g., R116Q) (16). The less profound effect of diet in KI flies or patients with severe PNPO deficiency is presumably due to the diminished capability of mutant PNPOs in converting dietary VB6 to PLP.

On the other hand, although mild PNPO mutants retain a capacity to convert dietary VB6 to PLP to a greater extent and it is less likely for them to cause acute deleterious effects, the fact that hR116Q flies exhibited a shortened life span on the normal diet (Fig. 3B) suggests that the deleterious effect of mild PNPO deficiency can be cumulative. While R95H and D33V mutations are rare in the general population, R116Q is relatively common; ~10% are carriers, and 1% are homozygous (52). Therefore, it will be important to examine whether mild PNPO deficiency caused by R116Q can be exacerbated by other genetic and/or environmental factors to cause epilepsy or other diseases in humans.

Developmental delay is one common symptom manifested by PNPO-deficient patients (3, 17, 19, 53), raising the question of whether specific developmental impairments lead to seizures. By providing flies with PLP during either the larval or adult stages, we have demonstrated that developmental impairments and seizures in adults are dissociable (Fig. 6). Our data also indicate that prenatal PLP supplementation may be beneficial for the development of fetuses who carry severe PNPO mutations.

A major finding from this study is the dominant-negative effect of the R95H mutation. Neonatal epileptic encephalopathy caused by PNPO deficiency is generally thought to be an autosomal recessive disease (Online Mendelian Inheritance in Man no. 610090), implying that PNPO mutant carriers do not show any overt phenotype. Indeed, in all reported PNPO deficiency cases, 78% are homozygous for a specific mutation, and 21% are compound heterozygotes (16), except for one patient reported to be a heterozygote (3). Yet, in KI flies, we found a readily detectable dominant-negative effect from the hR95H allele (Fig. 7). The dominant-negative effect is presumably associated with heterodimer formation between hR95H and hWT. Structural studies have shown that amino acid R95 (arginine 95) is required for the cofactor FMN binding (14, 54) and thus, PNPO enzyme activity (14). An altered FMN binding site may lead to global conformation changes and hence, a partially or fully inactive enzyme. It is tempting to predict that mutations that do not impede dimerization but affect FMN binding can have a dominant-negative effect. Therefore, human carriers of such
a category of PNPO mutations could be susceptible to epilepsy and related disorders.

We find a unique pattern of spontaneous DLM burst firings in some \( h^{D33V} \) flies. The individual variability could be due to many factors, including stochastic events in the developmental processes into adults (Fig. 3A). Notably, distinct electroencephalogram (EEG) patterns have been reported in patients carrying the D33V mutation, which often exhibit a unique pattern of temporal sharp waves or multifocal sharp waves instead of the burst suppression in the majority of PNPO-deficient patients (5). The molecular and neural mechanisms associated with this peculiar mutation underlying unique firing or EEG pattern remain to be elucidated in both Drosophila and humans. The amino acid D33 (aspartate 33) is located in the N terminus of the PNPO protein, and the N terminus of PNPO is often not retained in crystal samples (Fig. 7B) (14); thus, it is often challenging to examine D33V in structural studies. Our molecular studies on \( h^{D33V} \) KI flies show a decrease in the mRNA level and consequently, the protein level of PNPO (Fig. 2A–C). However, it seems unlikely that the decreased PNPO protein level is a major determining factor for the distinct firing in \( h^{D33V} \) flies since a decreased PNPO protein level was also observed in \( h^{R116Q} \) flies (Fig. 2A), which exhibit only regular seizure discharges (Fig. 6 H–J). It is worth noting that \( h^{R116Q} \) flies have a higher level of protein decrease and a greater level of enzymatic activity loss than \( h^{D33V} \) flies; thus, there is a possibility that firing patterns are associated with the degree of PNPO deficiency. Future studies should investigate whether the D33V mutant protein alters neuronal or circuit functions in unique ways and whether different extents of PNPO deficiency can affect the various categories of neural function in a severity-dependent manner.

The molecular and functional consequences of R116Q have been controversial. R116Q was first reported in patients with neonatal epileptic encephalopathy (3), suggesting that it is likely a severe mutation. Later clinical reports show that unlike seizures in patients carrying other PNPO mutations, seizures in R116Q patients can occur beyond the neonatal stage (3–5), indicating that R116Q is more likely to be a relatively mild mutation. The molecular consequence of R116Q is also inconclusive. An earlier study showed that R116Q deficiency-induced seizures. In contrast, two recent studies report decreases to 83% of the WT protein (3). In contrast, one recent study also observed in \( h^{R116Q} \) flies (Fig. 2A), which exhibit only regular seizure discharges (Fig. 6 H–J). It is worth noting that \( h^{R116Q} \) flies have a higher level of protein decrease and a greater level of enzymatic activity loss than \( h^{D33V} \) flies; thus, there is a possibility that firing patterns are associated with the degree of PNPO deficiency. Future studies should investigate whether the D33V mutant protein alters neuronal or circuit functions in unique ways and whether different extents of PNPO deficiency can affect the various categories of neural function in a severity-dependent manner.

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The neural mechanisms underlying PNPO deficiency-induced seizures remain to be determined. Our previous studies on spike pattern analyses from \( sgll^{PPS} \) and \( sgll \) knockdown flies have suggested that GABA dysfunction contributes to PNPO deficiency-induced seizures (27). In this study, we find that \( q^{D33V} \) flies are more vulnerable to GABA \(_A\) receptor blockade (Fig. 5G), suggesting that these flies may have a reduced GABAAergic tone, presumably arising from decreased GABA synthesis. In agreement with these results, reduced GABA levels have been recently reported in a zebrafish PNPO knockout model (57). Therefore, PNPO deficiency probably leads to a reduced GABAAergic tone, which decreases the firing threshold and/or promotes synchronous firing.

It should be noted that PLP exhibits considerable functional complexities that can complicate the inference of the neurotransmitter system responsible for the seizures and may account for the variable biochemical measurements among PNPO-deficient patients (17). In addition to being a cofactor and involved in the synthesis of several neurotransmitters, PLP has other cellular functions that can potentially modify the eventual expression of seizures, including altering immune function and acting as an adenosine triphosphatase antagonist at P2 purinoceptors (58). One interesting question is why seizures in PNPO-deficient patients generally do not respond to available antiepileptic drugs, including those targeting GABA signaling (59). One possibility is that GABA dysfunction leads to initiation of seizure-prone conditions, which may be functionally compensated by subsequent remodeling of neuron circuitry and modified involvement of other neurotransmitters, such as dopamine and serotonin. This notion is partially supported by the fact that some PNPO-deficient patients show initial responses to GABA-related drugs (5, 18, 19, 60), although seizures in them ultimately become drug resistant. Thus, future investigations into the complexity of neuronal network dynamics, the role of various PLP-dependent neurotransmitters, and other interactive biological functions of PLP are required to elucidate the process in generating PNPO deficiency-induced seizures.

In summary, our studies uncover a diversity of molecular defects of PNPO variants, reveal the role of the PNPO–allele–diet interaction in the phenotype expression, and highlight the contribution of PNPO deficiency to epilepsy in general. These data provide biological bases for understanding phenotypic variations in PNPO deficiency patients and have significant clinical implications in developing treatment strategies. These studies also demonstrate that KI Drosophila models are valuable for systematically analyzing the functional and molecular effects associated with each PNPO allele identified in epilepsy patients.

Materials and Methods

Generation of Ki Strains. Four different Ki strains were generated using CRISPR-Cas9 technology (61). The WT \( hPNPO \) cDNA was amplified from the human brain cDNA library (TaKaRa; catalog no. 637242) (27). Three mutations were introduced separately by mutagenesis. The single guide RNAs, Cas9 mRNAs, and donor constructs were injected into embryos from flies with a genotype of \( w^{1118} F^M t m A \); Bc/yt6A; TM3F106B, Hu, Tb (http://www.fungene.tech) (SI Appendix).

Drosophila Fly Husbandry. Flies were generated on either standard CYM or FB media (34). Flies used in all experiments were raised and tested at room temperature (−23 °C) in a 12:12-h light-dark cycle (SI Appendix).

Western Blotting. Total protein from adult heads was extracted, loaded for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and detected with rabbit anti–hPNPO and mouse anti–β-tubulin antibodies (SI Appendix).

qRT-PCR. Total RNA was extracted and reversely transcribed to cDNA for qPCR. Two pairs of primers were designed to target the \( hPNPO \) KI alleles (N- and C-terminal regions, respectively). A pair of primers was used to amplify rp49 (SI Appendix).

Immunohistochemistry. The protocol was adapted from the Flylight protocol (62). Briefly, brains were dissected, fixed, and stained with rabbit anti–hPNPO and mouse anti–β-3 tubulin antibodies. Signals for hPNPO were amplified with the Tyramide SuperBoost kit (Invitrogen; catalog no. B40926). DAPI was added into the wash buffer to stain the nucleus when needed. Images were taken using a Leica SPS-H-STED-CW confocal microscope and processed in Fiji (83) (SI Appendix).

Developmental Assay. A cohort of 2 to 3 flies per sex was set up in a single vial, or a group of 12 to 15 flies per sex was set up in a single bottle. Flies eclosed within 6 d from each cross were examined for the Balancer marker (SI Appendix).

Life Span and Survival Study. Fifteen to 20 male flies were maintained in vials filled with the standard CYM medium (the normal diet condition) or 4% sucrose in 1% agar (the sugar-only condition). Daily survival was recorded (SI Appendix).

Drosophila carrying epilepsy-associated variants in the vitamin B6 metabolism gene PNPO display allele- and diet-dependent phenotypes
Behavioral Recording. Single fly or multiple flies were recorded and tracked using lowaFLI Tracker (27, 64). The total distance traveled, average speed, percentage of active time, and SCC were further calculated as previously described (27) (SI Appendix).

Electrophysiology. Action potentials were recorded from DLM muscles as previously described (27, 65). Drugs were delivered via dorsal vessel injection (41, 66), and GF stimulation was delivered through tungsten electrodes inserted in each cercus (as previously described) (67). ECS discharges were induced by HFS across the brain delivered by the same electrodes (40, 65). The stimulation protocol consisted of a 2-s train of 0.1-ms pulses delivered at 200 Hz at a specified voltage (30 to 80 V) (SI Appendix).

Statistical Analysis. Statistical analysis was performed in MATLAB (R2019b) or R (version 3.6.1). Details on statistical analyses, including sample sizes, tests performed, and multiple test correction if necessary, are provided in the figures.

Data Availability. Custom scripts have been deposited in GitHub (https://github.com/lyenargAtulay/lowaFLI_tracker and https://github.com/lyenargAtulya SpikeTrainAnalysisToolkit). All study data are included in the article and/or supporting information.

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