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GENETIC AND NEURAL MECHANISMS OF PNPO DEFICIENCY IN VITAMIN B6-DEPENDENT EPILEPSY

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To my family and in memory of my grandmother, Zhilian Zhang.

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ABSTRACT

Vitamin B6 (VB6)-dependent epilepsy was first reported in 1954; however, the underlying genetic cause(s) was unknown until 2005 when the first mutation was identified in an autosomal recessive gene *pyridox(am)ine 5'-phosphate oxidase* (*PNPO*). Gene *PNPO* encodes a rate-limiting enzyme in the synthesis of pyridoxal 5'-phosphate (PLP), which is the biologically active form of VB6 and a co-factor required for the synthesis of several neurotransmitters including GABA. Since 2005, mutations in *PNPO* have been increasingly reported, mostly in neonatal epileptic encephalopathy (NEE) patients but also in early-onset epilepsy. Moreover, *PNPO* has been recently included as one of the sixteen epilepsy genes involved in the common epilepsies, suggesting that PNPO deficiency may contribute to epilepsy in general. In contrast to the increasingly recognized significance of PNPO deficiency in epilepsy, our understanding of the neurobiological mechanisms underlying PNPO deficiency associated symptoms is limited. Moreover, PNPO mutations and PNPO deficiency have never been systematically studied due to the lack of animal models.

Based on a nutritional conditional lethal phenotype, I have previously identified a *Drosophila PNPO* gene (*sugarlethal*, *sgll*) and a hypomorphic *sgll* allele ($sgll^{95}$). This identification opened up opportunities of using *Drosophila* as a model system to study PNPO deficiency and PNPO mutations identified in patients. In the first part of my thesis, I report the establishment of *Drosophila* models of PNPO-deficiency-induced epilepsy. Using both behavioral and electrophysiological approaches, we for the first time show that PNPO deficiency leads to seizures in an animal model as it does in humans. Moreover, seizures and the conditional lethality are correlated with low internal PLP levels and can be rescued by ubiquitous expression of wild-type (WT) human *PNPO* (*hPNPO*). Furthermore, examination of the spike patterns from electrophysiological recordings reveals a potential involvement of GABA dysfunction in seizures caused by PNPO deficiency. Lastly, cell type-specific *sgll* knock-down indicates an important role of brain-expressed *sgll* in survival and seizure prevention.

In the second part, I generated *hPNPO* knock-in (KI) lines using CRISPR/Cas9. In each KI line, the endogenous *sgll* gene was replaced by one of three disease-causing *hPNPO* cDNAs. Based on *in vitro* studies, these three mutant alleles show different severity of impaired PNPO activity. A WT *hPNPO* KI line was generated as a control. Establishing these KI lines has allowed me to discover that severe hPNPO deficiency leads to lethality in early development, intermediate hPNPO deficiency results in conditional lethality and seizures, whereas mild hPNPO deficiency shortens lifespan. At the molecular and cellular level, in addition to the reported impairment in catalytic activity, hPNPO mutations affect stability, cellular localization, or transcription of hPNPO. Lastly, I report a dominant-negative effect of R95H mutant protein on WT protein. Based on these fly studies and previously published structural studies by other groups, I predict that a number of other seizure-causing hPNPO mutations will likely have dominant-negative effects as well. Therefore, individuals who are heterozygous for these mutations may be susceptible to diseases.

The third part of my thesis is devoted to human studies. I tested whether mild PNPO deficiency confers susceptibility to epilepsy in adults. Specifically, I examined whether mild PNPO deficiency caused by R116Q mutation is over-represented in adult patients with generalized epilepsy. I focused on R116Q because it can cause NEE and early-onset epilepsy and it is common in the general population. So far, a total of 36 samples have been collected. Among these 36 samples, six are heterozygous for R116Q, so the allele frequency is 8.33 %, which is higher than that in any races in the general population. Power analysis based on the current effect size show that a total of $\sim$ 300 samples will be needed to reach a power level of 0.8.

Genes and mutations that cause human genetic diseases have been increasingly identified in patients since the introduction of whole genome sequencing and whole exome sequencing. Valid animal models are indispensable to functionally examining identified genes and mutations. My thesis work represents a concrete example of using *Drosophila* models to study the fundamental biology of disease-causing genes and to uncover the molecular and cellular

mechanisms of disease-causing mutations identified in patients. These studies expand our understanding of disease-causing genes and mutations and may also lead to better treatments in the future.

CHAPTER 1

INTRODUCTION

1.1 *Drosophila melanogaster* as a Model System for Studying Human Genetic Diseases

Since more than one century ago when Thomas H. Morgan identified the X chromosome-linked gene *white* in *Drosophila melanogaster* [1], *Drosophila* has been used as a model system for studying almost every aspect of basic biological processes. The completion of sequencing the *Drosophila* genome in 2000 [2] further underscored its utility for studying human genetic diseases. *Drosophila* has orthologs of $\sim$ 75 % of human disease-causing genes [3, 4].

Gene conservation is not the only reason that *Drosophila* is suitable for studying human genetic diseases. Previous studies have shown that all major signal transduction pathways are conserved between flies and humans [5]. Furthermore, flies have functional counterparts of almost all of human organs. *Drosophila* models have already been established for various human diseases such as neurological diseases, cancer, cardiovascular diseases, developmental disorders, and metabolic disorders [6, 7, 8, 9, 10].

While conservation in genes and functions between *Drosophila* and humans makes *Drosophila* suitable for studying human diseases, sophisticated genetic tools make it effective and attractive as a model system. For example, the generation of balancer chromosomes–a set of special chromosomes that prevent recombination through a series of DNA inversions–allows researchers to maintain recessive lethal mutations or sterile mutations on single chromosomes over generations, as well as to easily generate specific lines that carry double or triple mutations. In addition to balancer chromosomes, various transposons, such as *P* elements, *piggyBac*, and Minos transposons [11, 12], are used to target genes in the *Drosophila* genome to generate null mutations and tagged proteins and to provide a platform to track gene expression pattern. Furthermore, the UAS/Gal4 binary system [13] (Figure 1.1) and other binary systems [14, 15] further allow researchers to knockout/knockdown or overexpress a

gene in a spatiotemporal manner. Recently, the CRISPR/Cas9 technique has been used on flies [16, 17] to expedite the inactivation, tagging, and overexpression of any gene in the genome; and other genome modification approaches continue to offer further promises [18, 19, 20].

These genetic tools also make it feasible to perform both forward genetics and reverse genetics in flies. Forward genetics is a process of identifying the genotype by following a phenotype. In forward genetics, mutations are first introduced by chemicals (e.g. mutagen ethyl methanesulfonate, EMS) or transposons, but sometimes they may occur spontaneously, after which researchers screen for a particular phenotype. Thus, forward genetics is an unbiased approach and can be used for identifying previously unknown genes or characterizing previously uncharacterized mutations. By comparison, reverse genetics is a process from a genotype to a phenotype. Known mutations are created in fly genes first, and then they are followed by the examination of phenotypes. Both forward genetics and reverse genetics have achieved great success in *Drosophila* research. Numerous novel genes have been identified and functions of genes have been analyzed, including genes that are involved in human diseases such as epilepsy [21, 22].

1.2 Epilepsy and Epilepsy Studies in *Drosophila melanogaster*

1.2.1 Overview of Epilepsy

Epilepsy is one of the most disabling neurological disorders, and it is defined as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" [23]. Epilepsy can affect people of any age and has no geographic, social, or racial boundaries [24]. Globally, 70 million people have been diagnosed with epilepsy, with 2.4 million people being diagnosed each year [24]. Epilepsy can be classified into two main categories: generalized epilepsy if seizures are bilaterally distributed, and focal epilepsy if seizures are limited to one hemisphere of the brain [25]. About 60% of people with epilepsy

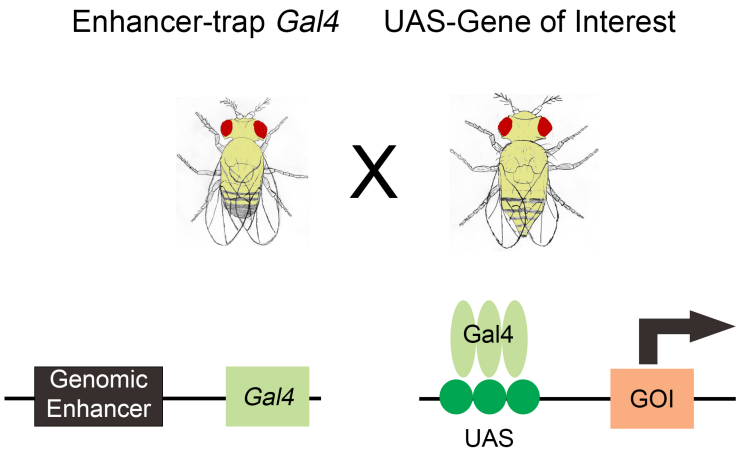

Figure 1.1: **The UAS-Gal4 binary system.** The UAS-Gal4 system is commonly used to control gene expression in *Drosophila* research. In this system, the upstream activation sequence (UAS) and its specific binding protein, Gal4, are separated into two fly lines such that one line has the Gal4 element that is controlled by a genomic enhancer or a promoter, and the other line has the UAS element that is placed to the upstream of a gene of interest (GOI). When flies from these two lines are crossed, Gal4 protein binds to UAS to drive the expression of GOI. Thus, the expression pattern of GOI is determined by the genomic enhancer or the promoter in the Gal4 line. Thousands of Gal4 lines that target different tissues or even different cell types have been established (https://bdsc.indiana.edu), which makes it possible to manipulate gene expression in a tissue- or cell type-specific manner. (Fly drawing: Bowen Cheng)

are classified as focal [25].

The underlying causes for epilepsy are heterogenous, and our understanding of the causes has continued to evolve over time. In 1975, the majority of people diagnosed with epilepsy were classified as 'idiopathic', that is, of unknown cause. Among epilepsies with known causes, trauma and stroke are two main contributors [26]. In 2014, however, the majority of the 'idiopathic' portion were classified as having a genetic basis [27]. Epilepsy genes, defined as genes that, when mutated, cause epilepsies or syndromes with epilepsy as the core symptom, have been increasingly identified. In fact, by 2016, a total of 84 genes had already been reported [28]. These genes encode ion channels, enzymes/enzyme modulators, transporters/receptors, and proteins with other functions. Lastly, it is worth to mention that there are another ~ 900 genes that are classified as epilepsy-associated genes. When mutated, epilepsy-associated genes cause not only epilepsy, but also many other symptoms such as brain malformations and physical abnormalities [28].

The mainstay of treatment for epilepsy is drug therapy. A number of animal models have been developed for drug screening, from electroshock seizure models in the early days to chemical-induced seizure models more recently [29, 30]. Using these models, more than forty different anti-epileptic drugs (AEDs) have been established for the treatment of epilepsy, half of which were developed in the last 30 years [29]. Despite the introduction of these new AEDs, however, the rate of the drug-resistant epilepsy, defined as seizures that are not controlled with two or more AEDs, has not been changed [31]. Indeed, there are still about 30 % of epilepsy patients who have to seek non-drug therapies, such as resective surgery, vagus nerve stimulation, dietary therapy, or deep-brain stimulation [32].

The unchanged drug-resistance rate indicates that newly identified drugs probably share the same molecular action sites as old ones, which mainly target voltage-gated sodium channels, voltage-gated calcium channels, γ-aminobutyric acid (GABA) systems, and glutamate receptors [33]. To develop new drugs with different targets, there is a need to establish animal models that have a diverse biological basis in epileptogenesis. The unchanged drug-resistant

rate also signifies the need to understand the fundamental biology of these drug-resistant epilepsies.

1.2.2 Epilepsy Studies in Drosophila melanogaster

As discussed above, *Drosophila melanogaster* is a particularly useful model system because of its rich genetic tools. Using *Drosophila* to study seizures started in the early 1970s, when Seymour Benzer's laboratory treated flies with EMS and isolated different mutants based on the behavioral phenotypes [34]. One type of mutant shows seizure-like activities, including wing-flapping, proboscis extension, abdominal muscle contractions, and leg-shaking when flies are disturbed by mechanical stimulation [35]. This type of mutant is now known as the Bang-Sensitive mutant, which forms the basis of the *Drosophila* seizure model [21, 22]. Other seizure models include Temperature-Sensitive mutants [36], spontaneous seizure mutants [37, 38], and, the most recently generated model, the human seizure-causing mutation knock-in flies [39, 40]. Similar to the mutant genes found in humans, mutant genes identified in *Drosophila* mainly encode ion channels, enzymes, and transporters [22, 41, 42, 43, 44, 45, 46].

Different approaches have been developed to facilitate seizure studies in flies. For example, the application of the high-resolution-video-recording system makes it possible to quantitatively characterize seizure behaviors [47, 48], the development of an electrophysiology method makes it possible to quantify seizure thresholds of different mutants (Figure 1.2) [22, 49, 50, 51, 52], and a newly developed data analysis method to analyze electrophysiological recordings further makes it possible to examine the stereotypes of electrical spikes associated with different genetic defects or behaviors [53].

Another key advantage of using *Drosophila* models to study seizures is the ability to perform studies that are less feasible in mammals. These studies include the ability to examine genetic or functional interactions among established mutants [35, 54] or to perform suppressor/enhancer screens to identify modifiers for known seizure genes [55, 56]. These modifiers themselves do not cause seizures; they only change seizure susceptibilities of seizure

genes [28, 57].

Lastly, the small body size of flies, their large progenies, and their low-maintenance cost makes it efficient and cost-effective to perform drug screening in *Drosophila* models. These models can be either larvae or adult flies. Different routes of drug administration approaches have been developed. For example, drugs can be mixed in the diet for chronic treatment [58, 59] or administered by injection for acute treatment [53, 60].

Ultimately, in combination with genetic manipulations, electrophysiological recordings, and drug treatments, *Drosophila* models have been and will continue to offer promises as a model system. They will continue to contribute to the uncovering of mechanisms underlying drug-resistant epilepsies, and be valuable tools in finding therapeutic options for these drug-resistant epilepsies.

1.3 Vitamin B6 (VB6) and VB6-dependent Epilepsy

1.3.1 Overview of Vitamin B6

Vitamin B6 (VB6) was discovered by Paul György and colleagues in 1934 [65, 66]. It was initially referred to as pyridoxine (PN), a name coined by György because the chemical is a pyridine derivative [67] (Figure 1.3). Later on, two more chemically related but less stable forms, pyridoxamine (PM), pyridoxal (PL), were identified [68, 69, 70]. PN, PM, and PL can be phosphorylated into their corresponding phosphorylated forms PNP, PMP, and PLP. These six compounds are now known as B6 vitamers.

The absorption, transportation, and metabolism of VB6 in different species have been extensively studied since its discovery. Now it is well known that bacteria, fungi, and plants can synthesize VB6 *de novo* while mammals and insects cannot so they have to rely on dietary VB6 [71]. Different forms of VB6 present in different diets: plant-derived food contains primarily PN, PNP, and PN-β-glucoside (PNG) while animal-derived food contains mainly PLP and PMP. In animals, non-phosphorylated forms of VB6 are absorbed in the small

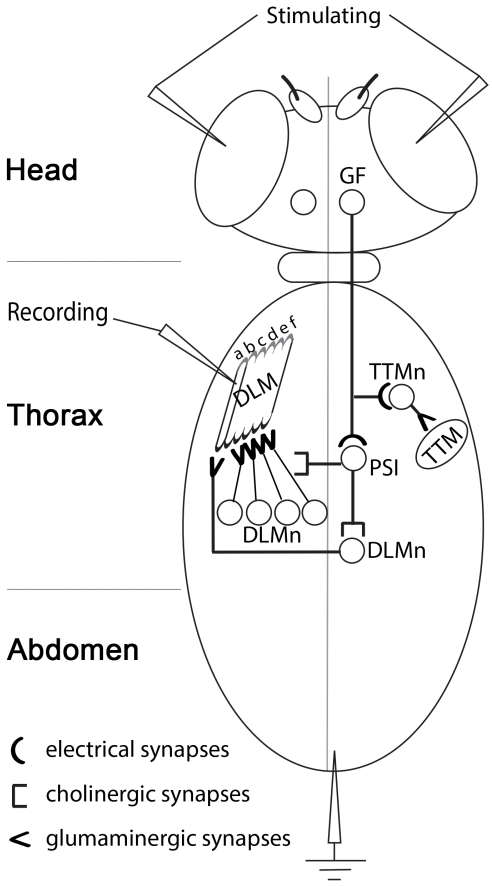

Figure 1.2: **A schematic presentation of the giant fiber (GF) pathway and the recording setup for electroconvulsive seizure.** The GF pathway is a jump-and-flight escape pathway in *Drosophila*, which comprise several elements: a pair of giant fiber (GF) neurons in the brain, peripherally synapsing interneuron (PSI), tergotrochanteral muscle motor neuron (TTMn), dorsal longitudinal muscle motor neuron (DLMn), tergotrochanteral muscle (TTM), and a stack of six dorsal longitudinal muscle fibers (DLMa-f) [61, 62, 63, 64]. A pair of bilaterally symmetrical GF interneurons in the brain projects to ipsilateral TTMn and PSI. The TTMn innervates ipsilateral TTM and the PSI makes synapses with one ipsilateral DLMn and four contralateral DLMns. The ipsilateral DLMn innervates both DLMa and DLMb and the contralateral four DLMns individually innervate DLMc-f. Different types of synapses are formed, which include electrical, cholinergic, and glutaminergic, as indicated in the Figure. For the recording of the electroconvulsive seizures, GF neurons in the brain are stimulated by high-frequency electrical impulses through a pair of stimulating electrodes inserted into two eyes, one in each, and responses from DLMa is collected via the recording electrode that is inserted into DLMa. A ground electrode is inserted into the abdomen. Seizures evoked by high-frequency stimulation are in an all-or-none manner. Essentially, when the stimulus is strong enough, wild-type (WT) flies can show seizure-like electrical activities. However, seizure-prone mutants will show seizures with low voltage stimulation, i.e., they have low seizure thresholds. For recording of spontaneous seizures, no stimulus is needed.

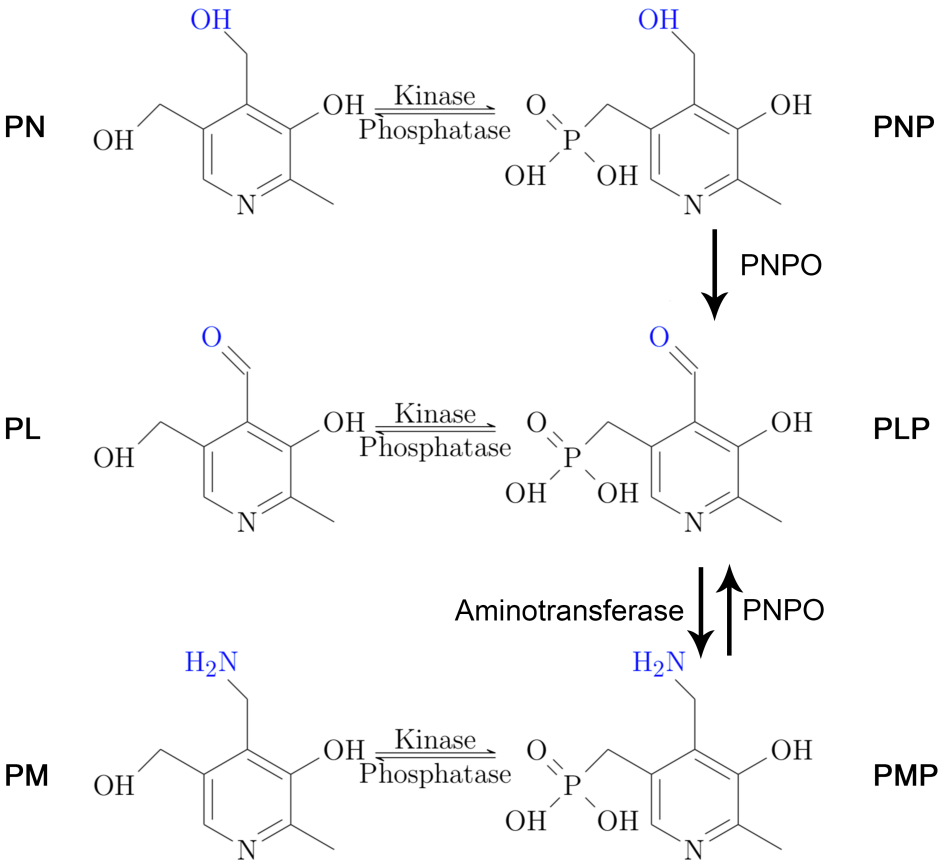

Figure 1.3: **B6 vitamers and their chemical structures.** Vitamin B6 comprises a group of six compounds, also known as B6 vitamers. They all contain a pyridine ring as their core but differ at the chemical groups at the pyridine 4' position. PN has a hydroxyl methyl group, pyridoxamine (PM) has an amino methyl group, and pyridoxal (PL) has an aldehyde group. The other three forms are their corresponding phosphorylated forms. PNPO: pyridox(am)ine 5'-phosphate oxidase.

intestine [72] after digestion. The phosphorylated forms of B6 and PNG need to be converted to non-phosphorylated forms first by intestinal phosphatase or PNG hydrolase, respectively, and then are absorbed [73].

Once inside cells of the intestine, B6 vitamers are transported to the liver where PN, PM, and PL are first re-phosphorylated by pyridoxal kinase (PK, EC 2.7.1.35) to PNP, PMP, and PLP, respectively. PNP and PMP are then oxidized by pyridox(am)ine 5'-phosphate oxidase (PNPO, EC 1.4.3.5) to PLP. Although previous studies show that these conversions mainly occur in the liver [74], recent cell studies suggest that they can also occur in the small intestine [75] and maybe in other organs as well, given that PNPO is ubiquitously expressed [76]. Before being released into the circulation from the liver, PLP becomes tightly bound to albumin in the plasma [77] or to hemoglobin in erythrocytes [78]. Thus, it is PLP-containing complexes instead of free PLP proteins that are transported to different tissues/organs, including the brain [79, 80]. The binding of PLP to albumin or hemoglobin is believed to prevent PLP from premature de-phosphorylation by tissue non-specific alkaline phosphatase (TNSALP).

To cross the blood-brain barrier and to enter neural cells, PLP has to be dissociated from albumin or hemoglobin first and then be de-phosphorylated to PL as TNSALP only uses free PLP as its substrate. The transportation of PL from outside to inside brain cells is mediated by a saturable mechanism [81]; however the identity of the responsible transporter(s) is still unknown. Intracellular PL is then converted back to PLP and "trapped" there. Similar processes occur to other cells.

PLP is a highly reactive aldehyde, which allows it to be functional as a versatile co-factor for more than 140 enzymes inside cells [82]. In the nervous system, PLP is required for the synthesis of several neurotransmitters such as GABA, dopamine, and serotonin [82]. On the other hand, the high reactivity of PLP also suggests that cells must have a very sophisticated mechanism to regulate the homeostasis of PLP. It is still unclear how cells protect PLP from being de-phosphorylated by TNSALP and in the mean time, prevent its reaction with

non-specific enzymes. Both PK and PNPO have PLP binding sites [83], indicating that they may function as chaperone proteins to deliver PLP to apoenzymes. However, PLP-dependent enzymes present not only in the cytoplasm but also in the mitochondria and peroxisomes [84] and PLP cannot permeate membrane; therefore, there must be different mechanisms for delivering PLP to different cellular compartments. These potential mechanisms are still being actively studied.

1.3.2 VB6-Dependent Epilepsy

Given that PLP is involved in the synthesis of neurotransmitters, it is not surprising that VB6 deficiency causes seizures. However, because VB6 is ubiquitously existent in diet, nutritional VB6 deficiency is uncommon in humans. Genetic VB6 deficiency was first recognized by Hunt *et al.* in 1954 [85] and the recognition was based on a "trial-and-error" process. In their paper, Hunt and his colleagues described a female infant who exhibited generalized twitching at three hours after birth. Seizures were initially slightly relieved by barbiturates, an anti-epileptic drug (AED) that potentiate inhibitory $GABA_A$ receptors and inhibit excitatory AMPA receptors [86]. However, on the fifth day of life, the baby developed severe generalized convulsions and did not respond to barbiturates or other AEDs. Because of seizures, the baby stopped oral feedings on day six and had to rely on parenteral fluids, in which multivitamin was provided. Once received multivitamin, the baby showed no seizures. With the testing and eliminating each ingredients in the multivitamin, Hunt *et al.* eventually found that it was PN that was responsible for the seizure cessation. This was the first case showing that a pathologic condition occurred spontaneously and responded to VB6 treatment.

Although in the following fifty years more cases with VB6-dependent epilepsy were reported [87, 88, 89, 90], and PN or PLP became a test treatment for drug-resistant epilepsies in neonates or in children, the genetic cause underlying this condition was unknown until 2005 when the first mutation was identified in gene *pyridox(am)ine 5'-phosphate oxidase* (*PNPO*) [91]. Patients with PNPO deficiency usually do not respond to PN but only to PLP. However,

despite well-controlled seizures with PLP, most patients still show neurodevelopmental disabilities.

Recently, two more genes related to VB6-dependent epilepsy were identified: one is *ALDH7A1* that encodes α-aminoadipic-semialdehyde-dehydrogenase/antiquitin, an enzyme involved in the L-lysine catabolism [92] and the other one is *PROSC*/*PLPBP* that encodes PLP homeostasis protein (PLPHP) [93]. Unlike PNPO, antiqutin and PLPHP do not affect the metabolism of VB6. They only affect the bioavailability of PLP, probably through different mechanisms. Loss of the enzyme function in antiquitin leads to the accumulation of the lysine intermediates aminoadipate semialdehyde and its cyclic equilibrium form piperideine 6-carboxylate (P6C). P6C sequesters and inactivates PLP, which leads to PLP deficiency [92, 94]. By comparison, PLP deficiency caused by mutations in PLPHP is probably mediated through a mitochondrion-related mechanism because a recent study shows that PLPHP is localized in mitochondria and is likely to play a role in mitochondrial metabolism [95]. The exact mechanisms underlying PLPHP-induced PLP deficiency are still unknown.

The increased identification of genes and mutations on one hand expand our understanding of VB6-dependent epilepsy. On the other hand, it also indicates an urgent need of valid animal models that can be used for studying the fundamental biology of identified genes and the pathophysiology of seizure-causing mutations. While in the past two years, zebrafish models have been established for antiquitin deficiency [94] and PLPHP deficiency [95], to my knowledge there are no animal models for PNPO deficiency except the *Drosophila* models presented here.

1.4 Identification of a *Drosophila PNPO* Gene (*sugarlethal*, *sgll*) and a Hypomorphic *sgll* Allele ($sgll^{95}$)

During a study [1] of the role of dopamine in the *Drosophila* feeding behavior, we observed that *Ddc*-Gal4 homozygotes could not survive on a 4% sucrose-only diet for more than 5 days, whereas wild-type flies could survive in the same condition for more than 10 days. This conditional lethal phenotype was observed in both female and male *Ddc*-Gal4 flies. To rule out the contribution of genetic background to the lethal phenotype, we backcrossed *Ddc*-Gal4 flies to w^{1118}, the control strain used in this study, for five generations by following the mini-white marker and then examined the survival of backcrossed flies on the sugar-only diet. Backcrossed homozygous *Ddc*-Gal4 flies still exhibited the lethal phenotype on 4% sucrose but they survived well on 5% yeast or 5% yeast plus 4% sucrose (Figure 1.4), suggesting that their death was caused either by their deficient sugar catabolism or by their oversensitivity to the lack of specific nutrients in the sugar-only diet.

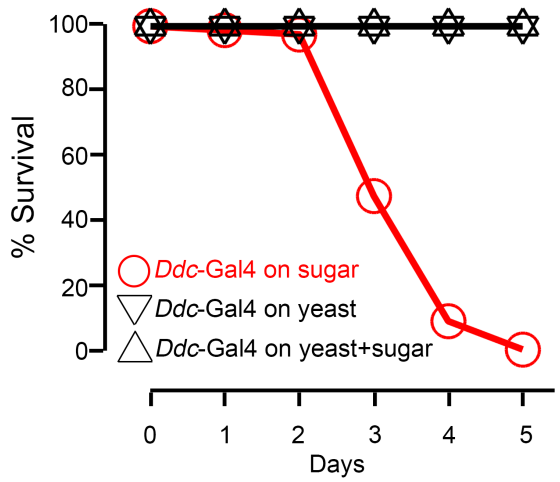

Figure 1.4: **Survival of *Ddc*-Gal4 flies on different diets.** *Ddc*-GAL4 flies survived well on 5 % yeast or 5 % yeast + 4 % sucrose but died on the 4 % sucrose-only diet.

1. Modified from Chi W, Zhang L, Du W, Zhuang X. A Nutritional Conditional Lethal Mutant Due to Pyridoxine 5'-Phosphate Oxidase Deficiency in *Drosophila melanogaster*. G3: Genes, Genomics, Genetics (2014)

To determine if the conditional lethal phenotype was due to the gene disruption caused by *P*-element insertion in *Ddc*-Gal4 flies, we performed precise excision by crossing the *Ddc*-Gal4 line with a Δ 2-3 transposase line. Flies with precise excision still showed the lethal phenotype when reared on the sugar-only diet, demonstrating that another gene was responsible for the lethality. We therefore named this gene *sugarlethal* (*sgll*).

To identify the causal gene, we performed forward genetic approaches (e.g. meiotic recombination mapping, deficiency mapping) and whole genome sequencing (Figure 1.5). Meiotic recombination mapping narrowed the region of interest to a region between phenotypic marker *th* (thread arista; cytogenetic locus 72C1-72D1) and *cu* (curled wings; 86D7-86D7); deficiency mapping localized the mutation to a 100 Kbp region, which contains 16 genes; and whole genome sequencing identified a c.95C>A mutation in CG31472 among these 16 genes. The homozygous c.95C>A substitution in the exon 2 of CG31472, designated $sgll^{95}$, results in the substitution of a neutral alanine to an acidic aspartate (p.Ala32Asp) in the protein sequence (Figure 1.6 A).

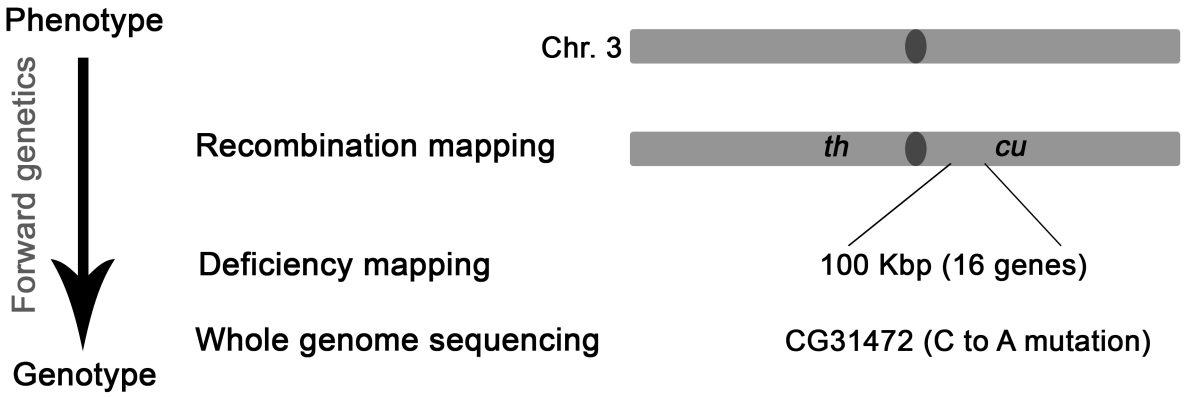

Figure 1.5: **Using forward genetic approaches and whole-genome sequencing to identify a missense mutation in gene *sgll*.**

Gene *sgll* spans a region of $\sim$ 1.4 Kbp in the genome (RefSeq: NM_169194.3 and NM_169195.4). It encodes two putative pyridox(am)ine 5'-phosphate oxidase (PNPO) proteins due to alternative transcriptional starts. These two proteins share the C-terminus but differ at the N-terminus, with RA variant containing nine extra amino acid residues (Figure 1.6 B

and C). Amino acid sequence alignment analysis showed more than 75% similarity (more than 40% identity) between SGLL and human PNPO (Figure 1.6 C). The alanine (mutated to aspartate in $sgll^{95}$) at +32 is also conserved between humans and flies. Crystal structure studies of human PNPO show that some amino acid residues are critical to the enzyme activity [96]. In the alignment analysis, we found that almost all these critical residues are conserved (highlighted in blue, Figure 1.6 C). Moreover, the C-terminus that is essential for the enzyme activity is also highly conserved between humans and flies [76] (Figure 1.6 C).

To confirm that the conditional lethality of $sgll^{95}$ flies was due to PNPO deficiency, we treated flies with pyridoxal 5'-phosphate (PLP) or pyridoxine (PN), the product or precursor of PNPO, respectively. We observed a dose-dependent survival from $sgll^{95}$ flies on either PLP or PN supplementation (Figure 1.7 A&B). Therefore, we conclude the following: the conditional lethal phenotype of $sgll^{95}$ flies is caused by the oversensitivity to the lack of vitamin B6 in the diet; SGLL is the *Drosophila* homolog of PNPO; and the c.95C>A mutation in SGLL only causes a partial decrease of the enzyme activity of PNPO.

The sugar lethal phenotype was also observed in ubiquitous *sgll* knockdown (KD) flies, which was improved by PLP, but not PN (Table 1.1), suggesting that PNPO activity in ubiquitous KD flies was too low to convert PN to PLP.

In summary, using forward genetic approaches and whole genome sequencing we identified a *Drosophila* homolog (*sugarlethal*, *sgll*) of human *PNPO* gene and identified a missense mutation in *sgll*. This mutation causes partial loss of function of *sgll*. The corresponding hypomorphic allele is designated as $sgll^{95}$.

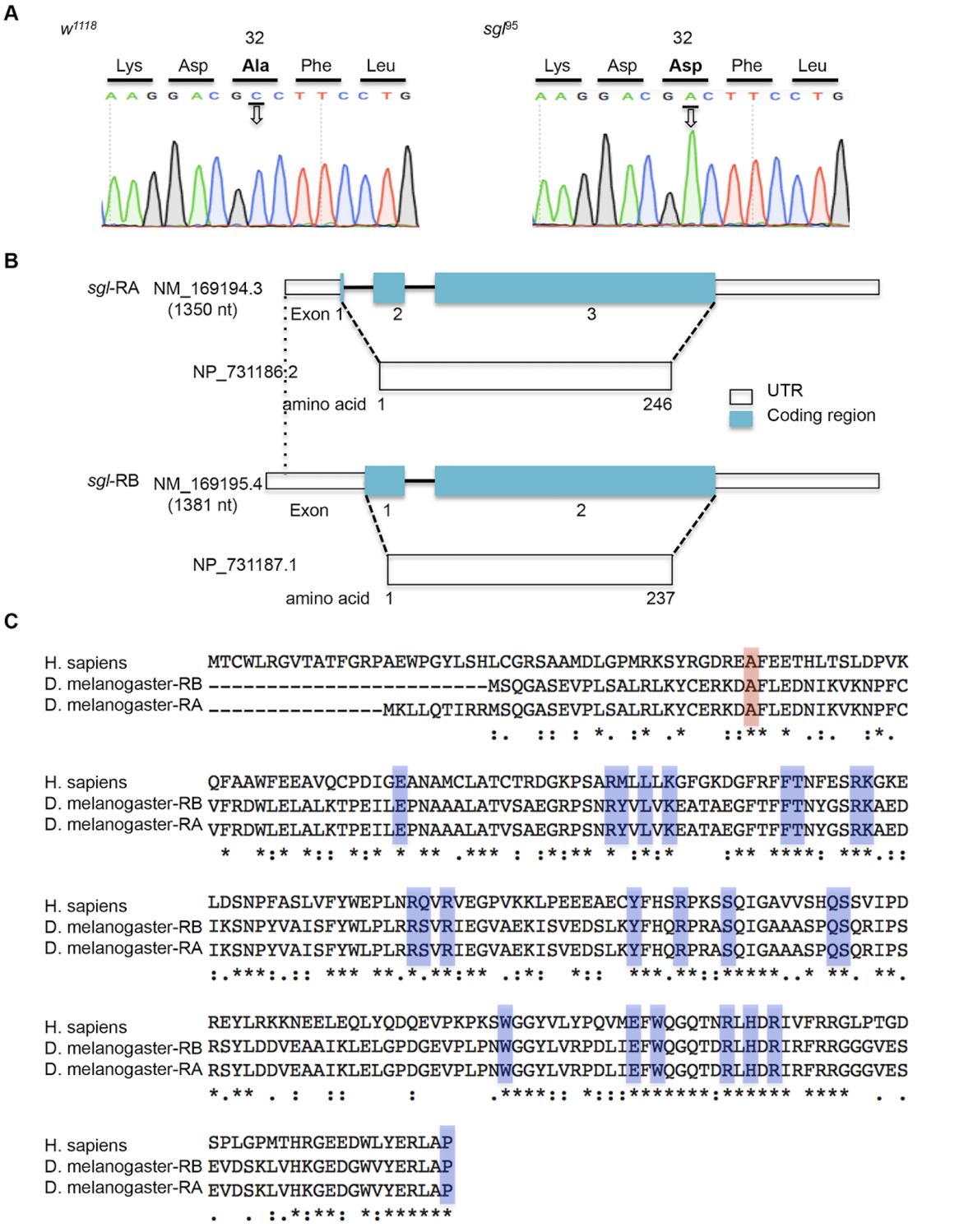

Figure 1.6: **Mutation, gene structure, and homology analysis.** (A) A homozygous missense mutation in *sgll* was validated by targeted Sanger sequencing. (B) Gene structure of *sgll*. (C) Amino acid sequence alignment analysis. Known essential residues for enzyme activity are shaded in blue. The alanine at +32 is highlighted with red. Human PNPO amino acid sequences were obtained from NCBI (RefSeq: NP_060599.1).Semi-conserved (.), conserved (:), and absolutely conserved (*) are shown.

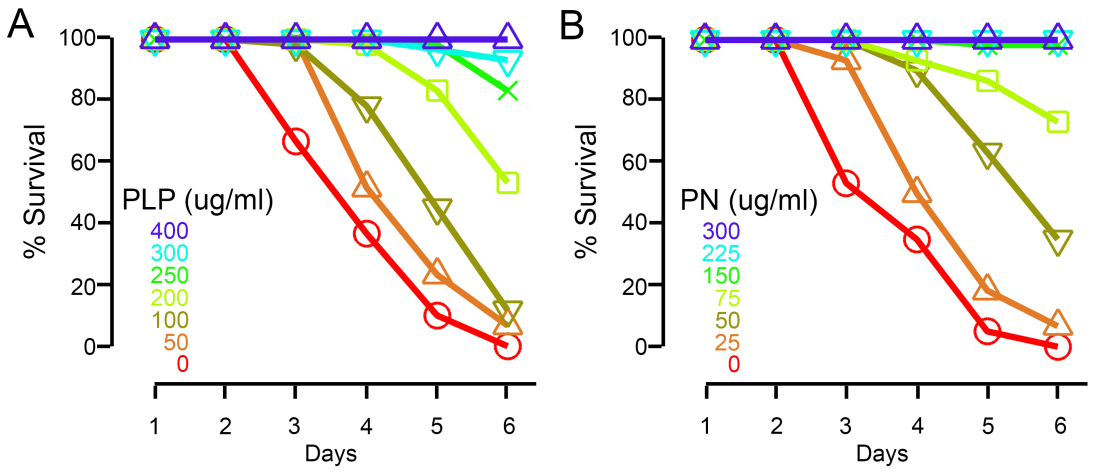

Figure 1.7: **Either PLP or PN supplementation rescued the lethal phenotype of *sgll*^{95} homozygotes.** PLP and PN were added to 4% sucrose and survival was checked daily. Compared with 100% death in the sugar-only group by day 6, flies with PLP supplement (A) and PN supplement (B) survived in a dose-dependent manner.

Table 1.1: **Survival rates of ubiquitous *sgll* KD flies under different conditions.**

Treatment	*actin*-Gal4/UAS-*sgll* RNAi	*actin*-Gal4/+	UAS-*sgll* RNAi/+
Control	0.58	1	1
PN	0.53	1	1
PLP	0.84*	1	1

n = 30-40 per group; $^*P < 0.05$ Chi-square test of homogeneity
Control: 4 % sucrose in 1 % agar; PN: 75 ng/ml PN plus 4 % sucrose in 1 % agar; PLP: 200 ng/ml PN plus 4 % sucrose in 1 % agar. The concentrations of PN and PLP were chosen based on their EC50 in their respective dose-response curves in rescuing *sgll*^{95} flies (Figure 1.7).

1.5 Dissertation Overview

The identification of a *Drosophila PNPO* gene and a *PNPO* deficient strain ($sgll^{95}$) opened up opportunities to study PNPO-deficiency-induced epilepsy, which has been increasingly reported in young patients but the underlying mechanisms have never been studied due to the lack of animal models. Moreover, it is unknown whether mild PNPO deficiency confers susceptibility to epilepsy in adults. In this dissertation, I first established *Drosophila* models of PNPO-deficiency-induced epilepsy (Chapter 2) and then studied the fundamental biology of PNPO and PNPO deficiency using knock-in *Drosophila* strains that carry human seizure-causing PNPO mutations (Chapter3). In chapter 4, I initiated a human study to test the hypothesis that mild PNPO deficiency contributes to adult-onset epilepsy.

CHAPTER 2

PYRIDOX(AM)INE 5'-PHOSPHATE OXIDASE DEFICIENCY INDUCES SEIZURES IN *DROSOPHILA MELANOGASTER*

2.1 Abstract

Pyridox(am)ine 5'-phosphate oxidase (PNPO)[1] is a rate-limiting enzyme in converting dietary vitamin B6 (VB6) to PLP, the biologically active form of VB6 and involved in the synthesis of neurotransmitters including GABA, DA, and serotonin. In humans, PNPO deficiency has been increasingly identified in neonatal epileptic encephalopathy and more recently also in early-onset epilepsy. Till now, little is known about the neurological mechanisms underlying PNPO-deficiency-induced seizures due to the lack of animal models. Previously we identified a c.95C>A missense mutation in *sgll* - the *Drosophila* homolog of human *PNPO* (*hPNPO*) and found mutant ($sgll^{95}$) flies exhibiting a lethal phenotype on a diet devoid of VB6. Here we report the establishment of both $sgll^{95}$ and ubiquitous *sgll* knockdown (KD) flies as valid animal models of PNPO-deficiency-induced epilepsy. Both $sgll^{95}$ and *sgll* KD flies exhibit spontaneous seizures before they die. Electrophysiological recordings reveal that seizures caused by PNPO deficiency have characteristics similar to that in flies treated with GABA antagonist picrotoxin. Both seizures and lethality are associated with low PLP levels and can be rescued by ubiquitous expression of wild-type *sgll* or *hPNPO*, suggesting the functional conservation of PNPO enzyme between humans and flies. Results from cell type-specific *sgll* KD further demonstrate that PNPO in the brain is necessary for seizure prevention and survival. Our establishment of the first animal model of PNPO deficiency will lead to better understanding of VB6 biology, the *PNPO* gene and its mutations discovered in patients, and can be a cost-effective system to test therapeutic drugs.

1. Citation for chapter: Chi W, Iyengar ASR, Albersen M, Bosma M, Verhoeven-Duif NM, Wu CF, Zhuang X. bioRxiv (2019)

2.2 Author Contributions

Figure 1: Data were collected by W.C. and analyzed by W.C.; **Figure 2**: Data were collected by W.C. and analyzed by W.C.; **Figure 3**: Data were collected by A.I. and W.C. and analyzed by A.I.; **Figure 4**: Flies were prepared by W.C., B6 vitamers were measured by M.A., M.B., N.V.D., and data was analyzed by W.C.; **Figure 5**: Panel A, B, C, and D were done by W.C. and Panel E was done by A.I.; **Figure 6**: Data were collected by W.C. and analyzed by W.C.

2.3 Introduction

Mutations in an autosomal gene encoding pyridox(am)ine 5'-phosphate oxidase (PNPO) cause neonatal epileptic encephalopathy (NEE, OMIM #610090), a severe neurological disease that leads to death if untreated [91]. Furthermore, PNPO mutations have more recently been identified in patients with infantile spasms [97] and early-onset epilepsy [98, 99, 100]. While the causal relationship between PNPO deficiency and NEE has been established on the molecular level since 2005 [91], the neurological mechanisms of how PNPO deficiency leads to NEE and related epilepsy syndromes remain to be established.

PNPO is a rate-limiting enzyme in the synthesis of vitamin B6 (VB6), which comprises a group of six different forms including pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP) [68, 101]. PNPO converts PNP and PMP to PLP, the biologically active form of VB6, which is a co-factor for more than 140 different enzymes that are involved not only in the metabolism of amino acids, glycogen, and unsaturated fatty acids but also in the synthesis of various neurotransmitters including γ-aminobutyric acid (GABA), dopamine, and serotonin [82]. PNPO activity is essential to humans and other animals because unlike plants, fungi, and bacteria, they cannot synthesize VB6 *de novo* [71]. PNPO is highly expressed in liver, brain, and kidney in mammals [76] and previous studies have implicated the liver as

the primary site of conversion of dietary VB6 to PLP [74], which is further transported by circulation to different tissues/organs, including the brain [79, 80]. However, the contribution of brain-expressed PNPO to seizures and lethality, is unknown.

In recent years, PNPO mutations have been increasingly reported in human patients [91, 98, 100, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116]. Seizures in PNPO deficient patients usually do not respond to conventional anti-epileptic drugs but is responsive exclusively to PLP, or to PN in patients with partial PNPO deficiency [105, 108, 111]. Despite its effectiveness in seizure control, PLP or PN treatment has little effect on other neurological defects observed in PNPO deficiency patients, such as developmental delay or intellectual disability [108, 112, 114]. Moreover, long-term PLP treatment causes hepatic cirrhosis [117, 118]. There is a need for valid animal models to study the fundamental biology of PNPO and VB6, their role in neurodevelopment and neurotransmission, and to explore other treatment options for PNPO deficiency.

Based on the nutritional conditional lethal phenotype, we have recently identified a *Drosophila melanogaster* homolog gene of human *PNPO sgll* and identified a mutant with partial PNPO deficiency ($sgll^{95}$) [119]. The $sgll^{95}$ flies survive well on a normal diet, but due to their low PNPO activity, they exhibit lethality when reared on a sugar-only diet (4% sucrose in 1% agar, i.e., diet devoid of VB6). This nutritional conditional lethal phenotype can be rescued by the supplementation of either PN or PLP. Here we report that human and *Drosophila* PNPO are functionally conserved and that PNPO deficiency in *Drosophila* causes seizures that are defined by both behavioral and electrophysiological parameters. These PNPO-deficiency-induced-phenotypes are associated with low levels of PLP and PNPO deficiency restricted to the neural tissue is sufficient to cause seizures and lethality.

2.4 Results

2.4.1 $sgll^{95}$ Flies Exhibit Seizure-like Behavior Before They Die

Consistent with our previous report [119], we found that $sgll^{95}$ homozygotes reared on a sugar-only diet, consisting of a 4% sucrose in a 1% agar, exhibited a striking lethality phenotype compared to w^{1118} counterparts. As shown in Figure 2.1 A, nearly all $sgll^{95}$ mutants had died after four days while no control flies had died. Before death (2 - 3 days on the sugar-only diet, green box in Figure 2.1 A), $sgll^{95}$ mutants displayed bouts of rapid 'wing-buzzing', and 'body-rolling', and later on, they often exhibited a 'wings-up' posture (Figure 2.1 B). These seizure-like behavioral phenotypes occurred spontaneously and did not appear to be initiated by mechanical shock [34, 35] or temperature stress [36, 120].

To examine these behaviors, we recorded individual $sgll^{95}$ or w^{1118} control flies walking in a 60 mm-wide open arena with a high-resolution video camera for 3 min (See Methods for details). Fly positions were then tracked with the IowaFLI Tracker [48], and the corresponding travel traces from each fly were plotted. We observed a smooth travel trace from each w^{1118} control fly (n = 22 on sugar; n = 19 on the normal diet) or from $sgll^{95}$ fly on the normal diet (n = 14). However, travel traces of $sgll^{95}$ flies reared on the sugar-only diet (n = 21) were heterogeneous with some traces similar to that of controls whereas others did not show smooth trajectories at all. Representative traces are shown in Figure 2.1 C.

To quantify these travel traces, we plotted each trace on a scatter plot where the speeds of a fly during a frame t are plotted against the speeds in the next frame t+1 (Figure 2.1 D) and then we calculated the speed correlation coefficients. The reasoning is that a smooth travel trace indicates consistent locomotion, i.e., similar speeds at two consecutive time points. On the scatter plot, all points from a smooth trace would land on the lower left to upper right diagonal line and give a high correlation coefficient of speed. On the opposite side, the less smooth travel traces will have their points spread out from the diagonal line and give lower correlation coefficients. Indeed, the average correlation coefficient is 0.654 ± 0.116 (Mean ±

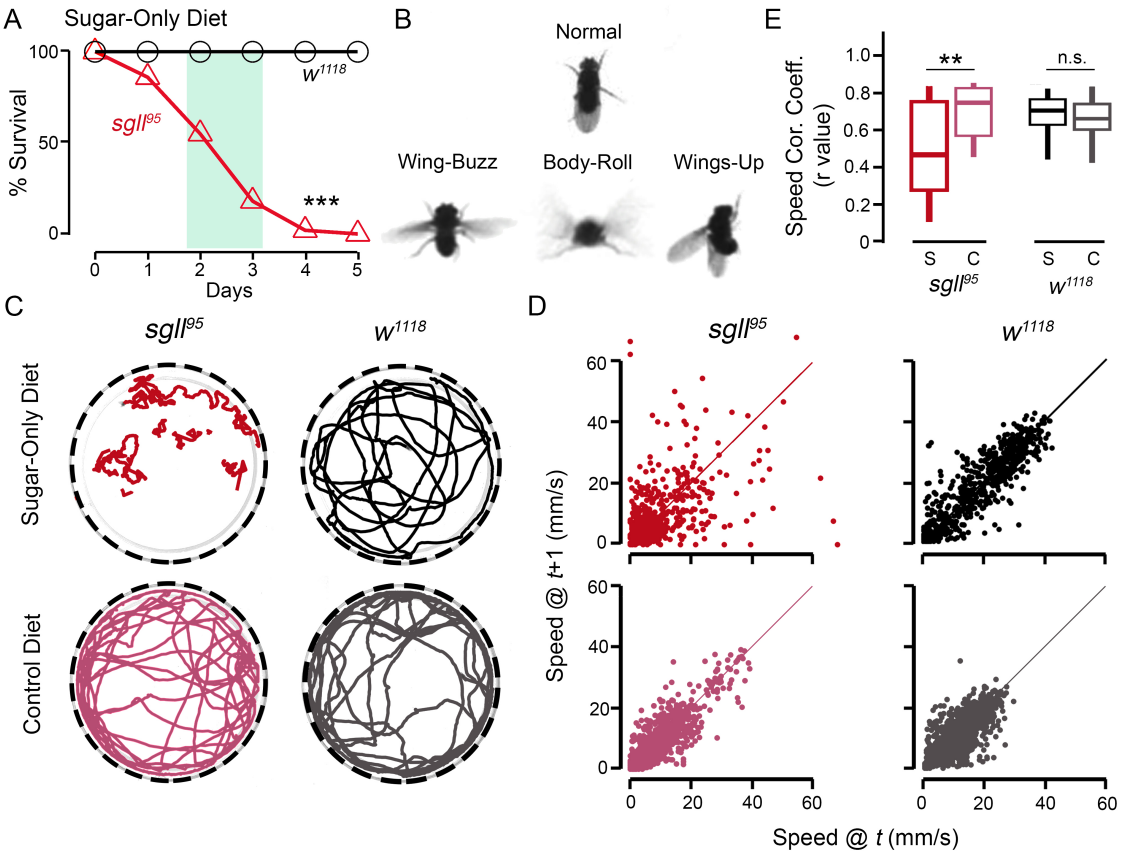

Figure 2.1: ***sgll*^{95} flies show seizure-like behavior before they die when reared on the sugar-only diet.** A) Survival of *sgll*^{95} and w^{1118} on the sugar-only diet. *** $P < 0.001$, Log-rank test, n = 100-101. The green block indicates the time window when flies were characterized for the seizure phenotypes. B) Representative images of normal and seizure-like behaviors from video recordings. C) Representative travel traces from video recordings. Each trace is from one fly. The fragmented trace from the *sgll*^{95} fly on the sugar-only diet is due to the faster movement of the fly than the frame window (50 ms). D) Speed correlation plots between the frame t and frame t+1. Each plot is corresponding to the one in panel C. E) Summary plot of the speed correlation coefficient (Speed Cor. Coeff.). S: sugar-only diet, C: normal diet, n.s.: $P > 0.05$, ** $P < 0.01$, Two-way ANOVA with Tukey's *post hoc*, n = 14-22 per genotype per condition.

SD), 0.672 ± 0.120, 0.701 ± 0.140, and 0.493 ± 0.260 for w^{1118} on the normal diet, w^{1118} on the sugar-only diet, $sgll^{95}$ on the normal diet, and $sgll^{95}$ on the sugar-only diet, respectively. Therefore, consistent with qualitative distinctions between $sgll^{95}$ and w^{1118} flies, we found significantly reduced speed correlation coefficients in $sgll^{95}$ when reared on the sugar-only diet (Figure 2.1 E, $P = 0.0047$, gene by condition interaction $P = 0.0065$).

2.4.2 Ubiquitous RNAi-mediated Knockdown of sgll Leads to Lethality and Seizure-like Behavior when Reared on the Sugar-only Diet

We have previously shown that ubiquitous *sgll* knockdown (KD, i.e. *actin*-Gal4/UAS-*sgll* RNAi) flies also show the conditional lethal phenotype, which can be rescued by PLP but not PN [119], indicating severe PNPO deficiency in these flies compared to that in $sgll^{95}$ flies. Indeed, *sgll* KD flies survived shorter than $sgll^{95}$ flies (compared males to males); all *sgll* KD flies died within four days (Figure 2.2 A) while all $sgll^{95}$ flies died within five days (Figure 2.1 A). Prior to death, *sgll* KD flies displayed seizure-like behavior similar to that in $sgll^{95}$ flies including the wing buzzes, wings-up posture and convoluted walking trajectories (Figure 2.2 B&C). The average speed correlation coefficient of *sgll* KD flies is 0.423 ± 0.240 (Mean ± SD), which is significantly lower than that from the parental controls (0.644 ± 0.139, and 0.645 ± 0.233 for *actin*-Gal4/+ and UAS-*sgll* RNAi/+, respectively. Figure 2.2 D; $P =$ 5.947e-05).

2.4.3 Spontaneous Seizure Discharges in sgll Mutants Reared on the Sugar-only Diet

To monitor electrophysiological activity and identify aberrant patterns in *sgll* mutants, we undertook an analysis of flight muscle activity in a tethered fly preparation [121, 122]. The isometric contractions of the dorsal longitudinal muscle (DLM) enable prolonged recordings of spiking activity with minimal damage to the muscle. Several studies have taken advantage

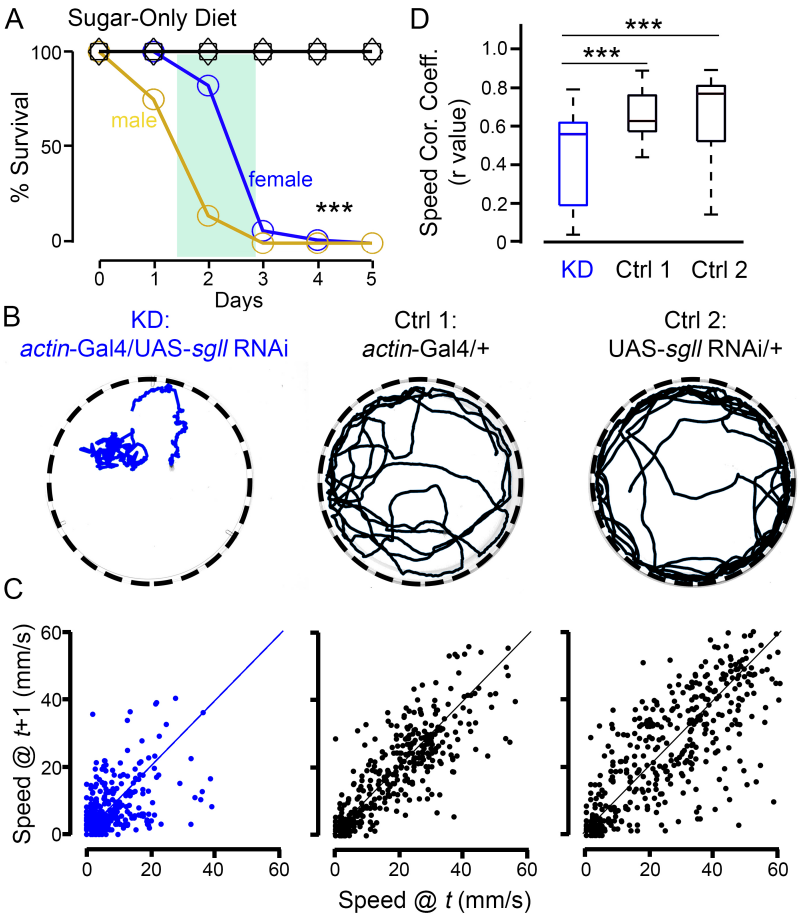

Figure 2.2: **Ubiquitous *sgll* KD flies show lethality and seizure-like behavior when reared on the sugar-only diet.** A) Survival of KD (blue and golden) and parental control flies (black square and black diamond) on the sugar-only diet. The green block indicates the time window in which the behavioral characterization was performed. *** $P < 0.001$, Log-rank test, n = 56-60 per genotype per sex. B) Representative travel traces from video recordings. C) Speed correlation plots between frame t and frame t+1. Each plot is corresponding to the one in panel B. D) Summary plot of speed correlation coefficient (Speed Cor. Coeff.). *** $P < 0.001$, One-way ANOVA with Tukey's *post hoc*, n = 27-34 per genotype per condition.

of this stable recording preparation to monitor aberrant spike discharges associated with seizures that occur spontaneously in mutant flies [37, 38], seizures that are triggered by high-frequency electroconvulsive stimulation [121, 49, 51] or by proconvulsant administration [53]. Importantly, the bouts of DLM spiking activity during seizure discharges appears to be temporally correlated with extracellular synchronous activity across different body-axes [51].

We recorded spontaneous DLM spiking activity not associated with flight from $sgll^{95}$, *sgll* KD flies and w^{1118} control flies, reared on either the normal diet or the sugar-only diet (Figure 2.3 A). Flies reared on the normal diet displayed short bouts of a few spikes associated with wing depression events during grooming activity [53]. Over the recording session ($\sim$ 240 s) the average firing frequency (total # spikes / recording duration) was $\sim$ 0.3 Hz, for the three genotypes tested (Figure 2.3 B). In a striking contrast, recordings from both $sgll^{95}$ and *sgll* KD flies showed high-frequency spike burst discharges qualitatively distinct from the grooming-related spiking seen in their counterparts reared on the normal diet (Figure 2.3 A), and there was also significantly more firing (median firing rate of: 2.03 Hz.- vs. 0.32 Hz.- for $sgll^{95}$, and 7.91 vs. 0.07 Hz for *sgll* KD flies, $P = 0.0045$ and $P = 1.138\text{e-}6$, for $sgll^{95}$ and *sgll* KD, respectively. Figure 2.3B. These spike bursts occurred simultaneously in the left and right muscles (data not shown), suggesting that they were triggered by relatively widespread events across the nervous system. Notably, our sample included $sgll^{95}$ and *sgll* KD flies with 'wings-up' as well as normal wing posture (Figure 2.1B). We found that between our recordings of $sgll^{95}$ and *sgll* KD flies, all wings-up flies displayed spike bursts (6 / 6), and several individuals with normal wing posture did that as well (5 / 8 flies), suggesting that the burst discharge phenotype likely initiates prior to appearance of the 'wings-up' posture.

An important characteristic of the DLM spike trains recorded was the highly variable nature of inter-spike intervals (ISIs). Within a spike train, the instantaneous firing frequency between two spikes (defined as the inverse of the ISI, i.e. ISI^{-1}) could range from less than 1 Hz to more than 100 Hz within a bout. To quantitatively delineate the grooming-related spike patterns, we employed a phase-space analysis of ISIs [53]. For the sequence of spikes within

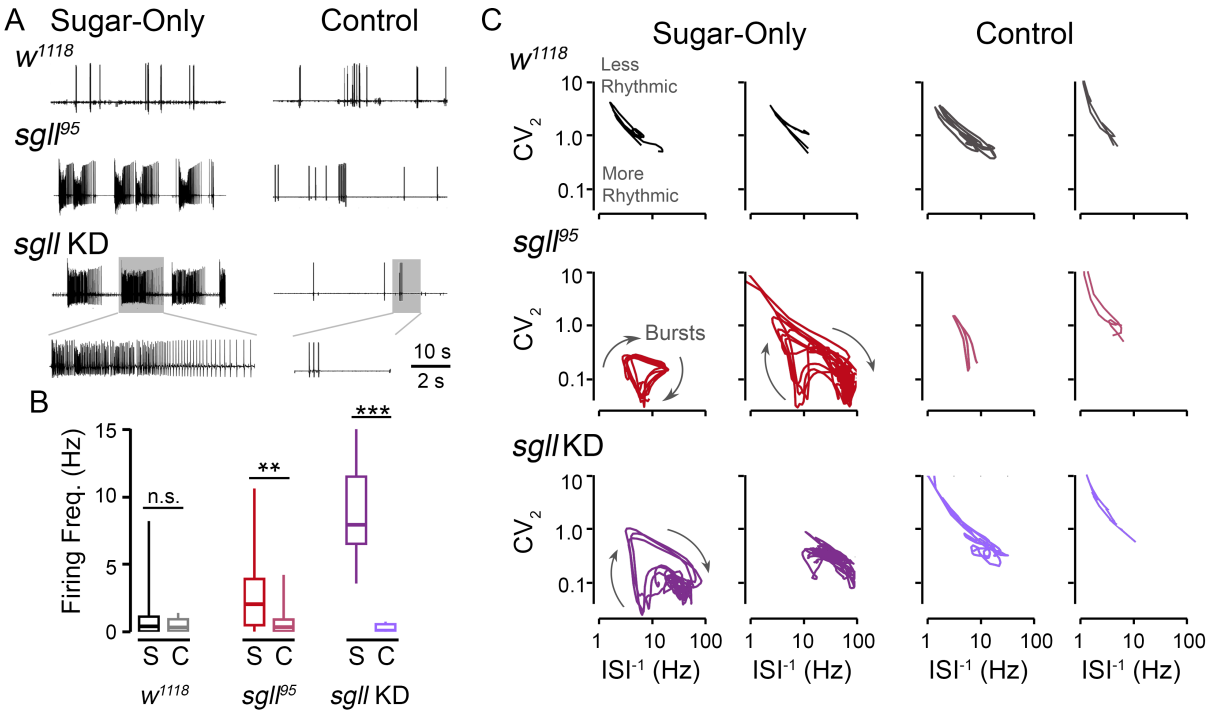

Figure 2.3: **Spontaneous spike discharges in flight muscles of *sgll* mutants.** A) Representative traces of DLM spiking in w^{1118} control flies, $sgll^{95}$ mutants, and ubiquitous RNAi knockdown of *sgll* by *actin*-Gal4 driver (i.e. UAS-*sgll* RNAi/*actin*-Gal4, indicated as *sgll* KD). Flies were reared on the sugar-only diet (Sugar-only, left column) or normal diet (Control, right column). Note the difference between the high-frequency spike discharges in the $sgll^{95}$ and *sgll* KD traces and the spiking in w^{1118} flies and *sgll* mutants on the normal diet (see expanded traces). The spiking in flies on the normal diet is associated with grooming behavior (Supplemental Video 1, also see reference (43)). B) Summary plot of the average firing frequency over the duration of the recordings (90 s) in the respective genotypes on 4% sucrose (S) or normal (C) diet. n.s.: $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, Rank-sum test. n = 7 -11 flies. (C) Plots of the trajectory of the instantaneous firing frequency (ISI^{-1}) vs. the instantaneous coefficient of variation (CV_2) during DLM spiking. For each genotype/condition, two representative trajectories are shown. The oscillations indicated in $sgll^{95}$and *sgll* KD trajectories correspond to individual burst discharges in the DLM trace.

the recording, we plotted the ISI^{-1} of each spike against the spike's instantaneous coefficient of variation, CV_2, a measure of variability between successive ISI^{-1} values (see Methods for computational details). The resulting ISI^{-1} vs CV_2 trajectories have been shown to clearly distinguish between spike patterns associated with flight, grooming and electroconvulsive stimulation-induced seizure discharges in *Drosophila* [53].

The ISI^{-1} vs. CV_2 trajectories of the burst discharges in *sgll* mutants on sugar-only diet were readily distinguished from the spiking activities in counterparts on the normal diet and in w^{1118} flies (representative trajectories shown in Figure 2.3 C). Specifically, trajectories of $sgll^{95}$ flies on the sugar-only diet display 'loops' consisting of the ISI^{-1} of the trajectory with abrupt acceleration (often peaking between 50 and 100 Hz) followed by a gradual deceleration characterized by relatively low CV_2 values. A few *sgll* KD flies displayed more extreme trajectories which were more compact, reflecting continuous high-frequency firing. In contrast, the ISI^{-1} vs. CV_2 trajectories of *sgll* mutants on the normal diet as well as that of w^{1118} flies generally ambled within a limited region of higher CV_2 values (> 0.5) reflecting a high degree of variability in successive spike intervals, consistent with observations of grooming spike patterns in other wild-type fly strains [53].

2.4.4 Seizures and Lethality are Correlated with Low Levels of Internal PLP

PNPO converts intracellular PNP and PMP to PLP (Figure 2.4 A), and PNPO deficiency is expected to result in a low level of PLP. However, both normal and decreased PLP levels have been reported in human PNPO deficient patients [114, 123]. Such inconsistency is conceivably due to confounding factors such as heterogeneous genetic backgrounds and different diets [98]. Since both genetic background and dietary conditions were well controlled in flies, we reasoned that by comparing the PLP level between $sgll^{95}$ and w^{1118} control flies we should be able to characterize the impact of PNPO deficiency on VB6 metabolism. Furthermore, seizures in human PNPO deficient patients cease with PLP or PN treatment [105, 108, 111, 112]. Our previous studies have also shown that PLP or PN supplementation can rescue the conditional

lethality of $sgll^{95}$ flies [119]. Therefore, to further correlate the PLP level with seizures and lethality, we measured PLP in flies reared on the sugar-only diet as well as the sugar-only diet supplemented with PN.

As shown in Figure 2.4 B, when reared on the sugar-only diet (S), $sgll^{95}$ flies had significantly reduced PLP compared to w^{1118} flies (red bar vs. black bar, $P = 4.020\text{e-}5$), which was significantly improved by PN supplementation (red vs. pink bar, $P = 0.0035$). On the other hand, PN supplementation did not change the PLP level in w^{1118} flies (black vs. gray bar, $P = 0.9126$), suggesting that PLP is highly regulated in normal flies. The regulation of PLP in w^{1118} flies is most likely mediated through the conversion from PLP to PL as indicated by a significantly increased PL level after PN supplementation in w^{1118} flies compared to $sgll^{95}$ flies (gene by treatment interaction: $P = 0.0115$; $P = 0.0004$ and $P = 0.0988$ the treatment effect in w^{1118} and $sgll^{95}$ flies, respectively). In comparison, PMP and PA levels show no difference between w^{1118} and $sgll^{95}$ flies before PN supplementation ($P = 0.6914$ and $P = 0.5305$ for PMP and PA, respectively). After PN supplementation, PA is significantly increased in w^{1118} flies compared to $sgll^{95}$ flies (gene by treatment interaction: $P = 0.0198$; $P = 0.0504$ and $P = 0.8017$ for the treatment effect in w^{1118} and $sgll^{95}$ flies, respectively), whereas PMP is similar between the two genotypes (gene by treatment interaction: $P = 0.4062$; $P = 0.5159$ and $P = 0.9980$ for the treatment effect in w^{1118} and $sgll^{95}$ flies, respectively).

2.4.5 PNPO is Functionally Conserved Between Humans and Flies

Amino acid sequence alignment analysis revealed that the protein product of *sgll* shares more than 75% similarity with human PNPO [119]. To further study if the PNPO enzyme is functionally conserved between humans and flies, we generated transgenic flies by sub-cloning cDNAs from wild-type (WT) human and *Drosophila PNPO* gene into a pUAST vector (UAS-*hPNPO* and UAS-*sgll*, respectively). We used the GAL4/UAS system [13] to drive the expression of transgenes. To examine the effect of the ubiquitous expression of each transgene,

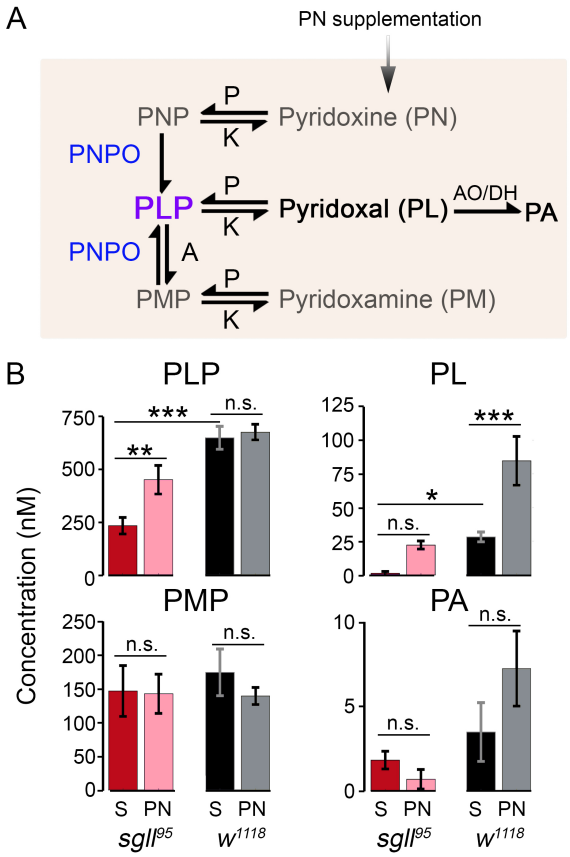

Figure 2.4: **B6 vitamer measurements in *sgll*^{95} and *w*^{1118} flies under different conditions.** A) A schematic representation of the interchangeable relationships among B6 vitamers from studies in mammals and humans. The conversion occurs primarily in the liver cells. After conversion, B6 vitamers (non-phosphorylated forms) are released into the circulation and transported to other tissues/organs. Among them, PL is the main form of VB6 for most tissues/organs. Excess PL is oxidized by aldehyde oxidase (AO) or NAD^+-dependent aldehyde dehydrogenase (DH) to pyridoxic acid (PA) in the liver or kidney. PA is then excreted in urine. In normal humans, abundant B6 forms are PLP, PL, and PA. Sometimes PM or PMP are detectable, whereas PN or PNP usually are undetectable [124, 125, 126, 127, 128]. PNP: pyridoxine 5'-phosphate, PLP: pyridoxal 5'-phosphate, PMP: pyridoxamine 5'-phosphate, PNPO: pyridox(am)ine 5'-phosphate oxidase, P: phosphatase, K: pyridoxal kinase, A: aminotransferase. B) Six B6 vitamers (PN, PNP, PL, PLP, PM, and PMP) and PA were measured in whole flies. The relative B6 vitamer composition in *w*^{1118} flies on the sugar-only diet is PLP > PMP > PL > PA. The other B6 vitamers were undetectable. Levels of various B6 vitamers in *sgll*^{95} and *w*^{1118} flies under the sugar-only diet (S) or the sugar-only diet supplemented with PN are shown in four panels. ** $P < 0.01$, *** $P < 0.001$, n.s.: $P > 0.05$; Two-way ANOVA with Tukey's *post hoc*, n = 3 biological replicates per genotype per condition.

we first backcrossed each UAS line and an *actin*-Gal4 line (genotype: *actin*-Gal4/*CyO*) to the $sgll^{95}$ background. We then crossed each backcrossed UAS line with the backcrossed *actin*-Gal4 line to generate flies in which the transgene was ubiquitously expressed on the $sgll^{95}$ background. The conditional survival rates of these flies (genotypes: *actin*-Gal4/UAS-*sgll*; $sgll^{95}/sgll^{95}$ flies and *actin*-Gal4/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$) and control flies (genotypes: +/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$, +/UAS-*sgll*; $sgll^{95}/sgll^{95}$, and *actin*-Gal4/+; $sgll^{95}/sgll^{95}$ and $sgll^{95}/sgll^{95}$) were examined.

In contrast to $sgll^{95}$ homozygotes that died within five days on the sugar-only diet (Figure 2.5 A, red curve), $sgll^{95}$ flies with ubiquitous expression of WT *sgll* or *hPNPO* (i.e. rescue flies) displayed no mortality (blue or green circle versus red point-up triangle; $P < 0.001$ for both genotypes compared to $sgll^{95}$ homozygotes) and they did not show any seizure-like behavior. To confirm that, we performed behavioral characterization on rescue flies reared on the sugar-only diet for two or five days. As shown in Figure 2.5 B&C&D, rescue flies behaved normally and had similar average speed correlation coefficients to $sgll^{95}$ flies on the normal fly diet. The average speed correlation coefficient on day 2 is 0.601 ± 0.1411 (Mean ± SD) and 0.702 ± 0.106 for *actin*-Gal4/UAS-*sgll*; $sgll^{95}/sgll^{95}$ (n = 14) and *actin*-Gal4/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$ (n = 11), respectively. The average speed correlation coefficient on day 5 is 0.722 ± 0.107 and 0.675 ± 0.160 for *actin*-Gal4/UAS-*sgll*; $sgll^{95}/sgll^{95}$ (n = 10) and *actin*-Gal4/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$ (n = 10), respectively. These coefficients are not significantly different from those in $sgll^{95}$ flies on the normal fly diet ($P = 0.0766$, $P = 0.9866$, $P = 0.6873$, and $P = 0.6852$, unpaired t-test). Furthermore, recordings of DLM activities from *actin*-Gal4/UAS-*sgll*; $sgll^{95}/sgll^{95}$ and *actin*-Gal4/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$ flies did not show the spiking burst discharges characteristic of $sgll^{95}$ flies (Figure 2.5 E, n = 7 for each genotype). Together, these data indicate that ubiquitous expression of *hPNPO* completely rescued the conditional seizures and lethality of $sgll^{95}$ homozygotes and demonstrate the functional conservation of PNPO enzyme in humans and flies.

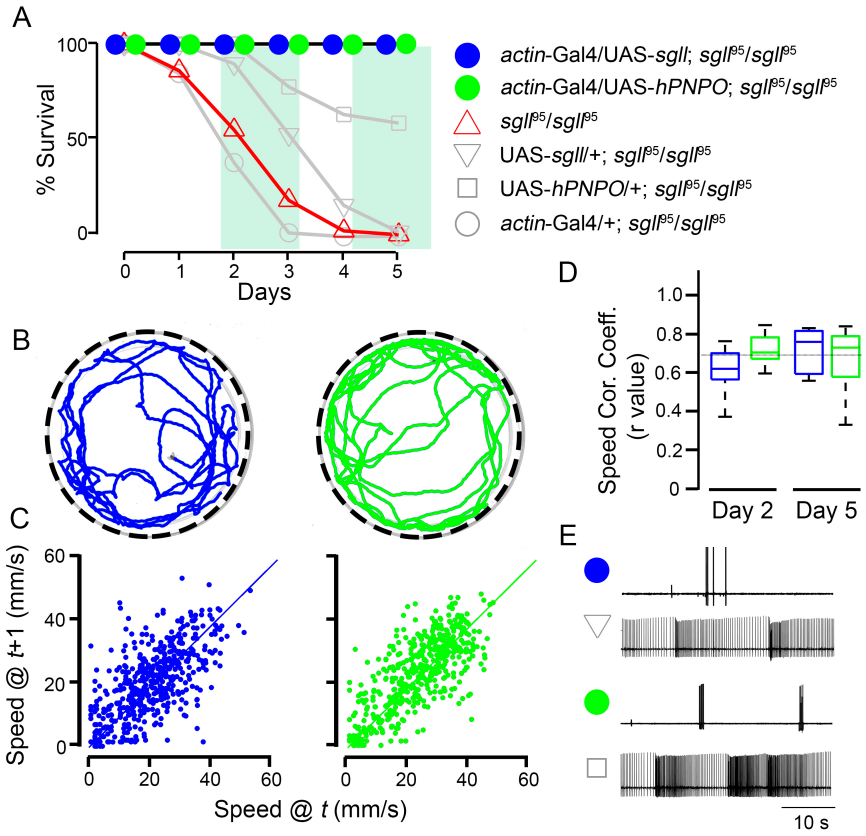

Figure 2.5: **Ubiquitous expression of WT *sgll* or *hPNPO* completely rescued the lethality and seizures of $sgll^{95}$ homozygotes.** A) Survival of flies with various genotypes on the sugar-only diet. Ubiquitous expression of WT *sgll* or *hPNPO* completely rescued the conditional lethality of $sgll^{95}$ homozygotes (blue or green circle vs. red point-up triangle, $P < 0.0001$, Log-rank test, n = 51-101). The curve of $sgll^{95}$ homozygotes was replotted from Figure 2.1A. Similar to $sgll^{95}$ homozygotes, *actin*-Gal4/+; $sgll^{95}/sgll^{95}$ (gray circle, n = 52) and UAS-*sgll*/+; $sgll^{95}/sgll^{95}$ (gray point-down triangle, n = 253) also died by day 5. Note that some UAS-*hPNPO*/+; $sgll^{95}/sgll^{95}$ flies survived by day 5 (gray square, total n = 314), which was presumably due to the expression of *hPNPO* in cells labeled by *Ddc*-Gal4 since the mutation in the $sgll^{95}$ allele was identified in the *Ddc*-Gal4 driver line [119]. The green block indicates the time window in which the seizure characterizations were performed. B) Representative travel traces from *actin*-Gal4/UAS-*sgll*; $sgll^{95}/sgll^{95}$ (left) and *actin*-Gal4/UAS-*hPNPO*; $sgll^{95}/sgll^{95}$ (right). C) Speed correlation plots between the frame t and frame t+1. Each plot is corresponding to the one in panel B. D) Summary plot of speed correlation coefficient (Speed Cor. Coeff.) measured on day 2 or day 5. The black dotted line indicates the average speed correlation coefficient of $sgll^{95}$ homozygotes on the normal diet (shown in Figure 2.1E). E) Representative spike traces of DLM spiking in rescue and their parental UAS lines.

2.4.6 PNPO in the Brain is Necessary for the Normal Brain Function

The brain obtains VB6 from the circulation [79, 80]. Previous studies have shown that PLP and PL are the primary forms of VB6 in the circulation [77, 78], raising the question whether PNPO in the brain is necessary for normal brain functions.

To answer this question, we generated neural-specific *sgll* KD flies using an *elav*-Gal4 driver [129]. KD and corresponding control flies were then tested on the sugar-only diet. We observed both lethality (Figure 2.6 A, $P < 0.001$) and seizures (Figure 2.6 B) in *sgll* KD flies, both of which are absent from controls, demonstrating that expression of PNPO in neurons is necessary for both seizure prevention and survival.

2.5 Discussion

The identification of a hypomorphic *PNPO* allele, $sgll^{95}$, in *Drosophila* by our earlier work [119] has opened up opportunities to study PNPO functions in an animal model for the first time. Here we report that PNPO deficiency causes seizures in *Drosophila*, similar to human conditions associated with PNPO deficiency. The seizure and lethality phenotypes are associated with low PLP levels and are completely rescued by VB6 supplementation and by ubiquitous expression of either WT *sgll* or WT human *PNPO* (*hPNPO*). Cell type-specific KD further suggests that PNPO in the brain is necessary for seizure prevention and survival.

The amino acid sequence of PNPO is evolutionarily conserved [119, 96]. The fact that PNPO deficiency induces seizures in flies as it does in humans (Figure 2.1-2.3) demonstrates that the biological function of PNPO is also conserved. Complete rescue of the conditional lethality and seizures of $sgll^{95}$ flies by ubiquitous expression of *hPNPO* further confirmed this notion (Figure 2.5). All these provide a solid foundation for studying the fundamental biology of PNPO (Figure 2.6) and for future characterizations of human-disease-associated hPNPO mutations in *Drosophila* models.

NEE caused by severe PNPO deficiency is lethal if untreated. However, favorable outcomes

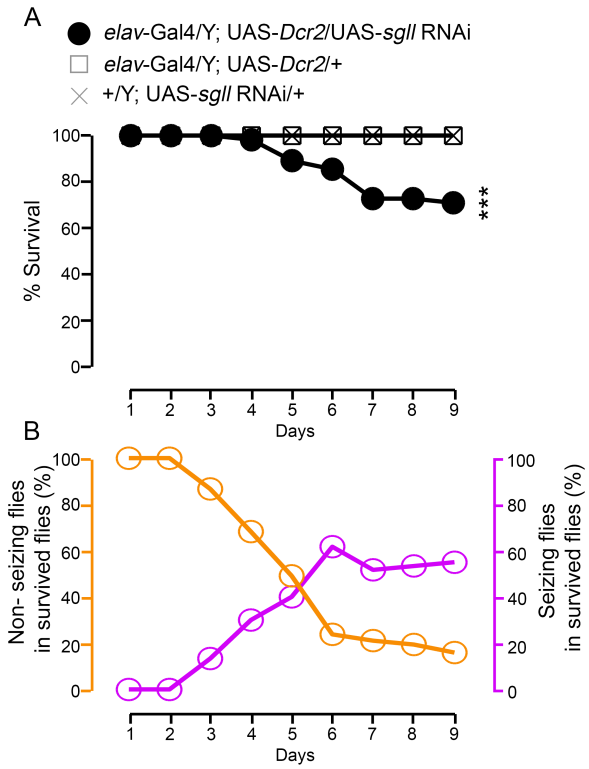

Figure 2.6: **Survival and seizure rate of neural-specific *sgll* KD (genotype: *elav*-Gal4/Y; UAS-*Dcr2*/UAS-*sgll* RNAi) and control flies (genotypes: *elav*-Gal4/Y; UAS-*Dcr2*/+ and +/Y; UAS-*Dcr2*/UAS-*sgll* RNAi) on the sugar-only diet.** A) Survival of *sgll* KD and control flies. *** $P < 0.001$, Log-rank test, compared to control lines, n = 55 - 60 per genotype. B) Nearly all survived *sgll* KD flies show seizure-like phenotype by day 9.

are still possible if patients could be diagnosed and treated timely [130]. A sensitive and specific biomarker will undoubtedly help the diagnosis. Given that PNPO determines the synthesis of PLP, the PLP level along with other B6 vitamers has been scrutinized in both PNPO deficient patients and individuals without PNPO deficiency. However, results have not been consistent for PLP level measurements [114], which can be attributed to the heterogeneous genetic background, different diets, as well as other uncontrolled factors. Recently, it has been shown that PNPO deficient patients have high plasma levels of PM [124], but the sample size is relatively small (n = 6) and majority of them (5 / 6) were on either PN or PN+PLP treatment. More samples are needed to validate this finding. In our fly models with well-controlled genetic background and environmental conditions, it is clear that PLP level is the best predictor of symptoms among B6 vitamers (Figure 2.4). It is worth noting that PMP instead of PM was detected in flies under the current conditions and the PMP level is not changed by PN supplementation and is not altered in $sgll^{95}$ flies compared to w^{1118} controls.

Little is known about the neurobiological mechanisms underlying PNPO-deficiency-induced seizures. In humans, different metabolites have been measured in PNPO deficient patients, including those involved in the synthesis of dopamine, serotonin, GABA, and other amino acids such as threonine and glycine. A potential contributor to the seizure phenotype in PNPO deficiency flies is an altered inhibitory central circuit. Notably, our spike pattern analysis suggests that seizures in PNPO deficient flies (Figure 2.3) share characteristics with spike discharges in wild-type flies administered with picrotoxin, a $GABA_A$ antagonist [53], supporting the notion of a potential role of dysfunctional GABAergic neurons as a consequence of PNPO deficiency. This is not surprising as PLP is required for GABA synthesis and GABA deficiency is known to be involved in the development of epilepsy.

This interpretation is also in agreement with results from animal models of other gene mutations in the VB6 pathway. Other than PNPO, several genes have been identified in VB6-dependent epilepsy patients, including *ALDH7A1* [92], *ALPL* [131], and *PROSC*

[93, 132], which encodes aldehyde dehydrogenase 7A1, tissue non-specific alkaline phosphatase (TNSALP), and proline synthetase co-transcribed homolog, respectively. These gene products affect the availability of PLP through different mechanisms. Knockout animal models have been generated to study the function of *ALPL* [133] and *ALDH7A1* genes [94]. Those studies suggest that reduced intracellular PLP and the consequent GABA deficiency are likely the main contributors to their seizure phenotype.

Seizures have long been studied in *Drosophila* [34]. Fly models based on mutations in a number of genes have been established and well characterized, including genes encoding sodium, potassium, and calcium channels [41, 42, 43], as well as genes encoding proteins that can affect the functions of ion transporters [44, 45, 46]. The characterization of PNPO mutants makes it possible to examine the genetic/functional interactions between PNPO and other proteins. For example, VB6 has been shown to control seizures in human patients who carry mutations in *KCNQ2* [134] or *CACNA1A* [135], both of which encode voltage-gated ion channels. However, the mechanism remains unknown.

Epilepsy affects more than 70 million people in the world with one-third of them not well-controlled by drug treatments [24, 31]. Now with the advent of next-generation sequencing, treatments may be designed based on genetic information. The power of such an approach is especially exciting for defects in genes encoding metabolic enzymes as treatments may include simple dietary changes. Thus, valid animal models are valuable tools for testing treatment options, and help to elucidate the fundamental biology of these causal genes.

2.6 Methods and Materials

2.6.1 Drosophila melanogaster Strains

Electrophysiology experiments were performed on flies bred on Frankel & Brosseau's media [136] at the University of Iowa. For all other experiments, flies were bred on the standard cornmeal-yeast-molasses medium from Fly kitchen at the University of Chicago. The $sgll^{95}$

line and its genetic control wild-type line, w^{1118}, were described before [119]. The UAS- *sgll* RNAi transgenic line (VDRC #105941), as well as its control, was obtained from the Vienna Drosophila Resource Center (VDRC). The Gal4 drivers *actin*-Gal4 (BDSC #4414) and *elav*-Gal4 (#25750) were obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana.

2.6.2 Generation of Transgenic Flies

To generate transgenic flies, we constructed UAS-*sgll* and UAS-*hPNPO* by sub-cloning the cDNAs of *Drosophila* wild-type *sgll* and human wild-type *PNPO*, respectively, into pUAST and injected into w^{1118} commercially (Rainbow Transgenic Flies, Inc). Specifically, fly WT *sgll* cDNA (RA form, see reference [119]) were amplified from w^{1118} cDNAs with primers: 5'- CGA ATT CGC CAC CAT GAA GTT GCT GCA AAC AAT TCG AAG G -3' and 5'- AGA GCT CCT AAG GAG CCA GCC GTT CGT ACA CCC A -3'; and human WT *hPNPO* cDNA was amplified from human brain cDNA library (TaKaRa, Cat #637242) with primers: 5'- CGA ATT CGC CAC CAT GAC GTG CTG GCT GCG GGG CGT CA -3' and 5'- GGA GCT CTT AAG GTG CAA GTC TCT CAT AGA GCC AGT CTT -3'. The sequences of constructs were confirmed by Sanger sequencing at the DNA Sequencing Facility at the University of Chicago.

2.6.3 Survival Study

We followed our previous method [119]. In brief, male flies, 1-3-day-old, were anesthetized briefly and transferred into a culture vial filled with 4% sucrose in 1% agar. Fifteen to twenty flies were grouped into a vial. Survival was checked daily.

2.6.4 Behavioral Recording and Data Analysis

For recordings in $sgll^{95}$ or rescue flies, 1-2-day-old male flies were anesthetized briefly and maintained on 4% sucrose in 1% agar for two or five days as indicated in the text. For recordings in ubiquitous *sgll* KD flies, we used female flies because male flies died sooner and gave a shorter time window to do recordings (Figure 2.2). Therefore, 1-2-day-old female *sgll* KD flies were anesthetized briefly and maintained on 4% sucrose in 1% agar for two days. After treatment, each individual fly was transferred without anesthetization to a 60-mm petri dish (Corning, Cat #430166) for recording. The dish was pre-filled with 1% agar to maintain humidity. Each fly was recorded for 3 min at 20 frames per second using a Flea3 video camera (FLIR Integrated Imaging Solutions, Inc). Videos were saved to the computer with FlyCapture (FLIR Integrated Imaging Solutions, Inc) and tracked with the IowaFLI Tracker [48]. After tracking, the travel traces and scatter plots were plotted in Matlab (R2018b, Mathworks, Natick, MA). For clarity, only the first five hundred frames were plotted in the scatter plots shown in Figures 2.1, Figure 2.5, and Figure 2.2.

2.6.5 Electrophysiological Recording of DLM Flight Muscle Activity

We followed the protocol from our previous publications [121, 51]. In brief, a single fly was shortly anesthetized on ice and glued to a tungsten wire between neck and thorax. After a 30-minute recovery period, two sharpened tungsten electrodes were inserted into the left and right thorax, one on each side, targeting the top-most dorsal longitudinal muscle (DLMa, [137]). A reference electrode was inserted into the abdomen of the fly. Electrical activity in each muscle were picked up with an AC amplifier (AM Systems Model 1800, Carlsbourg, WA) and digitized by data acquisition card (USB 6210 National Instruments, Austin TX) controlled by LabVIEW8.6 (National Instruments). Spike detection was done using a custom-written Matlab script. Following the conventions in our previous publication [53], the instantaneous firing frequency for an individual spike was defined as the reciprocal of the inter-spike interval between the current spike and the succeeding spike (ISI^{-1}). The instantaneous coefficient

of variation, CV_2 (71) for a pair of ISI^{-1} corresponding to adjacent interval i and i+1 was shown as :

$CV_2 = 2|ISI^{-1}{}_i - ISI^{-1}{}_{i+1}|/(ISI^{-1}{}_i + ISI^{-1}{}_{i+1})$

Lower CV_2 values indicate ISI^{-1} values with little variability, and higher CV_2 values correspond to irregular ISI^{-1} values [53].

2.6.6 B6 Vitamer Measurements

For measuring levels of B6 vitamers, a group of 20 male $sgll^{95}$ or w^{1118} flies, 1-to 3-day-old, were picked into vials supplied with 4% sucrose in 1% agar, which was supplemented with or without 500 ng/ml of PN. The concentration of 500 ng/ml was selected based on our previous studies [119] showing that all flies survive under this condition. Forty-eight hours later, whole flies in each vial were homogenized in cold trichloroacetic acid. After centrifugation, the supernatant in each vial was then collected into new Eppendorf tubes, which were frozen at -80 until B6 vitamer measurements. The concentration of each B6 vitamer in fly lysates was quantified by Ultra-Performance-Liquid-Chromatography-Tandem-Mass-Spectrometry (UPLC-MS/MS) [138].

2.6.7 Statistical Analysis

Statistical analysis was performed using R (version 3.3.2). Details on statistical analyses, including sample sizes, tests performed, exact P values, and multiple tests correction if necessary, are provided within the figure legends or in the text describing each figure.

2.7 Acknowledgements

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2.8 Supplementary Information

2.8.1 Supplementary Figures

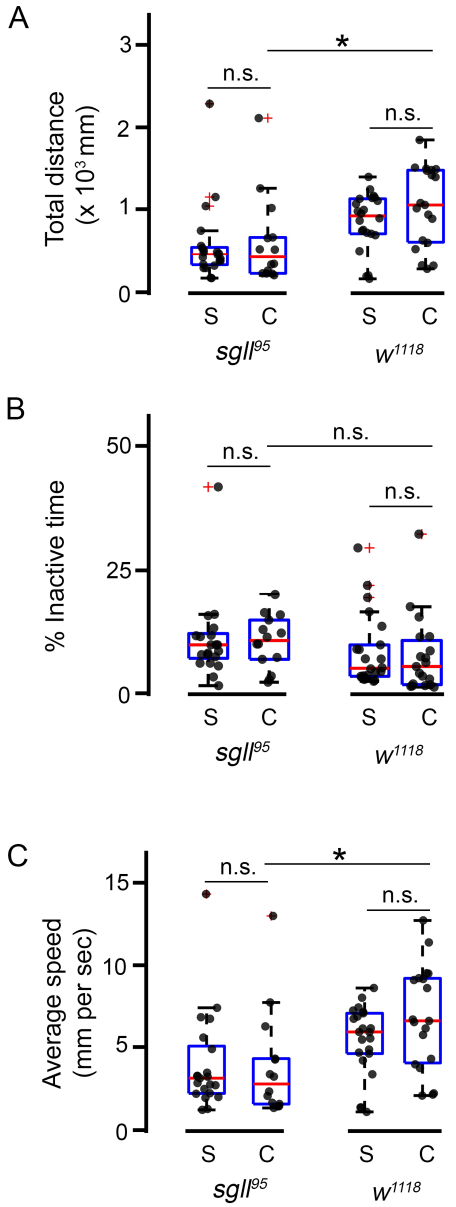

Figure 2.7: **Total distance, percentage of inactive time, and average speed of *sgll*^{95} and *w*^{1118} flies under different conditions.** The *sgll*^{95} or w^{1118} flies were individually recorded for 3 min at 20 frames per second (See Methods for details, also see Figure 2.1 C&D). Total distance traveled (A), percentage of inactive time (B), and average speed were calculated from the first 3300 frames. S: sugar-only diet, C: normal diet, * $P < 0.05$, n.s.: $P > 0.05$; Two-way ANOVA with Tukey's *post hoc*, n = 14-22 per genotype per condition.

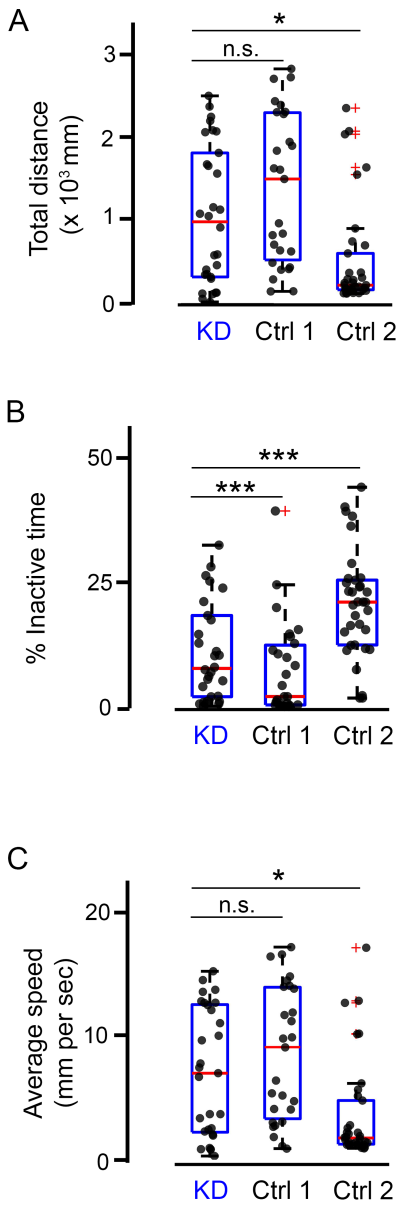

Figure 2.8: **Total distance, percentage of inactive time, and average speed of ubiquitous *sgll* KD flies and control flies on the sugar-only diet** Flies from each genotype were individually recorded for 3 min at 20 frames per second (See Methods for details, also see Figure 2.2 B&C). Total distance traveled (A), percentage of inactive time (B), and average speed were calculated from the first 3300 frames. KD: *actin*-Gal4/UAS-*sgll* RNAi; Ctrl1: *actin*-Gal4/+; Ctrl2: UAS-*sgll* RNAi/+. n.s.: $P > 0.05$; * $P < 0.05$, *** $P < 0.001$, One-way ANOVA with Tukey's *post hoc*, n = 27-34 per genotype per condition.

CHAPTER 3

FUNCTIONAL AND MOLECULAR CHARACTERIZATIONS OF HUMAN SEIZURE-CAUSING MUTATIONS IN PYRIDOX(AM)INE 5'-PHOSPHATE OXIDASE IN *DROSOPHILA* MODELS

3.1 Abstract

Pyridox(am)ine 5'-phosphate oxidase (PNPO), a rate-limiting enzyme in the synthesis of vitamin B6, is encoded by an autosomal recessive gene *PNPO*. Mutations in *PNPO* have been increasingly reported in neonatal epileptic encephalopathy (NEE) patients and more recently also in early-onset epilepsy. Moreover, *PNPO* has also been included as one of the sixteen epilepsy genes involved in the common epilepsies. In contrast to the increasingly recognized significance of PNPO deficiency in epilepsy, our knowledge of the fundamental biology of PNPO deficiency and *PNPO* gene is limited. Previously we have shown that PNPO deficiency causes seizures in flies as it does in humans, and that seizures can be rescued by ubiquitous expression of human *PNPO* (*hPNPO*). Here we report using *Drosophila* as an *in vivo* model system to study hPNPO mutations identified in patients. We generated one wild-type (WT) and three mutant knock-in (KI) lines using CRISPR/Cas9. In each line, the *Drosophila PNPO* gene (*sugarlethal*, *sgll*) was replaced with WT hPNPO cDNA or one of the three mutant cDNAs– R116Q, D33V, and R95H– that represent different levels of PNPO deficiency. We examined these KI flies for different phenotypes. We showed that PNPO deficiency had pleiotropic effects on the phenotypes in a severity-dependent manner: severe PNPO deficiency affected development, intermediate PNPO deficiency caused conditional lethality and seizures, and mild PNPO deficiency shortens lifespan. We also showed that PNPO mutations could affect hPNPO at both protein and mRNA levels. Lastly, from genetic studies, we found that R95H mutant allele had a dominant-negative effect on *hPNPO* but not on *sgll*. Based on our

fly studies and previously published structural studies by other groups, we predicted that a number of seizure-causing hPNPO mutations would have dominant-negative effects. Taken together, this study expands our understanding of *hPNPO* gene, hPNPO mutations, and hPNPO deficiency. It provides a concrete example that gene-by-environment interactions can cause diseases. It also indicates that *Drosophila* can be used as a prenatal diagnostic tool to examine the functional consequences of newly identified hPNPO mutations. Lastly, the established KI lines can be used to study other biological questions related to PNPO deficiency and to explore treatment options for PNPO deficient patients.

3.2 Introduction

Pyridox(am)ine 5'-phosphate oxidase (PNPO, EC 1.4.3.5) catalyzes the oxidation of pyridoxine 5'-phosphate (PNP) and pyridoxamine 5'-phosphate (PMP) to pyridoxal 5'-phosphate (PLP), which is a rate-limiting step in the biosynthesis of vitamin B6 [73]. Mutations in PNPO can cause neonatal epileptic encephalopathy (NEE), a devastating disease that usually leads to death if untreated [91]. Recently, PNPO mutations have also been reported in early-onset epilepsy [98, 99, 100]. It remains to be determined how different PNPO mutations lead to different seizure onsets.

The fundamental biology of PNPO has been studied since the 1970s [139, 140, 141, 142, 143]. PNPO is evolutionarily conserved [144]. Functional PNPO is a homodimer [145] and binds to two molecules of flavin mononucleotide (FMN) [146]. Crystal structures further show that in the dimerized complex, two monomers form a narrow cleft and along the interface there are two symmetrical cavities for two FMNs, one in each cavity, serving as an immediate electron receptor during the catalysis of PNP or PMP to PLP [96, 144, 146]. Both dimerization and binding to FMN are required for the enzyme activity. The formation of a dimer provides a structural basis for a potential dominant-negative effect of a mutant protein on the wild-type(WT) PNPO, which, however, has not been examined.

Since 2005 when the first human PNPO (hPNPO) mutation was identified in NEE patients

[91], more than 30 different mutations have been reported to date [147]. Biochemical studies have been performed for some mutations, showing that hPNPO mutations result in a range of 0 % to 83 % of residual activities [91, 98, 104, 114]. Such variations in the residual activities are likely due to their differential effects on the binding to substrates or to FMN, or on the thermal stability of hPNPO [99, 148]. While these studies nicely show that hPNPO mutations indeed directly affect the function of the enzyme by reducing its activity, it remains unknown whether hPNPO mutations can also indirectly affect the enzyme function through, for example, affecting the protein stability or its localization. Furthermore, the effect of hPNPO mutations on other aspects of fitness have never been systematically studied. Thus, there is a need to develop *in vivo* systems to study hPNPO mutations and to examine the functional consequences of hPNPO deficiency.

Previously, we identified a *Drosophila PNPO* gene (*sugarlethal*, *sgll*) and a hypomorphic *sgll* allele [119]. We showed that PNPO deficiency caused seizures in flies as it does in humans, and that seizures were rescued by ubiquitous expression of hPNPO [149]. In this study, we generated human-seizure-causing-mutation knock-in (KI) fly lines, and characterized these KI lines from different levels. We found that hPNPO deficiency affected development, conditional survival, and lifespan of KI flies in a severity-dependent manner. Moreover, we found that hPNPO mutations could affect transcription, protein stability, and protein localization. Through genetic interaction studies, we further showed that R95H mutation had a dominant-negative effect on WT hPNPO. Based on our fly studies and previously published structural studies by other groups, we predicted that a number of seizure-causing hPNPO mutations that affect FMN binding but not dimerization would have dominant-negative effects. Taken together, this study expands our knowledge on *hPNPO* gene, hPNPO mutations, and hPNPO deficiency. It provides a concrete example that gene-by-environment interactions can cause diseases. Moreover, this study also indicates that *Drosophila* can be used as a prenatal diagnostic tool to examine the functional consequences of newly identified hPNPO mutations. Lastly, the established KI lines are useful for studying other biological questions related to

PNPO deficiency and for exploring treatment options for PNPO deficient patients.

3.3 Results

3.3.1 Generation of Human-Seizure-Causing-PNPO-Mutation Knock-in Fly Strains

Human *PNPO* (*hPNPO*) gene spans a region of $\sim$ 8 Kbp in the genome and contains 7 exons (www.ensembl.org; Supplemental Figure 3.10 A). To date, a total of 35 different *hPNPO* mutations have been identified in NEE patients and patients with early-onset epilepsy. Majority of these mutations are missense mutations (58 %). Some mutations, especially missense mutations, have been studied biochemically in *in vitro* systems for their effects on the enzymatic activity [91, 98, 104, 114] (Data was summarized in Supplemental Figure 3.11). To systematically and functionally study seizure-causing mutations in hPNPO, we generated knock-in (KI) lines using CRISPR/Cas9 (See Methods for details). We chose three hPNPO mutations R116Q, D33V, and R95H to represent different levels of PNPO deficiency; corresponding mutant enzymes reduce the enzyme activity to $\sim$ 80%, 40%, and 20% of WT, respectively [98, 104]. In each KI line, the endogenous *sgll* gene was replaced by one of these three mutant *hPNPO* cDNAs (Figure 3.1 A). A WT hPNPO KI line was also generated as a reference. These four KI alleles were designated as sgll-R116Q, sgll-D33V, sgll-R95H, and sgll-WT, respectively. Mutation in each allele was confirmed by Sanger sequencing of the PCR products amplified using a pair of primers that specifically target *hPNPO* cDNA sequence and using genomic DNA extracted from each line as the substrate (Figure 3.1 B). All KI lines were maintained over a TM6B, Hu, Tb balancer (referred to as TM6B hereafter) to avoid the homozygous lethality.

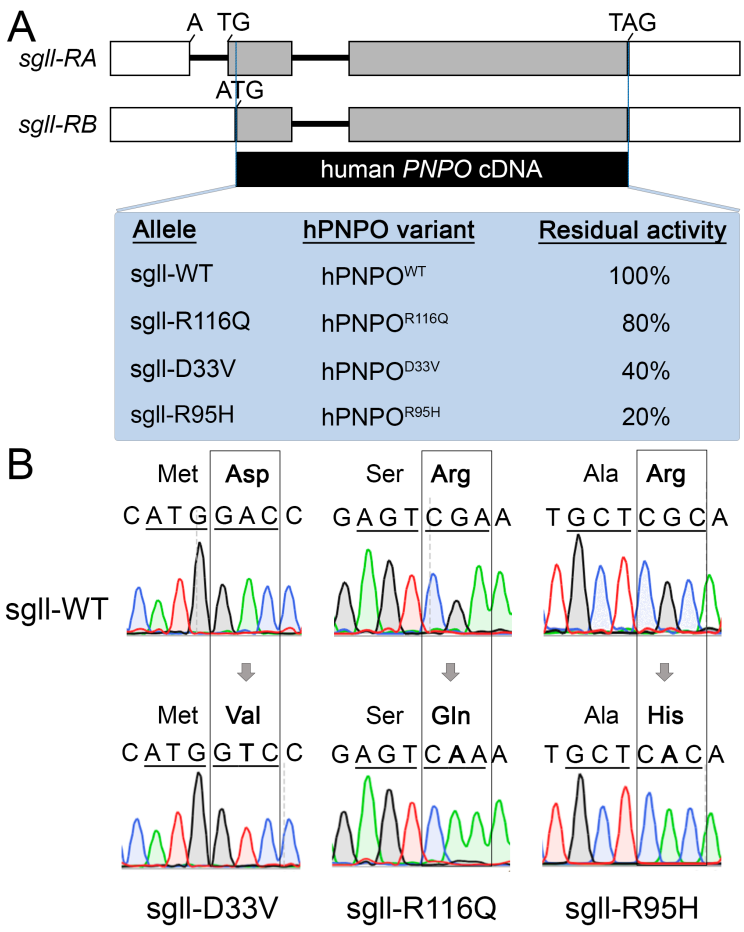

Figure 3.1: **Generation of knock-in(KI) lines that carry human-seizure-causing-mutations in hPNPO.** A) Gene *sugarlethal* (*sgll*) encodes two putative transcripts: RA and RB (www.flybase.org). Both of them can be detected by RNA-seq (www.modencode.org). However, RB is globally expressed and has a relatively high expression level in comparison to RA (Supplemental Figure 3.12). Protein products of these two transcripts share the C-terminus and differ in the N-terminus, with RA containing nine extra amino acids. Since the first exon in RA may play a regulatory role in the expression level and/or the expression pattern of *sgll*, we replaced RB with various *hPNPO* cDNAs in KI lines. Gray boxes indicate exons, white boxes UTRs, and black lines introns. The KI allele that corresponds to WT, R116Q, D33V, and R95H is designated as sgll-WT, sgll-R116Q, sgll-D33V, and sgll-R95H, respectively. The residual activity of each hPNPO variant was from published studies, which was measured biochemically under *in vitro* conditions [98, 104]. B) Sequencing chromatograms confirmed the presence of the targeted mutation in each line.

3.3.2 D33V and R95H Mutations, when Homozygous, Cause Lethality During Development

All balanced KI lines were viable and fertile. However, no homozygous KI pupae or adult flies were observed from sgll-R95H/TM6B breeding bottles, based on the marker Tubby (Tb) or Humeral (Hu), respectively. The absence of homozygous sgll-R95H pupae or adult flies was observed even after the KI line was backcrossed to w^{1118} for five generations, suggesting that this phenotype was most likely due to the R95H mutation. To systematically study the effects of these three hPNPO mutations on development, we self-crossed the heterozygous KI lines and examined the number of KI homozygous adult flies. The KI homozygosity ratio was further calculated for each line. The expected homozygosity ratio is 1/3, as homozygous TM6B results in embryonic lethality due to a lethal, recessive gene associated with it.

Consistent with our initial observations, no homozygous sgll-R95H flies were emerged from breeding vials (Table 3.1). Since we did not observe lethality in pupae, therefore, we conclude that sgll-R95H flies died before the pupal stage. The KI homozygosity ratio from sgll-WT breeding is 0.377, similar to the expected value, suggesting that hPNPO functions as *Drosophila* PNPO, which has already been shown in our previous studies [149]. Compared to sgll-WT, sgll-D33V generated fewer KI homozygous flies, suggesting that D33V also has a deleterious effect on development.

To further confirm the effect of D33V or R95H on the development was due to the mutation, we performed complementation tests (See Methods for details). Each KI line was crossed to Df(3R)ED5223/TM6C,Sb, a Deficiency (Df) line in which the *sgll* gene is included in the deleted region. In the meantime, each KI line was also crossed to Df(3R)BSC221/TM6B,Tb, a control Df line that has a deletion region adjacent to that in the Df line used in the above experimental group. Homozygous breeders were used from sgll-WT, sgll-R116Q, and sgll-D33V lines and heterozygous breeders from the sgll-R95H line due to the homozygous lethality. F1 flies from above breeding were examined for genotypes and the ratio of KI/Df flies was calculated.

Results showed that when each of KI lines bred to the experimental Df line, fewer than expected KI/Df flies and no KI/Df flies were generated from sgll-D33V and sgll-R95H breeding, respectively (Table 3.2). As a comparison, when each of KI lines bred to the control Df line, neither of them affected the KI/Df ratio. Therefore, these results confirmed that the absence or the reduced number of KI homozygotes in sgll-R95H or sgll-D33V breeding, respectively, was due to the mutation, that is, R95H and D33V affect the development of KI flies.

3.3.3 KI Flies Show Conditional Lethality and Seizures

Previously we showed that PNPO deficiency caused lethality and seizures in flies when reared on the sugar-only diet [149]. Since the residual activities of R116Q, D33V, and R95H are known, we reasoned that by examining the lethality of KI flies on the sugar-only diet, we could study the correlation between the residual activity of the mutant protein and the conditional survival of KI flies. We first tested homozygous flies from three KI lines: sgll-WT, sgll-R116Q, and sgll-D33V. The residual activity of corresponding variants is 100 %, 80 %, and 40 %, respectively. Based on the residual activities, we predicted that flies from sgll-WT and sgll-R116Q would survive well and flies from sgll-D33V might show lethality. Indeed, we observed no lethality from sgll-WT and sgll-R116Q flies (n = 73-89 per genotype), at least within 11 days, suggesting that mild PNPO deficiency does not affect the conditional survival of flies in a short-time period. We also observed that sgll-D33V flies survived relatively well, at least within 9 days; a few flies died on day 11 (total n = 85, Figure 3.2 A), suggesting that sgll-D33V flies that survived the developmental stages are more likely to have mild PNPO deficiency.

To expand the spectrum of PNPO deficiency and to study the genetic properties of PNPO mutations, we further generated heterozygous and trans-heterozygous flies. These flies have two different alleles (Figure 3.2 A). We predicted that none of heterozygous flies should show lethality because PNPO mutations had been considered as recessive mutations (www.omim.org), and that among all trans-heterozygous genotypes, the predicted order of

Table 3.1: **Number of adult flies emerged from each heterozygous breeding group**

KI line	$N_{KI/KI}$	$N_{KI/TM6B}$	Homozygosity Ratio	EV	*P*-value*
sgll-WT/TM6B	348	576	0.377	0.333	——
sgll-R116Q/TM6B	609	955	0.389	0.333	0.556
sgll-D33V/TM6B	507	1051	0.325	0.333	0.011
sgll-R95H/TM6B	0	648	0	0.333	< 2.2e-16

EV: Expected value for the ratio of the number of KI/KI adult flies to the total number of adult flies; * Chi-square test of homogeneity, compared to the sgll-WT/TM6B breeding group.

Table 3.2: **Number of adult flies emerged in each breeding group in the complementation tests**

Deficiency line	KI line	$N_{Balancer^-}$	$N_{Balancer^+}$	Ratio	EV	*P*-value*
Df(3R)ED5223 /TM6C,Sb	sgll-WT	125	118	0.514	0.5	0.653
	sgll-R116Q	219	246	0.471	0.5	0.211
	sgll-D33V	606	888	0.406	0.5	2.969e-13
	sgll-R95H/TM6B	0	315	0	0.333	< 2.2e-16
Df(3R)BSC221 /TM6B,Tb	sgll-WT	130	144	0.526	0.5	0.398
	sgll-R116Q	273	269	0.504	0.5	0.864
	sgll-D33V	344	344	0.5	0.5	1.000
	sgll-R95H/TM6B	200	359	0.358	0.333	0.220

Note: The deficiency line Df(3R)ED5223/TM6C,Sb has the *sgll* gene deleted while the deficiency line Df(3R)BSC221/TM6B,Tb contains the *sgll* gene. EV: Expected value for the ratio of the number of Balancer$^-$ flies to the total number of adult flies; Balancer$^-$: flies have no Balancers and Balancer$^+$: flies have Balancers; *Chi-square goodness-of-fit test, compared to EV in each group.

the lethality would be: sgll-D33V/sgll-R95H > R116Q/sgll-R95H > sgll-R116Q/sgll-D33V. Indeed, as shown in Figure 3.2, sgll-D33V/sgll-R95H trans-heterozygotes showed the most dramatic lethal phenotype: they started dying on day 4 and $\sim$ 80 % of them had died by day 11 (total n = 139). Compared to sgll-D33V/sgll-R95H, sgll-R116Q/sgll-R95H survived better and sgll-R116Q/sgll-D33V flies survived much better. The death rate of sgll-R116Q/sgll-R95H and sgll-R116Q/sgll-D33V is $\sim$ 20 % (total n = 218) and $\sim$ 1 % (total n = 147) by day 11, respectively. Therefore, the conditional survival rates of these flies correlate well with the residual activity measured in *in vitro* studies. Surprisingly, however, flies from one heterozygous group sgll-WT/sgll-R95H showed lethality (n = 128). The mechanisms underlying the lethality of sgll-WT/sgll-R95H flies were further studied (see Section 3.3.5).

Previously we showed that $sgll^{95}$ and ubiquitous *sgll* KD flies exhibited seizures before their death [149]. Not surprisingly, we also observed seizure-like behaviors in KI flies before they died. These seizure-like behaviors include wing-buzzing, body-rolling, and leg-shaking, similar to that observed in $sgll^{95}$ and ubiquitous *sgll* KD flies [149], indicating that hPNPO mutations lead to PNPO deficiency in KI flies.

3.3.4 R116Q Homozygous KI Flies Have a Shortened Lifespan on the Normal Diet

R116Q mutation has been identified in epilepsy patients at various ages [98, 99], indicating that its ultimate effect may be influenced by other factors. In KI flies, we did not observe any deleterious short-term effects of R116Q (i.e., R116Q affected neither development nor conditional lethality) and we asked whether R116Q could have a long-term effect. To answer this question, we examined the lifespan of sgll-R116Q homozgyotes on the normal diet. Homozygous flies from sgll-D33V or sgll-WT in the same condition were used as comparisons. As shown in Figure 3.3, compared to sgll-WT flies, sgll-R116Q flies have a slightly shortened lifespan but the change is statistically significant (median: 71 vs. 74 days for sgll-R116Q and sgll-WT, respectively. n = 63-79 per genotype). By comparison, sgll-D33V flies have a

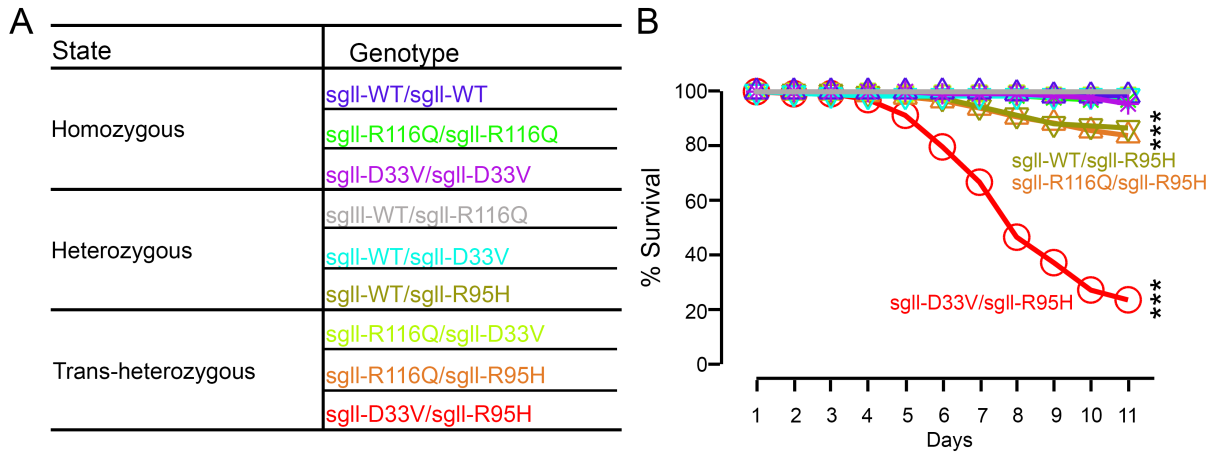

Figure 3.2: **Survival of KI flies on the sugar-only diet.** A) Genotypes of flies were tested. B) Survival of each genotype. *** $P < 0.001$, compared to sgll-WT/sgll-WT, Log-rank test.

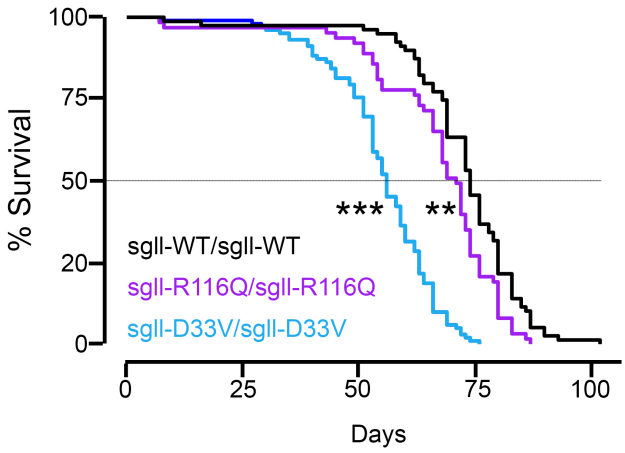

Figure 3.3: **Mild hPNPO deficiency reduces lifespan.** Homozygous flies from each genotype were tested on the normal diet. Flies were generated from breeding of homozygotes. ** $P < 0.01$, *** $P < 0.001$, Log-rank test, compared to sgll-WT/sgll-WT.

dramatically reduced lifespan (median: 56 vs. 74 days for sgll-D33V and sgll-WT, respectively. n = 79-102 per genotype). Taken together, these results demonstrate that PNPO activity is essential for a normal lifespan, and that mild PNPO deficiency may not have a short-term effect but will have a long-term effect.

3.3.5 hPNPO^{R95H} Has a Dominant-Negative Effect on hPNPO^{WT} but Not on SGLL

We observed lethality from sgll-WT/sgll-R95H flies when reared on the sugar-only diet (Figure 3.2 B). The lethality could be due to: 1) haploinsufficiency, or 2) a dominant-negative effect of the R95H mutant protein (hPNPO^{R95H}) on the WT hPNPO protein (hPNPO^{WT}). To test these two alternative possibilities, we compared the conditional survival rate of sgll-WT/sgll-R95H flies to that of sgll-WT/Df flies in which the *sgll* gene is deleted in the Df line. Since there is only one *hPNPO* allele in sgll-WT/Df flies, we reasoned that if the lethality was due to haploinsufficiency, then sgll-WT/sgll-R95H flies would survive similar to, if not better than, sgll-WT/Df flies, given that the residual activity of hPNPO^{R95H} is $\sim$ 18% of hPNPO^{WT} [104]. If the lethality was due to a dominant-negative effect of hPNPO^{R95H} on hPNPO^{WT}, however, then sgll-WT/sgll-R95H flies would survive worse than sgll-WT/Df flies. As shown in Figure 3.4 A, sgll-WT/sgll-R95H flies survived much worse than sgll-WT/Df flies (n = 133-136 per genotype). The median survival rate on the sugar-only diet is 19 and 27 days for sgll-WT/sgll-R95H and sgll-WT/Df, respectively, demonstrating that the lethality of sgll-WT/sgll-R95H flies was due to the dominant-negative effect.

Previous studies show that functional hPNPO is a dimer [96, 145]. Thus, the dominant-negative effect of hPNPO^{R95H} on hPNPO^{WT} is most likely mediated through the formation of a heterodimer between hPNPO^{R95H} and hPNPO^{WT}. Results from western blots show that the hPNPO protein level in sgll-WT/sgll-R95H flies is about twice as much as that in sgll-WT/Df flies (Figure 3.4 B & C), confirming that hPNPO^{R95H} is expressed as expected.

Human and *Drosophila* PNPOs share $\sim$ 40 % of identity and they have different lengths

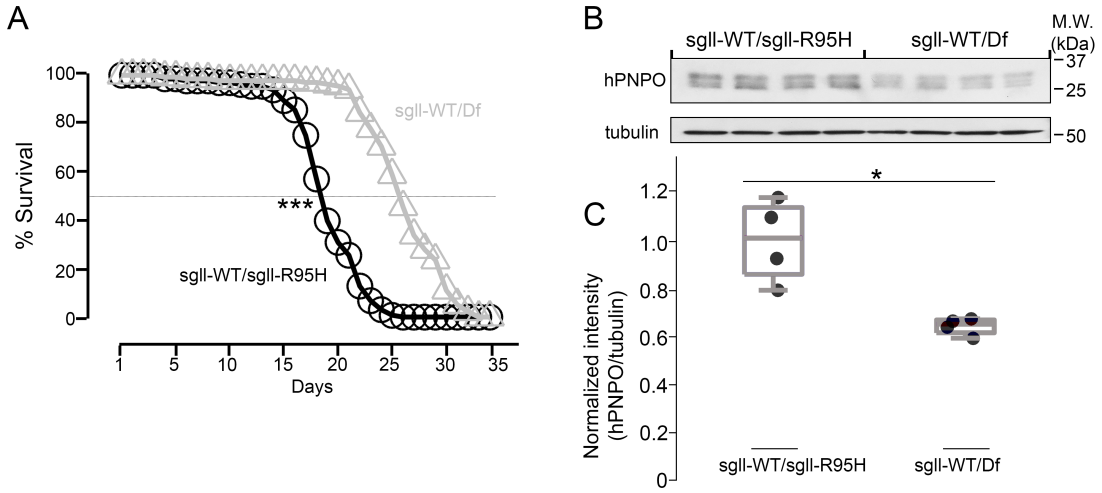

Figure 3.4: **sgll-R95H has a dominant-negative effect on sgll-WT.** A) Survival of sgll-WT/sgll-R95H and sgll-WT/Df flies on the sugar-only diet. *** $P < 0.001$, Log-rank test. B) Western blot of adult fly head homogenate from sgll-WT/sgll-R95H and sgll-WT/Df flies. Four biological replicates were used for each genotype. Tubulin is the loading control. The predicted molecular weight for hPNPO is 30 KDa. However, we observed three bands, between molecular weight (M.W.) 25 and 37 KDa, on the blot. The molecular nature of these bands are unclear. One possibility is that the production of multiple bands was due to proteolysis of the full length of hPNPO. Previous studies have also reported multiple bands during the process of hPNPO purification [96]. This "proteolysis" is resistant to protease inhibitors and not affected by temperature changes [96]. C) Quantifications of the intensity of hPNPO bands from four biological replicates in each genotype. All three bands were included in the quantification. *** $P < 0.05$, unpaired student t-test.

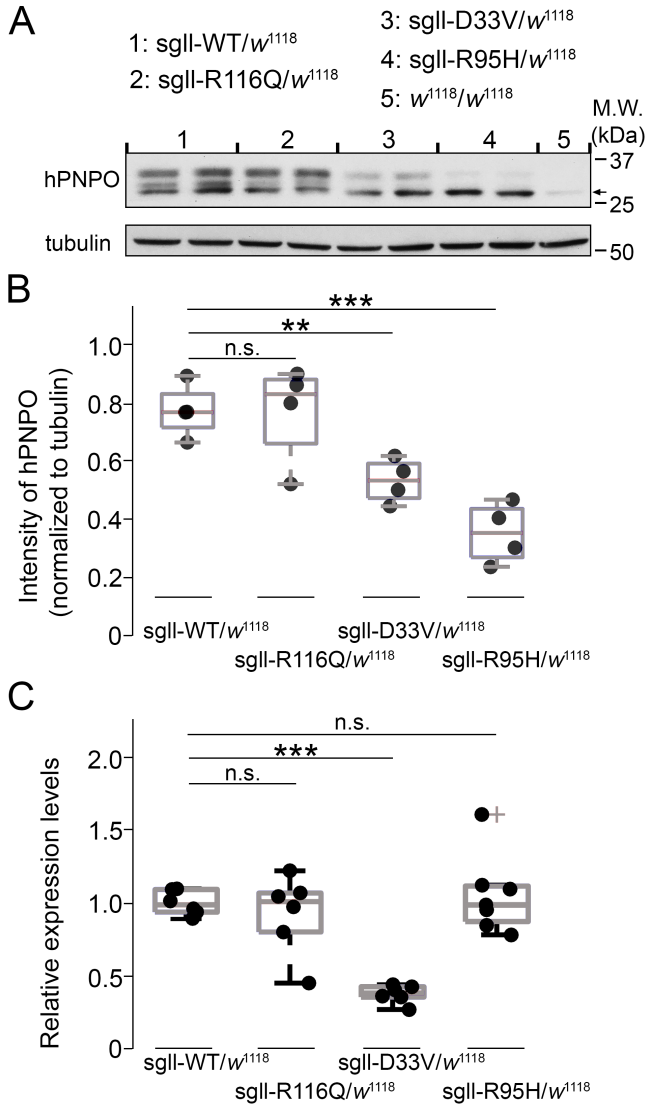

Figure 3.5: **R95H mutation affects the protein stability of hPNPO and D33V affects the transcription of hPNPO.** A) Western blot of adult fly head homogenate from each genotype. Two biological replicates were used for each KI/w^{1118} flies, where the KI allele was sgll-WT, sgll-R116Q, sgll-D33V, or sgll-R95H. One sample from w^{1118} flies was loaded as the antibody control. Tubulin is the loading control. The arrow indicates a band also seen in w^{1118} flies, which is most likely a non-specific band (see Supplemental Figure 3.13). The bottom band from KI/w^{1118} flies are a mixture of this non-specific band and a specific band from hPNPO (see Figure 3.4 B). B) Quantifications of hPNPO (the top band) from four biological replicates (see Supplemental Figure 3.14 for the other two replicates). C) Quantification of the mRNA level of hPNPO in adult fly heads by qRT-PCR using a pair of PCR primers that target the C-terminus of *hPNPO* cDNAs (see Supplemental Figure 3.15 A for the relative position of the PCR primers and Supplemental Figure 3.15 B for qRT-PCR results from a pair of primers that target the N-terminus of *hPNPO* cDNAs). n = 6-7 biological replicates per genotype.

[119]. Thus, it is less likely for them to form a dimer, i.e., it is less likely for hPNPO^{R95H} to have a dominant-negative effect on the *Drosophila* WT PNPO (SGLL). To test that, we generated sgll-R95H/w^{1118} flies and examined their survival on the sugar-only diet. We predicted that these flies would survive well. As a comparison, we also generated and tested sgll-R116Q/w^{1118}, sgll-D33V/w^{1118}, and sgll-WT/w^{1118} flies. Indeed, similar to the other three genotypes (n = 70-144 per genotype), sgll-R95H/w^{1118} flies survived well within 11 days (n = 80). Since the residual activity of hPNPO^{R95H} is very low, the survival of these flies is conceivably due to the functional homodimer formed by SGLL.

3.3.6 R95H Mutation Affects the Protein Stability of hPNPO

Western blot results show that the hPNPO^{R95H} protein is expressed and stabilized in sgll-WT/sgll-R95H flies (Figure 3.4 B & C). It is unclear, however, whether the stabilization of hPNPO^{R95H} was due to the formation of the heterodimer with hPNPO^{WT} or due to the stability of hPNPO^{R95H} itself. Since homozygous sgll-R95H flies are lethal, it is impossible to study that in sgll-R95H homozygotes. The fact that hPNPO^{R95H} and SGLL do not form a heterodimer, indirectly confirmed by the survival studies, suggests that we could study the stability of hPNPO^{R95H} in these flies by examining the hPNPO level using a hPNPO-specific antibody.

To do that, we first validated the antibody such that it cannot recognize SGLL. We tested the antibody in ubiquitous *sgll* KD and control flies because we know that the KD efficiency in ubiquitous *sgll* KD flies is more than 90 % on the mRNA level (Supplemental Figure 3.13 A), and that ubiquitous *sgll* KD flies must have a dramatic reduced SGLL level based on the seizure and survival studies [149]. We observed a band between molecular weight 25 and 37 KDa, which is similar to the predicted size. But the intensity of this band did not change in ubiquitous *sgll* KD flies, compared to two controls (Supplemental Figure 3.13 B). Therefore, we conclude that this antibody does not recognize SGLL.

Then we used this antibody to detect hPNPO in sgll-R95H/w^{1118} flies. The hPNPO

in sgll-WT/w^{1118}, sgll-R116Q/w^{1118}, and sgll-D33V/w^{1118} flies were detected as well. We observed a reduced hPNPO level in sgll-R95H/w^{1118} flies compared to that in sgll-WT/w^{1118} flies (Figure 3.5 A & Supplemental Figure 3.14). Quantification results show that the reduction is statistically significant (Figure 3.5 B). Given that hPNPO^{R95H} is expressed and stabilized in sgll-R95H/sgll-WT flies, we reasoned that the decreased hPNPO^{R95H} level in sgll-R95H/w^{1118} flies was more likely due to the instability of hPNPO^{R95H} than due to the reduced translation of it. To confirm that, we performed qRT-PCR to measure the mRNA level of hPNPO in sgll-R95H/w^{1118} flies, which was then compared to that in sgll-WT/w^{1118} flies. As shown in Figure 3.5 C, the mRNA level of *hPNPO* in sgll-R95H/w^{1118} flies is comparable to that in sgll-WT/w^{1118} flies. Taken together, we conclude that hPNPO^{R95H} itself is not stable but its dimerization with hPNPO^{WT} can stabilize it.

3.3.7 D33V Mutation Decreases the mRNA Levels of hPNPO

We observed $\sim$ 50 % reduction of hPNPO levels in sgll-D33V/w^{1118} flies compared to sgll-WT/w^{1118} flies (Figure 3.5 A & B), which can be attributed to decreased transcription or decreased mRNA stability (Figure 3.5 C). The reduced mRNA and protein levels were also observed in homozygous sgll-D33V flies (Supplemental Figure 3.16), which confirmed the effect of D33V on the transcription of *hPNPO* gene or on the stability of its mRNA. It is worth noting that since we knocked-in *hPNPO* cDNA instead of genomic DNA, there is a possibility that this effect is specific to *hPNPO* cDNA.

3.3.8 R116Q Mutation Increases the Terminal Localization of hPNPO

Results from western blot and qRT-PCR show that R116Q mutation does not affect hPNPO either on the protein level or on the mRNA level (Figure 3.5 A–C & Supplemental Figure 3.16). Next, we asked whether R116Q mutation could affect the protein localization of hPNPO in the brain. To answer this question, we performed immunohistochemistry staining in adult fly brains and detected hPNPO with the anti-hPNPO antibody. We used an antibody

(nc-82) that recognizes Brunchpilot (Brp), a marker of presynaptic active zones [150], as a counterstain.

Staining results from sgll-WT homozygotes show that hPNPO^{WT} is ubiquitously expressed in the brain and it mainly stays in the soma, as indicated by little overlap between hPNPO staining and Brp staining (Figure 3.6). Yet, there are some overlaps between hPNPO and Brp stainings, suggesting that a fraction of hPNPO^{WT} protein is either transported from soma to or translated locally in the terminal structures (Figure 3.7). In the soma, hPNPO staining does not overlap with DAPI, a nuclear marker, suggesting that hPNPO is a cytosolic protein (Figure 3.7).

In contrast to the faint hPNPO staining in the terminal structures in sgll-WT flies, we observed strong hPNPO staining in the same structure in sgll-R116Q brains (n = 7, see Figure 3.8 & Supplemental Figure 3.17 for representative images). The staining pattern of hPNPO in sgll-D33V (n = 5) or sgll-R95H/TM6B flies (n = 3) is similar to that in sgll-WT flies (images not shown), suggesting that these two mutations have no effects on hPNPO protein localization.

3.4 Discussion

Previously we identified a *Drosophila PNPO* gene, *sugarlethal* (*sgll*) and a hypomorphic *PNPO* allele (*sgll*^{95}) [119]. We reported seizures in PNPO deficient flies and showed that seizures were rescued by ubiquitous expression of *hPNPO* [149]. Here we report the functional and molecular characterization of human seizure-causing mutations in *hPNPO* in *Drosophila*. We generated three KI *Drosophila* lines that carry human seizure-causing hPNPO mutations: R116Q, D33V, and R95H, and we characterized these three KI lines from different levels. We showed that PNPO deficiency had pleiotropic effects on the phenotypes in a severity-dependent manner: severe PNPO deficiency affected development, intermediate PNPO deficiency caused conditional lethality and seizures, and mild PNPO deficiency shortened lifespan. We also showed that PNPO mutations could affect hPNPO at both protein and mRNA levels. Lastly,

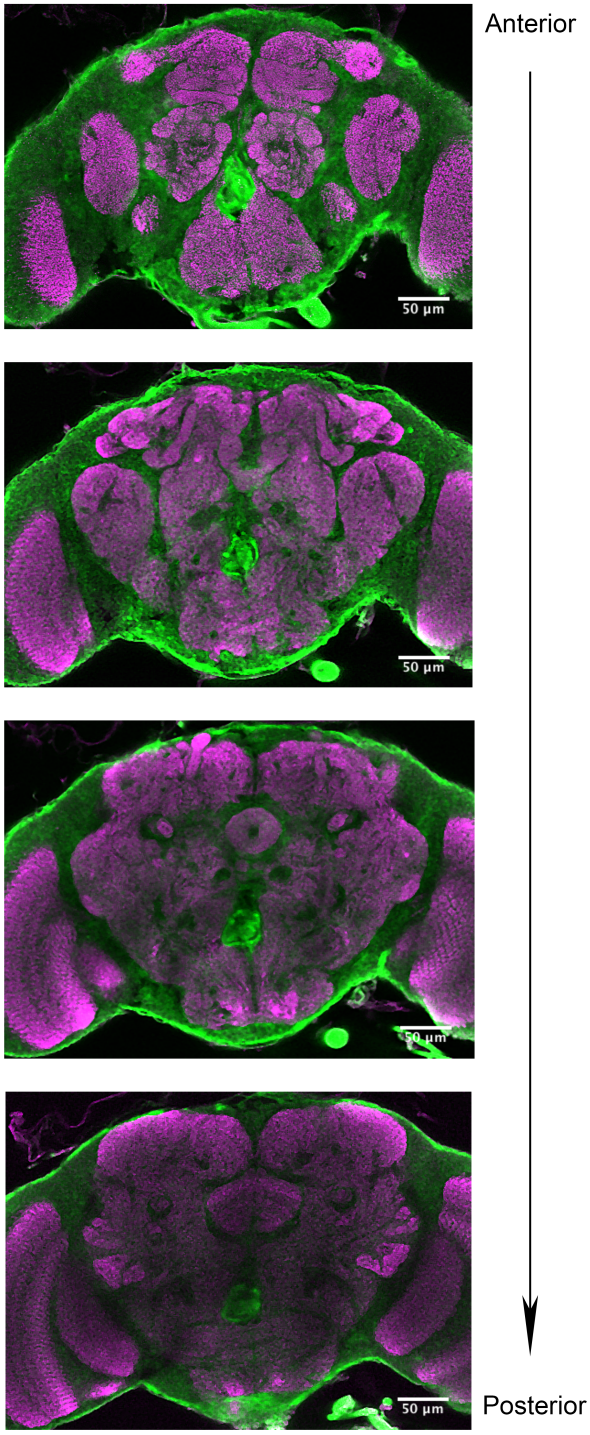

Figure 3.6: **hPNPO^{WT} is ubiquitously expressed in the adult brain.** Color representation: magenta indicates Brp staining and green hPNPO staining. Brp staining was used as a counterstain. Four images are shown, to represent the whole adult brain. Images are arranged from anterior to posterior.

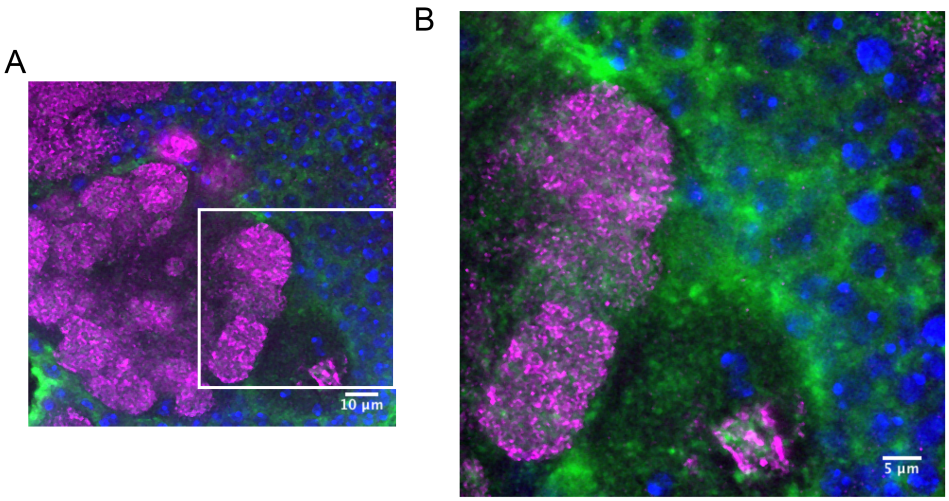

Figure 3.7: **hPNPO^{WT} is a cytosolic protein.** A) Color representation: magenta indicates Brp staining; green indicates hPNPO staining; blue indicates DAPI staining. B) Higher magnification of the selected region in panel A.

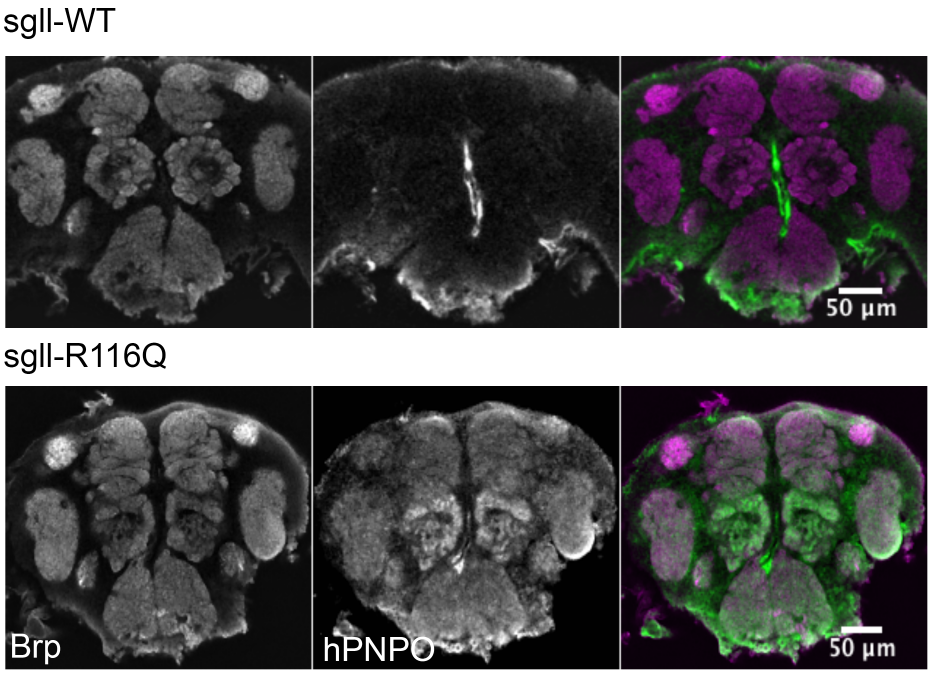

Figure 3.8: **R116Q increases the terminal localization of hPNPO.** Immunohistochemistry staining of hPNPO in the adult fly brain. The Brp staining was used as a counterstain, which stains terminal structures. Images from the anterior part are shown. Images for other parts of the brain are shown in Supplemental Figure 3.17.

from genetic studies, we found that the R95H mutant allele had a dominant-negative effect on *hPNPO* but not on *sgll*. Based on our fly studies and previous published structural studies [96], we predicted that a number of hPNPO mutations that affect FMN binding but not dimerization would have dominant-negative effects.

3.4.1 PNPO Activity is Required for Development

Expression of *sgll* can be detected throughout all developmental stages from embryos to pupae and it maintains at a high level in adult flies (www.modencode.org, see Supplemental Figure 3.12), suggesting that PNPO activity is required for development. Developmental data from R95H and D33V KI flies confirms it and further shows that the lack of or insufficient amount of PNPO activity before the pupae stage leads to lethality (Table 3.1). Yet, it remains to be determined the exact larval stage in which PNPO plays a significant role in the development. Given that PLP, the product of PNPO, is involved in more than 140 biochemical reactions [82], it also remains to be determined whether the lethality during development was solely due to PNPO deficiency in the brain or due to that in peripheral tissues. Further dissection of the developmental role(s) of PNPO can also bring insights to our understanding of the reason that NEE patients usually have developmental delay or intellectual disability even after seizures are controlled [98, 112].

3.4.2 Mild PNPO Deficiency has a Long-Term Effect

We have previously shown that PNPO deficiency causes conditional lethality and seizures [119, 149], which were also observed in KI flies (Figure 3.2). While these studies focused on the short-term role of PNPO, the long-term role of PNPO on fitness has never been studied. Using KI flies, we show that R116Q reduces the lifespan of corresponding KI homozygotes (Figure 3.3), suggesting that the effect of mild PNPO deficiency can accumulate. This cumulative long-term effect may interact with other genetic and/or environmental factors to cause seizures (and maybe other diseases) and such interactions may explain the wide range

(from 3 hours to 38 months) of age of seizure onset reported in patients who are homozygous for R116Q [98, 99].

3.4.3 hPNPO Mutations Can Potentially Regulate the Function of hPNPO at Multiple Levels

Previous *in vitro* studies show that hPNPO mutations affect the function of hPNPO by directly reducing the enzyme activity [91, 104, 98, 114]. Our *in vivo* studies show that hPNPO mutations may also affect the function of hPNPO through altering protein localization (Figure 3.8 & Supplemental Figure 3.17), decreasing protein stability (Figure 3.5 & Supplemental Figure 3.14), and affecting transcription (Figure 3.5 C & Supplemental Figure 3.15 B & Supplemental Figure 3.16 C). These extra layers of regulation suggest that it is necessary to study hPNPO mutations using *in vivo* models.

3.4.4 hPNPO Mutations that Affect FMN Binding but Not Dimerization May Have Dominant-Negative Effects

NEE caused by PNPO deficiency is an autosomal recessive disease (# 610090, www.omim.org), indicating that PNPO mutant carriers will not show any phenotype. Indeed, in all reported PNPO deficiency cases, 78 % of them are homozygous for a certain mutation and 21 % are compound heterozygous. One patient is a heterozygote [98]. However, in our KI flies, we found that hPNPO^{R95H} had a dominant-negative effect on hPNPO^{WT}, which rendered sgll-R95H/sgll-WT flies susceptible to VB6 deficiency (Figure 3.4). This finding implies that R95H carriers in humans may also be susceptible to VB6 deficiency.

The dominant-negative effect of hPNPO^{R95H} on hPNPO^{WT} is most likely mediated through the formation of a heterodimer between them, although the direct evidence of such an interaction is lacking for now. In the heterodimer of hPNPO^{R95H} and hPNPO^{WT}, one out of two FMN binding sites will lose its binding to FMN as amino acid R95 (arginine 95)

is required for FMN binding [96]. Since FMN binding is required for the enzyme activity [96, 145], loss of FMN binding will lead to a partially or fully inactivated enzyme. Whether the hPNPO^{R95H}/hPNPO^{WT} heterodimer is partially or fully inactive is unknown.

Based on these studies, we predict that mutations that affect FMN binding but not dimerization are likely to have dominant-negative effects. These mutations include R95C, R116Q, R138H, and R141C that have already been identified in patients (Supplemental Figure 3.10 B), and may also include others that are not reported in patients. These mutations deserve further investigation, especially R116Q as it is common in the general population [151, 152].

3.4.5 Summary

Taken together, data from KI flies demonstrate pleiotropic effects of PNPO efficiency on the phenotypes: severe PNPO deficiency affects development, intermediate PNPO deficiency causes conditional seizures and lethality, and mild deficiency shortens lifespan (Summarized in Figure 3.9). While the phenotypes in flies with severe PNPO deficiency is solely caused by PNPO mutations, the phenotypes in flies with intermediate and mild PNPO deficiency is affected by both PNPO mutations and environmental factors. In addition, data from KI flies also demonstrate that hPNPO mutations can affect the function of hPNPO at multiple levels: not only can hPNPO mutations affect the PNPO function directly through decreasing the enzyme activity but also indirectly through reducing the protein stability, altering the protein localization, and decreasing the mRNA levels. Lastly, data from KI flies demonstrate that R95H mutation has a dominant-negative effect. We predict that some other mutations may also have dominant-negative effects.

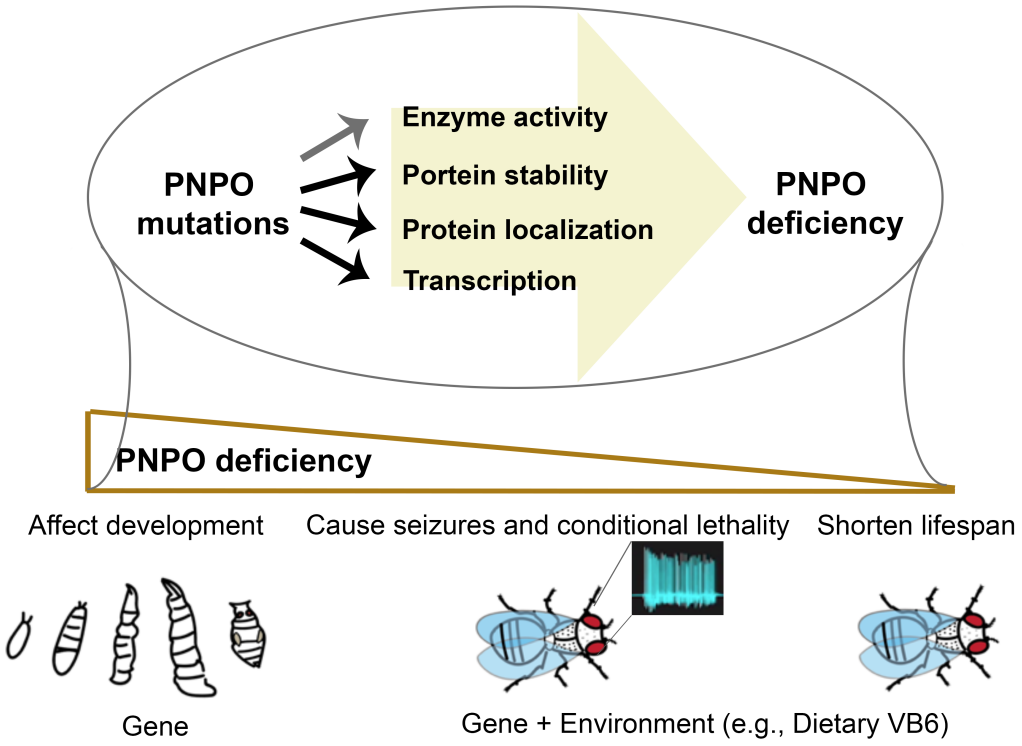

Figure 3.9: **Pleiotropic effects of PNPO mutation and PNPO deficiency.** Other than directly reducing the enzyme activity as shown in previous studies [91, 98, 104, 114], PNPO mutations can also affect protein stability, localization, and transcription. These factors will work together to determine the final outcome of PNPO deficiency. PNPO deficiency leads to different phenotypes depending on the severity of it: severe PNPO deficiency leads to lethality during development, intermediate PNPO deficiency causes conditional lethality and seizures, and mild PNPO deficiency shortens lifespan.

3.5 Methods and Materials

3.5.1 Drosophila melanogaster Strains

The w^{1118} strain was maintained in the laboratory [119]. Two deficiency lines were from the Bloomington Drosophila Stock Center (Df(3R)ED5223/TM6C,Sb # 9076 and Df(3R)BSC221/TM6B,Tb #9698). Flies were bred on the standard cornmeal-yeast-molasses medium from Fly Kitchen at the University of Chicago.

3.5.2 Generation of Knock-in Strains

Four different knock-in (KI) lines were generated using CRISPR/Cas9 [16]. The fly *sgll* gene was replaced by either WT *hPNPO* cDNA or one of the three mutant *hPNPO* cDNAs. The WT *hPNPO* cDNA was amplified from human brain cDNA library (TaKaRa, Cat #637242) [149]. The c.98A > T mutation (corresponding to p.D33V), c.284G > A mutation (corresponding to p.R95H), and c.347G > A mutation (corresponding to p.R116Q) was introduced separately by mutagenesis. The sequence of each cDNA was confirmed by Sanger sequencing at the DNA Sequencing Facility at the University of Chicago. KI lines were generated using CRISPR/Cas9 [153, 154]. sgRNA were designed with CRISPR Optimal Target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) [154] and *in vitro* transcribed by following the published protocol [153]. Cas9 mRNAs were *in vitro* transcribed from plasmid MLM3613 (Addgene plasmid #42251). Donors were various hPNPO cDNAs assembled in the pBluescirpt SK(-) vector. The sgRNAs, cas9 mRNAs and donor constructs were injected into embryos from flies with a genotype: w^{1118}/*FM7a* (BL785); *Bc/CyO*; *TM3/ TM6B, Hu, Tb* (http://www.fgbiotech.com).

The mutant alleles were initially maintained in heterozygotes with the *TM6B, Hu, Tb* balancer, but heterozygotes were gradually taken over by KI homozygotes in WT and R116Q breeding bottles. The introduction of the mutation was confirmed by Sanger sequencing of PCR products amplified with a pair of PCR primers that specifically target hPNPO cDNA [149].

3.5.3 Developmental Assay and Complementation Test

For the heterozygous breeding experiment, male and female heterozygous flies from each line were bred. For the complementation experiment, female flies, either homozygous (sgll-WT, sgll-R116Q, sgll-D33V) or heterozygous (sgll-R95H), and male flies from one of the Deficiency line were picked for breeding. A cohort of 2-3 such flies per sex were set up for each combination. F1 flies within 6 days from each cross were examined for the Balancer

marker. The ratio shown in the tables was calculated from results of 6 -20 vials.

3.5.4 Survival Study

Survival analysis was described before [119, 149]. In brief, fifteen to twenty male flies, 1-2-day old, were maintained in vials filled with 4% sucrose in 1% agar. Daily survival was recorded.

3.5.5 Western Blotting

Male flies, 1-2-day old, were used. Total protein from heads was extracted and quantified. A total of 50 μg protein from each sample was loaded for SDS-PAGE. Separated proteins were electrophorectically transferred to PVDF membrane. After blocking, the membrane was incubated with primary antibodies and then the secondary antibodies (Supplemental Table 3.3). Signals were detected with chemiluminescence (ThermoScientific).

3.5.6 qRT-PCR

Total RNA was extracted from male heads using RNA extraction kit (Zymo Research, Cat #R2030). After the removal of genomic DNA (DNA-free kit, AMBION, Cat #AM1906), total RNA was then used for cDNA synthesis using SMARTScribe Reverse Transcription Kit (TaKaRa, Cat #639537). Two pair of primers for the *hPNPO* KI alleles were used: one pair (KI_N) targets the N-terminus of and the other pair (KI_C) targets the C-terminus of *hPNPO* cDNA. Specific primers were used to amplify the house-keeping gene *ribosomal protein 49* (*rp49*). Primers sequences are shown in the Supplemental Table 3.4.

3.5.7 Immunohistochemistry Staining and Confocal Imaging

Immunohistochemistry staining was performed to examine the protein expression pattern. The protocol was adapted from Flylight protocol of Janelia Farm. In brief, brains of 1-2-day old flies were dissected in cold S2 media and fixed with 2% PFA for 55 min. After brief rinse,

brains were blocked with 5% normal donkey serum and incubated with primary antibodies (Supplemental Table 3.3). Signals for hPNPO were then amplified with Tyramide SuperBoost kit (Invitrogen, Cat # B40926) by following the manufacturer's protocol. After incubation with the secondary antibodies and washes, brains were mounted on a double-frosted glass and covered with cover glasses. DAPI was added into the wash buffer to stain nucleus when needed. Images were taken using Leica SP5-II-STED-CW confocal microscope in the Integrated Light Microscopy Core Facility at the University of Chicago, and processed in Fiji (http://fiji.sc).

3.6 Supplementary Information

3.6.1 Supplementary Figures and Tables

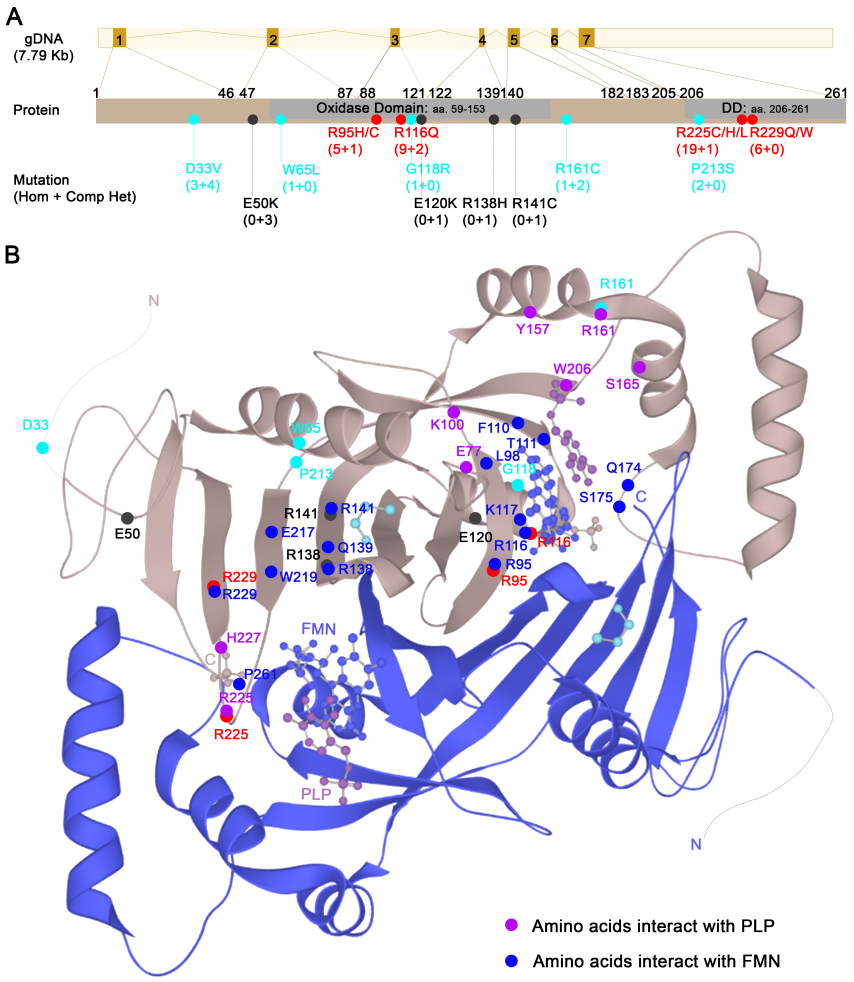

Figure 3.10: **Gene and protein structure of *hPNPO* and missense mutations identified in hPNPO.** A) Gene *hPNPO* spans a region of $\sim$ 8 Kbp in the genome and contains 7 exons, which encodes a 261-aa protein. The hPNPO protein comprises two domains: one oxidase domain in the middle and one dimerization domain at the C-terminus. Until now, a total of 35 mutations have been identified in patients with neonatal epileptic encephalopathy (NEE) or early-onset epilepsy. These mutations are from a variety of types including missense mutations, indels, splicing errors, frameshifts, stop codon gain/loss, and start codon loss. Among different types of mutations, majority of them are missense mutations, which are labelled on the protein sequence. We further grouped missense mutations into three categories: red dots are common mutations, cyan are less common, and black are rare and were only identified in compound heterozyotes. The first number in the parenthesis is the number of reported homozygous cases and the second number compound-heterozygous cases. B) A 3-D structure of dimerized hPNPO. Dimerization is required for PNPO activity. Each monomer binds to one molecule of flavin mononeucleotide (FMN) (blue stick-ball structure embedded in each PNPO monomer), a co-factor required for PNPO activity, and one molecular of PLP (purple stick-ball structure embedded in each PNPO monomer). Two FMN binding sites are localized in the dimerization interface. Amino acids that interact with FMN or PLP are labeled on the structure with blue dots or purple dots, respectively. The 3-D structure was adapted from protein data bank (https://www.ebi.ac.uk/pdbe/entry/pdb/1NRG).

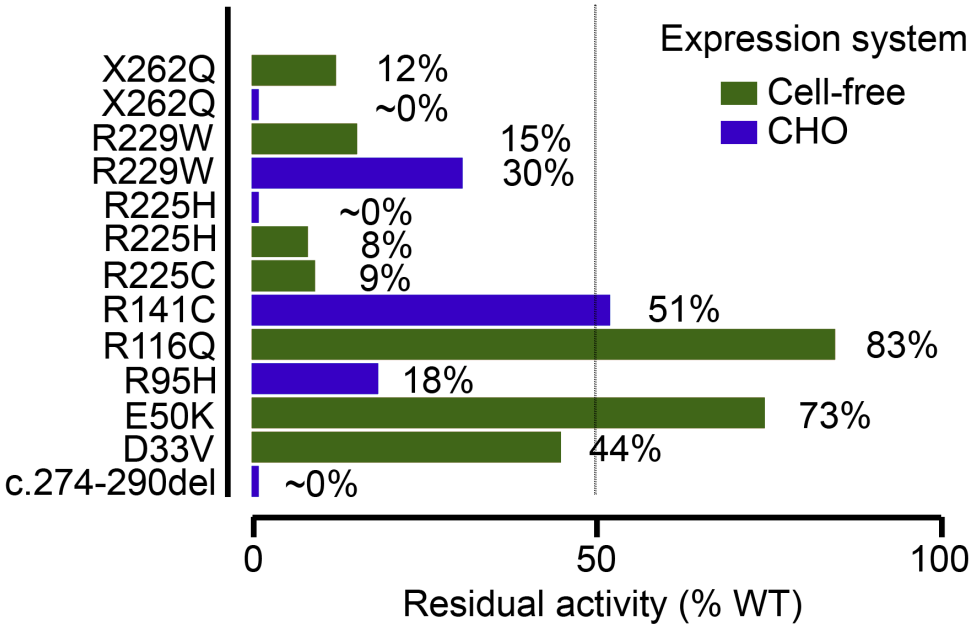

Figure 3.11: **Summary plot of the residual activity of various hPNPO mutants from published studies.** Different hPNPO mutants were expressed in either Chinese Hamster Ovary (CHO) cells (blue bar) or cell-free system (green bar). The residual activity of each mutant was measured *in vitro* with PMP as the substrate [98, 104, 114].

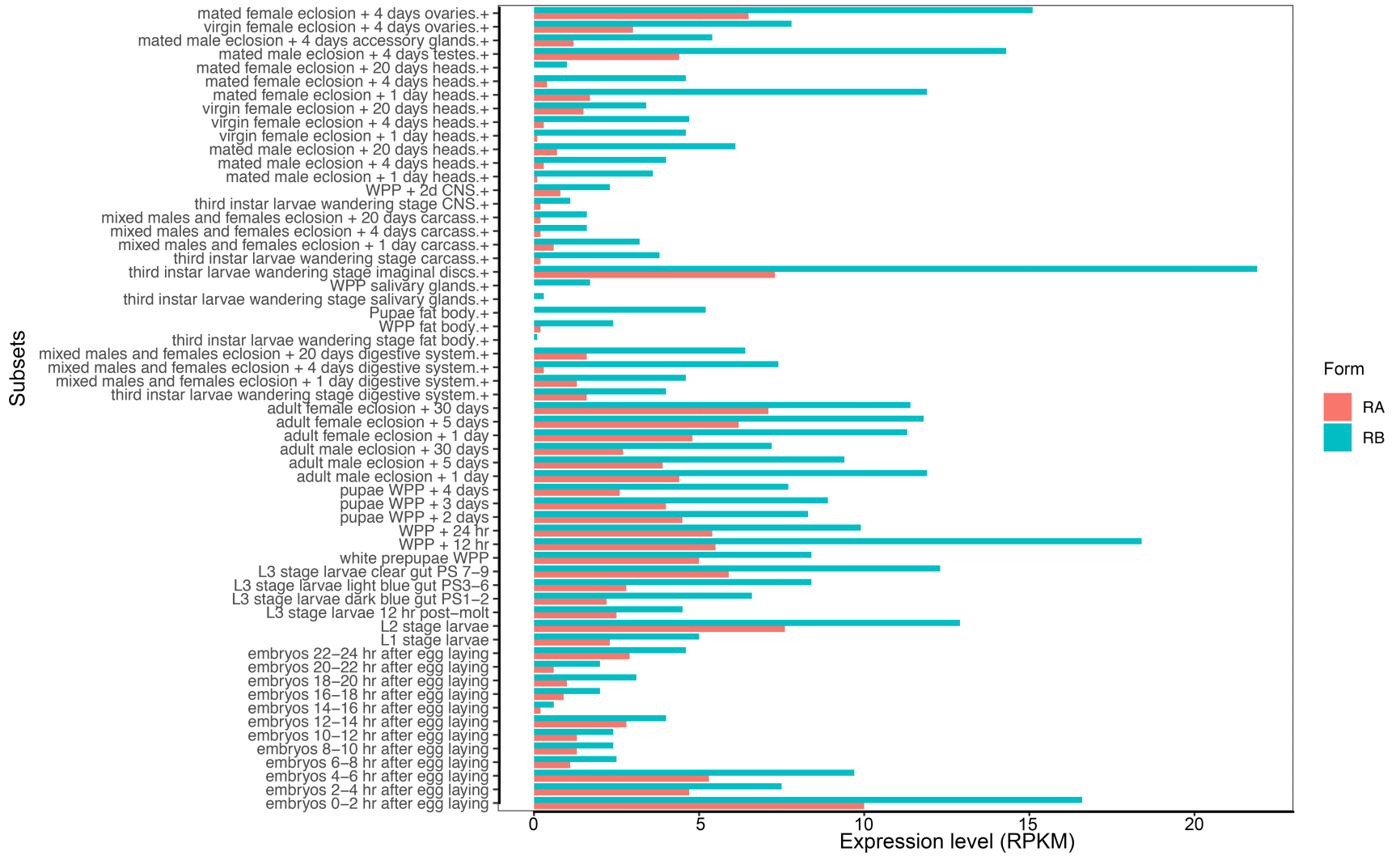

Figure 3.12: **Expression level of form RA versus RB of *sgll*.** Expression level of form RA or RB in different stages and tissues was measured using RNAseq. Data is from modEncode (www.modencode.org). Gene *sgll* is expressed throughout the development stage. Its expression is high at the early stages of embryos, slightly decreased at the late stages of embryos, and maintained at a high level in the following developmental stages and in adults. This pattern holds true for both RA and RB forms. However, the expression level of RB is relatively higher than RA.

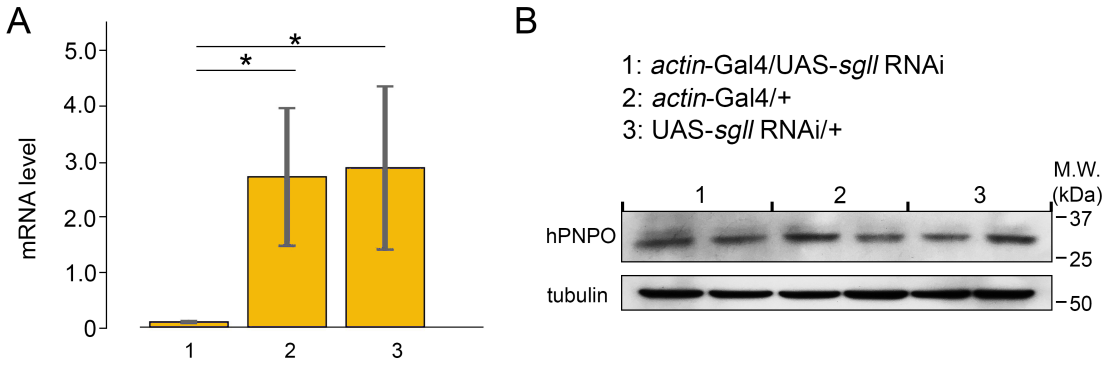

Figure 3.13: **The antibody used for detecting hPNPO does not recognize SGLL.** A) qPCR results from ubiquitous *sgll* KD and control flies. n = 4 biological replicates per genotype. Error bar = Mean ± SD. * $P < 0.05$, One-way Anova with Tukey's *post hoc*. Primer sequences are shown in the Supplemental Table 3.4. B) Western blots from fly head homogenates with various genotypes. Two biological replicates were used for each genotype. Tubulin is the loading control. We observed one bind from these genotypes, the size of which is similar to the predicted molecular weight for SGLL (∼ 27 KDa). However, the band intensity in *sgll* KD flies (genotype: *actin*-Gal4/UAS-*sgll* RNAi) is as same as that in two controls (genotypes: *actin*-Gal4/+ and UAS-*sgll* RNAi/+), indicating that this band is less likely to be SGLL.

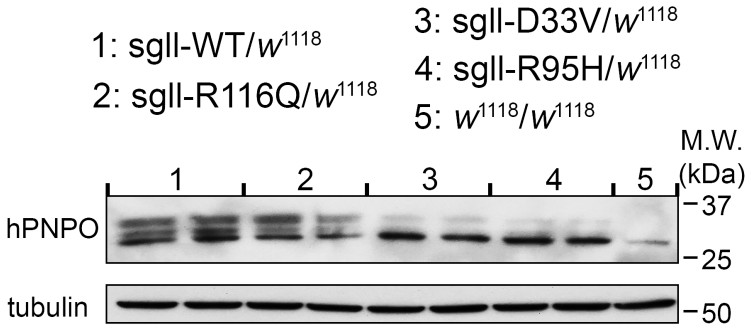

Figure 3.14: **R95H mutation affects the protein stability of hPNPO (the 2nd blot).** Western blot of adult fly head homogenate from each genotype. Two biological replicates were used for each KI/w^{1118} flies, where the KI allele was sgll-WT, sgll-R116Q, sgll-D33V, or sgll-R95H. Tubulin is the loading control. Quantifications of this blot along with the other blot (Figure 3.5 A), a total of four biological replicates per genotype, are shown in Figure 3.5 B.

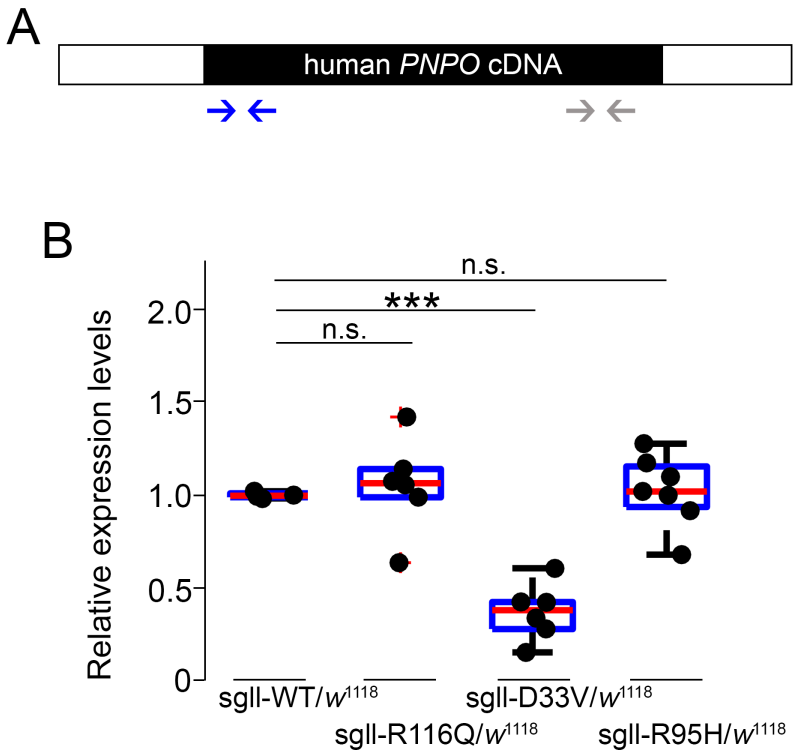

Figure 3.15: **Primer design and qRT-PCR results from KI/w^{1118} flies with primers that target the N-terminus of *hPNPO* cDNA.** A) Two pairs of PCR primers were designed to target either the N-terminus of *hPNPO* cDNA or the C-terminus of *hPNPO* cDNA. B) qRT-PCR results from primers that target the N-terminus of *hPNPO* cDNA, n = 6-7 biological replicates per genotype. n.s.: $P > 0.05$,*** $P < 0.001$, unpaired t test, compared to sgll-WT/w^{1118}. qRT-PCR results from primers that target the C-terminus of *hPNPO* cDNA are shown in Figure 3.5 C).

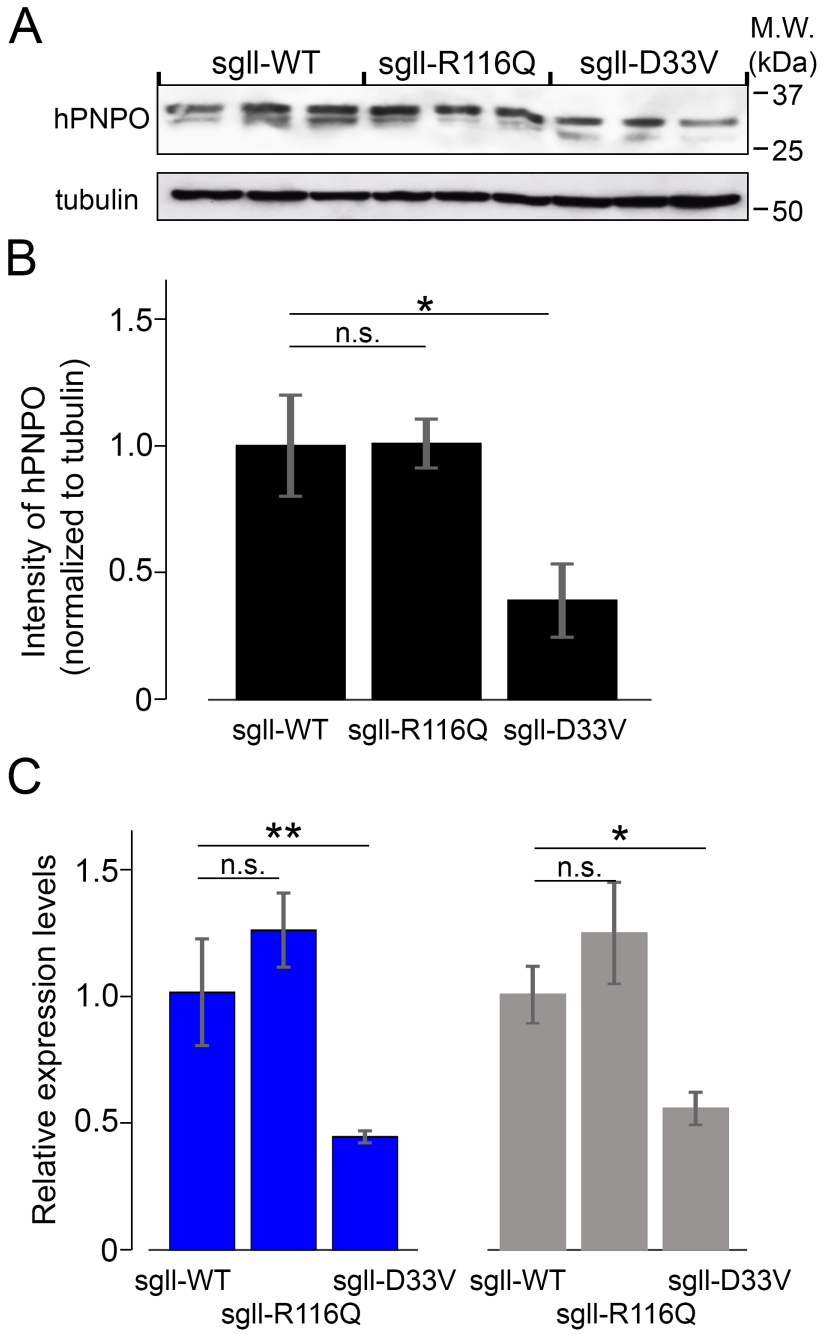

Figure 3.16: **Decreased *hPNPO* mRNA and protein levels in sgll-D33V homozygotes.** A) Western blot of adult fly head homogenate from sgll-WT, sgll-R116Q, and sgll-D33V. Three biological replicates were used for each genotype. Tubulin is the loading control. B) Quantifications of hPNPO in panel A. C) qRT-PCR results from primers that target the N-terminus (left panel) and the C-terminus (right panel) of *hPNPO* cDNA in homozygous KI flies, n = 3 biological replicates per genotype. Error bar = Mean ± SD. n.s.: $P > 0.05$,* $P < 0.05$, ** $P < 0.01$, unpaired t test, compared to sgll-WT/sgll-WT.

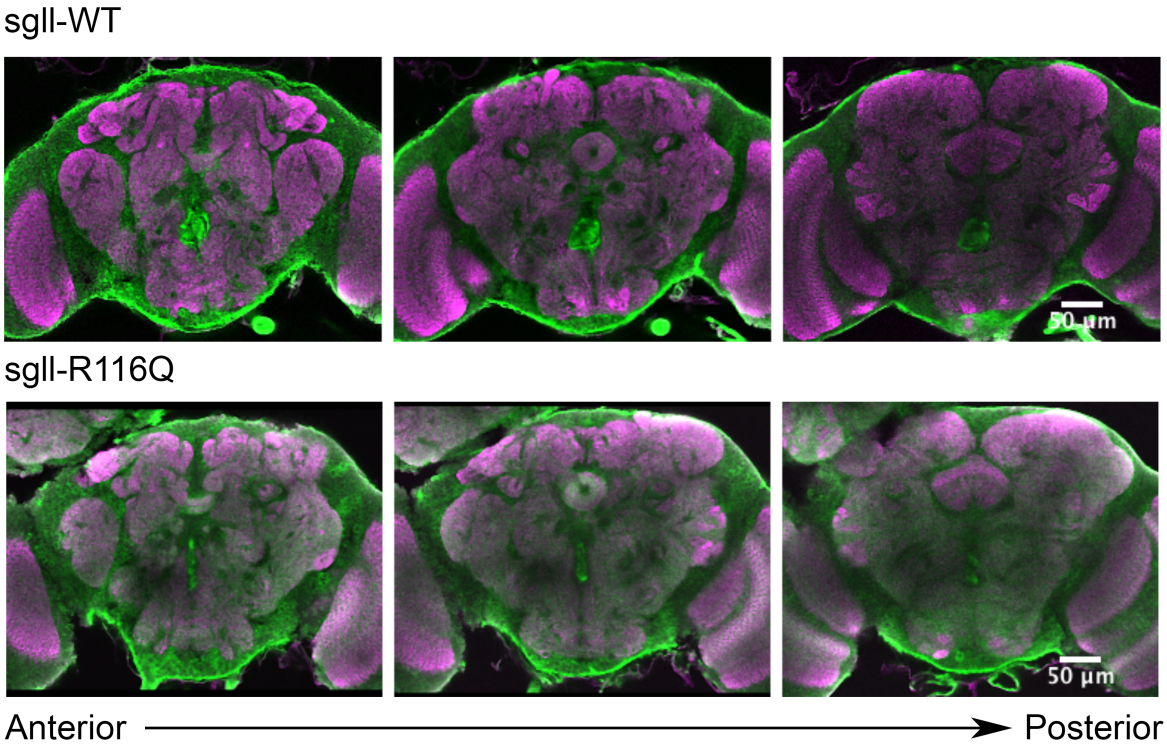

Figure 3.17: **R116Q increases the terminal localization of hPNPO.** Immunohistochemistry staining of hPNPO in adult fly brain. The Brp staining was used as a counterstain, which stains terminal structures. Images from the different parts of the brain are shown for these two genotypes. The most anterior images are shown in Figure 3.8.

Table 3.3: **Antibodies used in KI studies**

Antibody	Source	Cat #	Dilution
Rabbit anti-hPNPO	Novus	NBP1-87302	1:500 (WB); 1:300 (IHC)
Mouse anti-β tubulin	DSHB	E7	1:500
Goat anti-Rabbit-HRP	Jackson	111-035-144	1:10,000
Goat anti-Mouse-HRP	Jackson	115-035-003	1:10,000
Mouse anti-Brp	DSHB	nc-82	1:10
Flurophore conjugated Donkey anti-primary antibody species	Life Technologies	A21206; A21202; A31573; A31571	1:400
Flurophore conjugated Donkey anti-primary antibody species	Jackson	715-585-150; 711-585-152	1:400

DSHB: Developmental Studies Hybridoma Bank at University of Iowa; Jackson: Jackson ImmunoResearch

Table 3.4: **PCR primers for qRT-PCR**

Target	Sequence	Reference Figure(s)
KI-N	Forward: 5' - ATG ACG TGC TGG CTG CG -3' Reverse: 5' - ACC ACA CAG GTG ACT GAG GTC - 3'	Figures 3.15 & 3.16
KI-C	Forward: 5' - CTC AGG TGA TGG AGT TCT GGC A -3' Reverse: 5' - CTA AGG TGC AAG TCT CTC ATA GAG CC - 3'	Figures 3.5 & 3.16
rp49	Forward: 5' - GCT AAG CTG TCG CAC AAA TG -3' Reverse: 5' - GTT CGA TCC GTA ACC GAT GT - 3'	Figures 3.5 & 3.15 & 3.16
sgll	Forward: 5' - AAG TTG CTG CAA ACA ATT -3' Reverse: 5' - GGA TTC TTC ACC TTG ATG - 3'	Figure 3.13

CHAPTER 4

DOES MILD PNPO DEFICIENCY CONTRIBUTE TO ADULT-ONSET EPILEPSY?

4.1 Abstract

Pyridox(am)ine 5'-phosphate oxidase (PNPO, EC 1.4.3.5) is a rate-limiting enzyme in the biosynthesis of vitamin B6. While it is well known that PNPO deficiency causes neonatal epileptic encephalopathy (NEE) and early-onset epilepsy, the contribution of PNPO deficiency to adult-onset epilepsy has never been examined. Here we aimed to study whether PNPO deficiency is over-represented in adult patients with generalized epilepsy, specifically we focused on R116Q, a mutation/variant that affects the enzyme activity, has been identified in NEE and early-onset epilepsy patients, and is common in the general population. We collected SNPs information from these patients by Sanger sequencing PCR products amplified from *hPNPO* coding exons. So far, we have collected a total of 36 samples and among them, 6 are heterozygous for R116Q. Thus, the allele frequency of R116Q in the current samples is 8.33 %, which is higher than that in any races in the general population. Power analyses based on the current effect size showed that a total of $\sim$ 300 samples will be needed to reach a power level of 0.8.

4.2 Introduction

Pyridox(am)ine 5'-phosphate oxidase (PNPO, EC 1.4.3.5) is a rate-limiting enzyme in the biosynthesis of vitamin B6 [73]. While it is well known that PNPO deficiency causes neonatal epileptic encephalopathy (NEE) and early-onset epilepsy [147], it remains to be determined whether it contributes to epilepsy in adults.

Mutations in PNPO have been increasingly reported in NEE and early-onset epilepsy (Supplemental Table 4.5) since 2005 when the first mutation was identified [91]. *In vitro*

biochemical assays show that most mutations identified in NEE patients reduce the PNPO enzymatic activity to below 50% of the wild-type (WT) enzyme, suggesting that these mutations are severe PNPO mutations [91, 104, 98, 114] (Figure 4.1, Supplemental Figure 3.11). In addition, it seems that the total number of reported alleles and the ages of seizure onset are correlated with their residual activities (Figures 4.1 & 4.2, Supplemental Table 4.4), indicating that the lower the residual activity of one mutant is, the more likely the corresponding mutation is associated with NEE. As expected, due to their deleterious effects on fitness, the allele frequencies of these severe PNPO mutations are extremely low in the general population (www.gnomad.broadinstitute.org).

On the other hand, mild PNPO mutations may contribute to susceptibility to epilepsy in general. For example, PNPO has been associated with generalized epilepsy [155] and recently, it was included as one of the sixteen epilepsy genes involved in the common epilepsies [57]. The connection between mild PNPO deficiency and generalized epilepsy was also suggested by treatment response to PLP or PN in some idiopathic intractable epilepsy patients [156] and in epilepsy patients caused by genetic defects in other genes [134, 135]. In fact, PNPO variants that cause mild PNPO deficiency are rather common in the general population [151, 152]. One of such variants is R116Q, which has an average allele frequency of 5.5 % in the general population. The exact number varies among difference races (Table 4.1). The average allele frequency of R116Q is at least 200 times higher than that of other potential deleterious mutations in PNPO in the general population (Figure 4.3), suggesting that R116Q is the main contributor to mild PNPO deficiency in the general population.

R116Q may render individuals susceptible to epilepsy by interacting with other genetic or environmental factors. R116Q has been reported in NEE and early-onset epilepsy [98, 99]. However, R116Q is more likely to be a mild mutation because unlike other severe mutations identified in NEE patients, it only slightly decreases the enzyme activity [98]. Moreover, in our studies, R116Q affects neither development nor the conditional survival of knock-in (KI) flies (Data in Chapter 3). Thus, it is likely that R116Q cannot cause diseases by itself, but it

can increase the susceptibility to diseases of its carriers or homozygous individuals when they are challenged with other genetic defects and/or environmental factors.

Here we aimed to test the hypothesis that mild PNPO deficiency, particularly caused by R116Q mutation, is over-represented in adult generalized epilepsy patients. We have collected a total of 36 samples to date and 6 are positive for R116Q; thus, the allele frequency of R116Q in the current samples is 8.33 %, which is higher than that in any races in the general population. Power analyses based on the current effect size, calculated from the average allele frequency, showed that a total of 328 samples will be needed to reach a power level of 0.8. More samples will be needed.

4.3 Methods and Materials

4.3.1 Patients Recruitment

Research protocol used in this chapter was submitted to the International Review Board at the University of Chicago for approval before being implemented in this study. Adult generalized epilepsy patients were recruited from the Neurology section of the University of Chicago Hospital.

4.3.2 Saliva Collection and Genomic DNA Extraction

Saliva samples from patients were collected by health professionals with saliva collection kits (DNA genotek, ORAgene, Cat # OGR-500). Samples collected with these kits are stable at room temperature for years. Genomic DNA (gDNA) was purified with prepIT (DNA genotek, prepIT L2P, Cat # PT-L2P) by following the manufacturer's protocol. Basically, saliva samples were incubated in a water incubator at 50 °C for 1.5 hr. After incubation, 500 μl of the mixed sample was transferred to a 1.5 ml microcentrifuge tube and 20 μl of PT-L2P was added. Samples were mixed by vortexing for 5 sec and incubated on ice for 10 min, followed by centrifugation at RT for 5 min at 15,000g. Supernatant was then transferred with a pipette tip

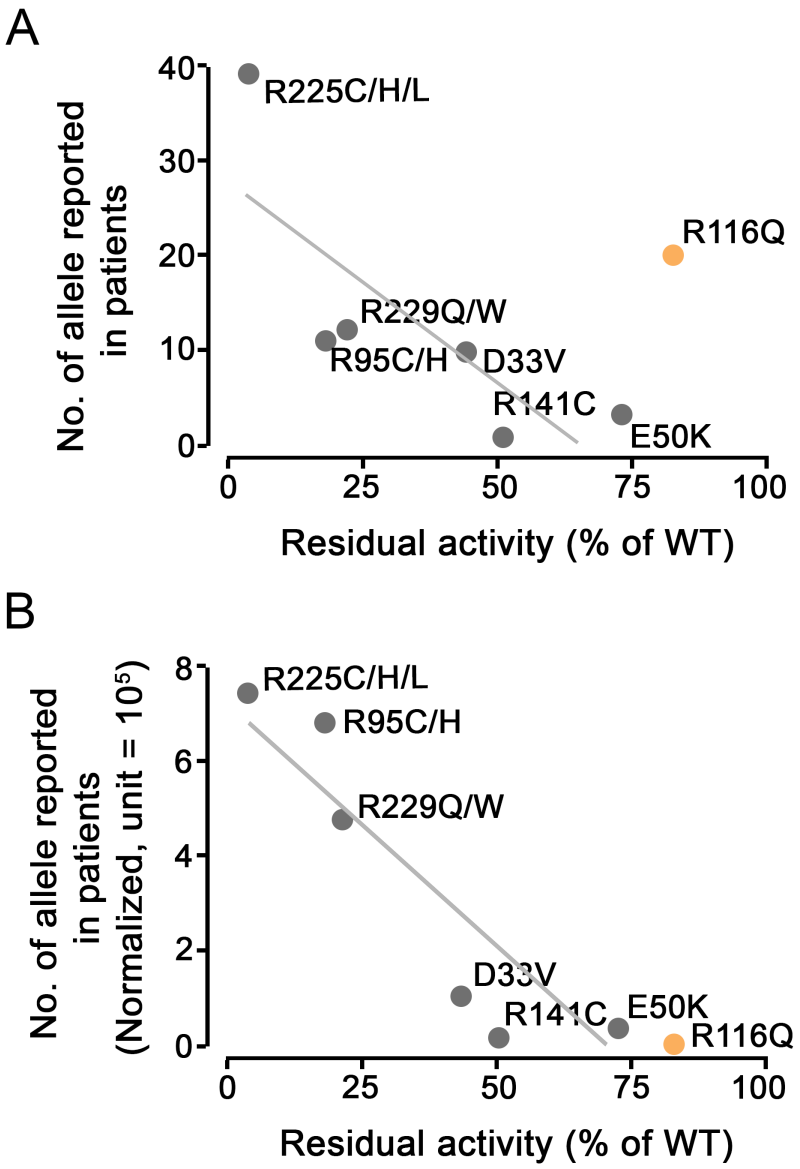

Figure 4.1: **Neonatal epileptic encephalopathy patients have severe PNPO mutations.** There is a negative correlation between the residual activities and the number of each mutant allele reported in patients. A) Data without R116Q was fitted to a linear model ($P = 0.06$); B) Data normalized to allele frequencies in the general population was fitted to a linear model ($P = 0.003$).

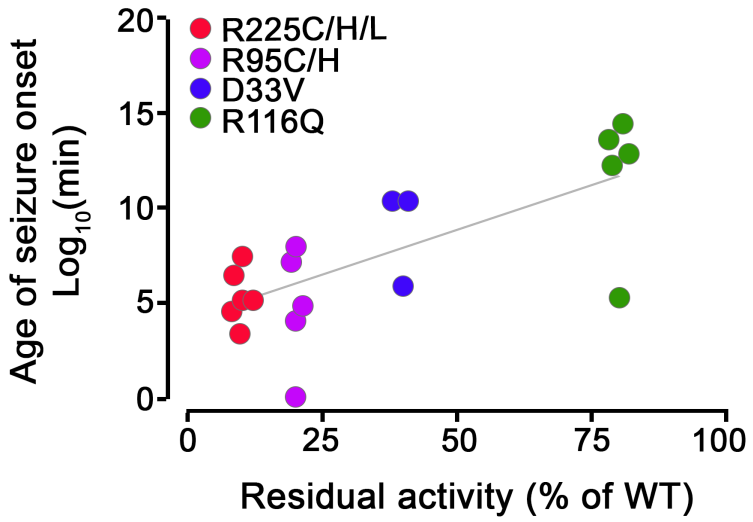

Figure 4.2: **Age of seizure onset is positively correlated with residual activity.** Ages of seizure onset are on a $\log_{10}$ scale; data was fitted to a linear model ($P = 0.0002$). See Supplemental Table 4.4 for detailed information.

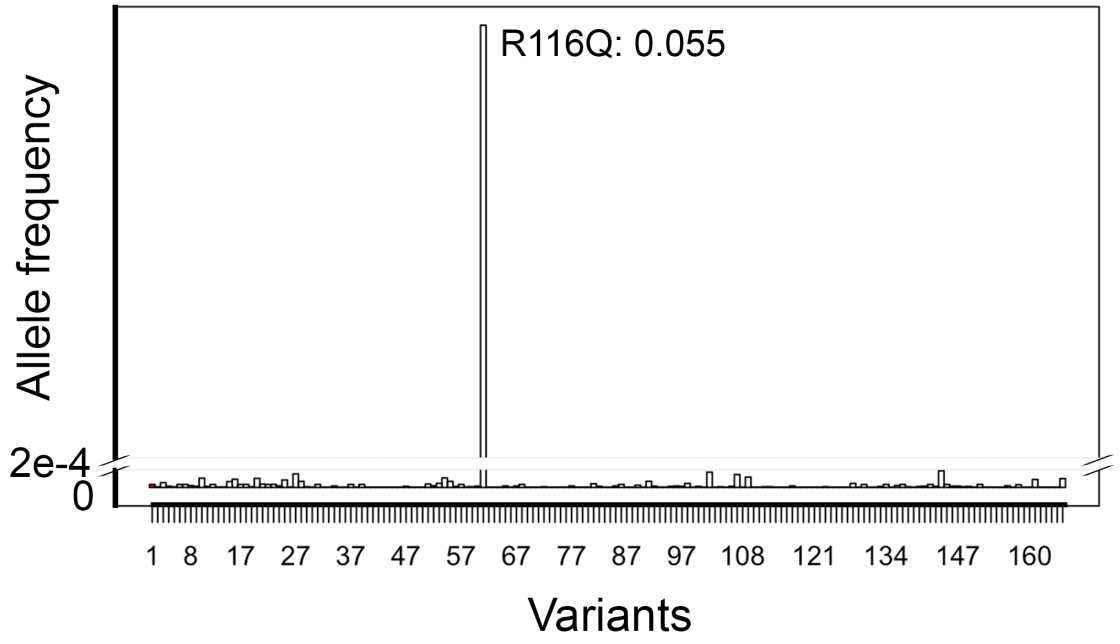

Figure 4.3: **Allele frequency of each deleterious PNPO variant in the general population, which consists of 141,456 non-epileptic individuals.** Deleterious mutations, which includes missense, frameshift, splice error, stop codon gain/loss, and start codon loss, are shown in the plot. Other than R116Q, none of them are homozygous. In all R116Q alleles, 6.8 % are from homozygotes. Data is from gnomAD (www.gnomad.broadinstitute.org) [151, 152].

into a fresh microcentrifuge tube and 600 μl of 100% ethanol was added. Samples were mixed gently by inversion 10 times and left on the bench at RT for 10 min, then were centrifuged at RT for 2 min at 15,000g. After centrifugation, the supernatant was removed and the DNA pellet was washed with 250 μl of 70% ethanol, followed by air drying. The DNA pellet was then dissolved in 100 μl Tris-EDTA solution. To ensure complete rehydration, DNA samples in TE were incubated at 50 °C for 1 hr with occasional vortexing. The concentration and quality of gDNA were examined with the spectrophotometer (ThermoScientificNanoDrop). DNA solution was stored at -20 °C for long-term storage.

4.3.3 PCR Amplication of PNPO Coding Exons and Obtaining SNP Information by Sanger sequencing

PCR was performed with the following primers (Table 4.3) that target seven coding exons of *PNPO*. Final PCR products were sequenced at the DNA core facility at the University of Chicago. Sequencing results were then blasted to human genomic DNA (GRCh38.p7) to obtain the SNP information of each coding exon. In addition, the corresponding chromatogram was inspected for the heterozygosity of each SNP. The SNP information of each sample were recorded and analyzed.

4.4 Results

Till now, a total of 36 samples have been collected. The basic information (sex, current age, age of seizure onset, and drug resistance) of these patients is not yet available.

We identified three variants in these samples. They are Ser55, R116Q, and Leu184. Both Ser55 and Leu184 are synonymous variants and R116Q is the only missense one. Among 36 samples, 6 patients are heterozygous for R116Q. Thus, the allele frequency of R116Q in the current samples is 8.33 % (6 / 72) (see Supplemental Figures 4.4 & 4.5), which is higher than that in any races in the general population (Table 4.1).

To estimate how many samples will be needed to achieve a certain power level, power analysis was performed based on the current effect size. Levels of power and corresponding sample sizes are summarized in Table 4.2.

To examine the statistical significance, we compared the allele frequency of 8.33 % to that of each race (Table 4.1), which is inaccurate because we know that our samples are mixed with several populations.

4.5 Discussion

We hypothesized that mild PNPO deficiency conferred susceptibility to epilepsy in adults. We aimed to test the hypothesis in adult epilepsy patients by examining if mild PNPO deficiency, particularly caused by R116Q, is over-represented.

To date, we have collected a total of 36 samples and obtained a positive rate of 8.33 %. Power analyses based on the current effect size demonstrated that about 300 samples would be needed to reach a power level of 0.8. The exact sample size will depend on the composition of races in samples because the R116Q allele frequency ranges from 0.02 % in the East Asian population to 7.2 % in the European Non-Finnish population.

The wide variation among different populations indicates that the race information of each patient is important. Currently, we collected race information based on self-reporting, which may not be accurate, but is the most convenient and cost-effective way. Alternatively, we can use whole genome sequencing to identify the race of each patient because each population has its signature haplotype (The International HapMap Consortium). This approach is rigorous, but is expensive and computationally intensive. Lastly, depending on the effect size of R116Q, if it is large enough, we can ignore the exact race information of each patient and treat all patients as European Non-Finnish, a race that has the highest allele frequency in the general population. We will compare the R116Q allele frequency from samples to that from European Non-Finnish in the general population. If the difference is significant in this comparison, then the difference should be significant for any race composition.

Table 4.1: **R116Q allele frequency in the general population and *P* values calculated for each race**

Population	Allele frequency in gnomAD	*P*-value
African	0.0114	0.0001
Ashkenazi Jewish	0.0538	0.1458
East Asian	0.0002	0
South Asian	0.0596	0.1857
European Finnish	0.0667	0.2921
European Non-Finnish	0.0720	0.3503
Latino	0.0369	0.0393
Other	0.0620	0.2457
Total	0.0547	0.1557

Table 4.2: **Power analysis to estimate the sample size based on the current effect size**

Population	Power = 0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95
African	40	45	50	56	64	73	85	105
Ashkenazi Jewish	194	218	245	275	311	356	416	515
East Asian	30	34	38	42	48	55	64	79
South Asian	275	309	346	389	440	503	589	728
European Finnish	460	516	579	651	736	842	986	1219
European Non-Finnish	745	837	939	1055	1194	1365	1598	1976
Latino	90	101	113	127	144	164	192	238
Other	322	362	406	457	516	591	691	855
Total	205	230	258	290	328	375	439	542

All above discussed approaches intend to use the R116Q allele frequency in the general population as the control. Alternatively, we can use samples collected from non-epileptic sibling(s) of each patient as controls. One disadvantage of this approach is that we may not be able to collect all control samples since a qualified sibling may not be available for every patient.

Lastly, we are currently focusing on generalized epilepsy patients, especially those with drug-resistance for two reasons: 1) patients in this category will benefit the most from our studies in the future if our hypothesis is true; 2) VB6 treatment has been shown effective in patients with genetic defects in ion channels [134, 135]. Patients in other categories may be studied in the future.

4.6 Supplementary Information

4.6.1 Supplementary Figures and Tables

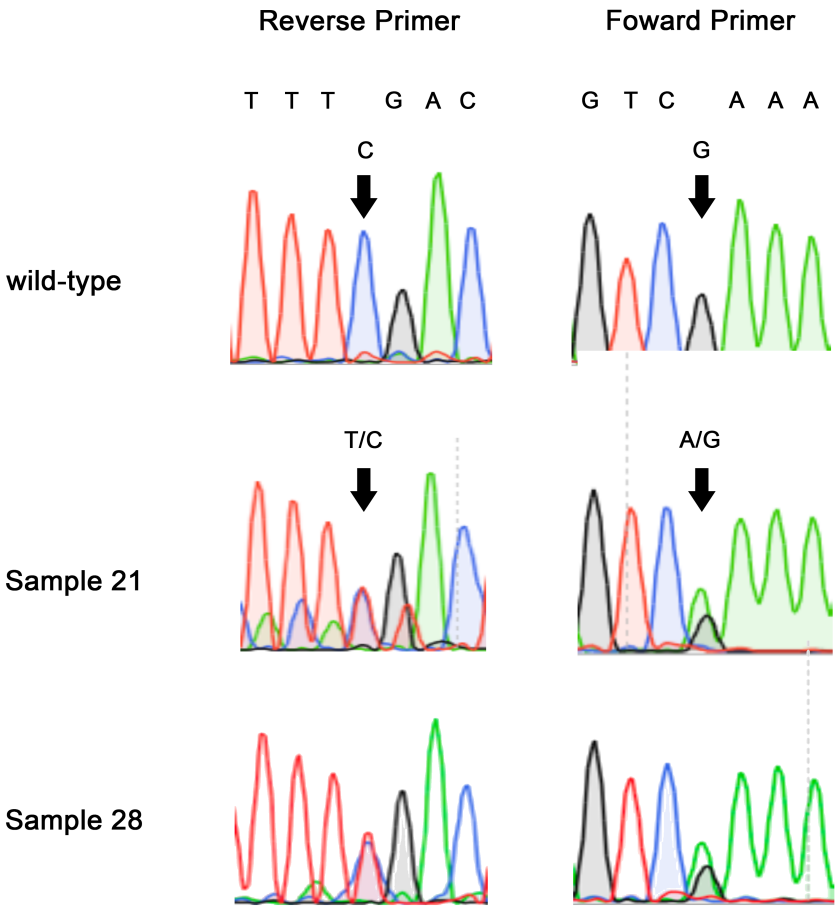

Figure 4.4: **Chromatograms of positive samples.** More samples are shown in Figure 4.5

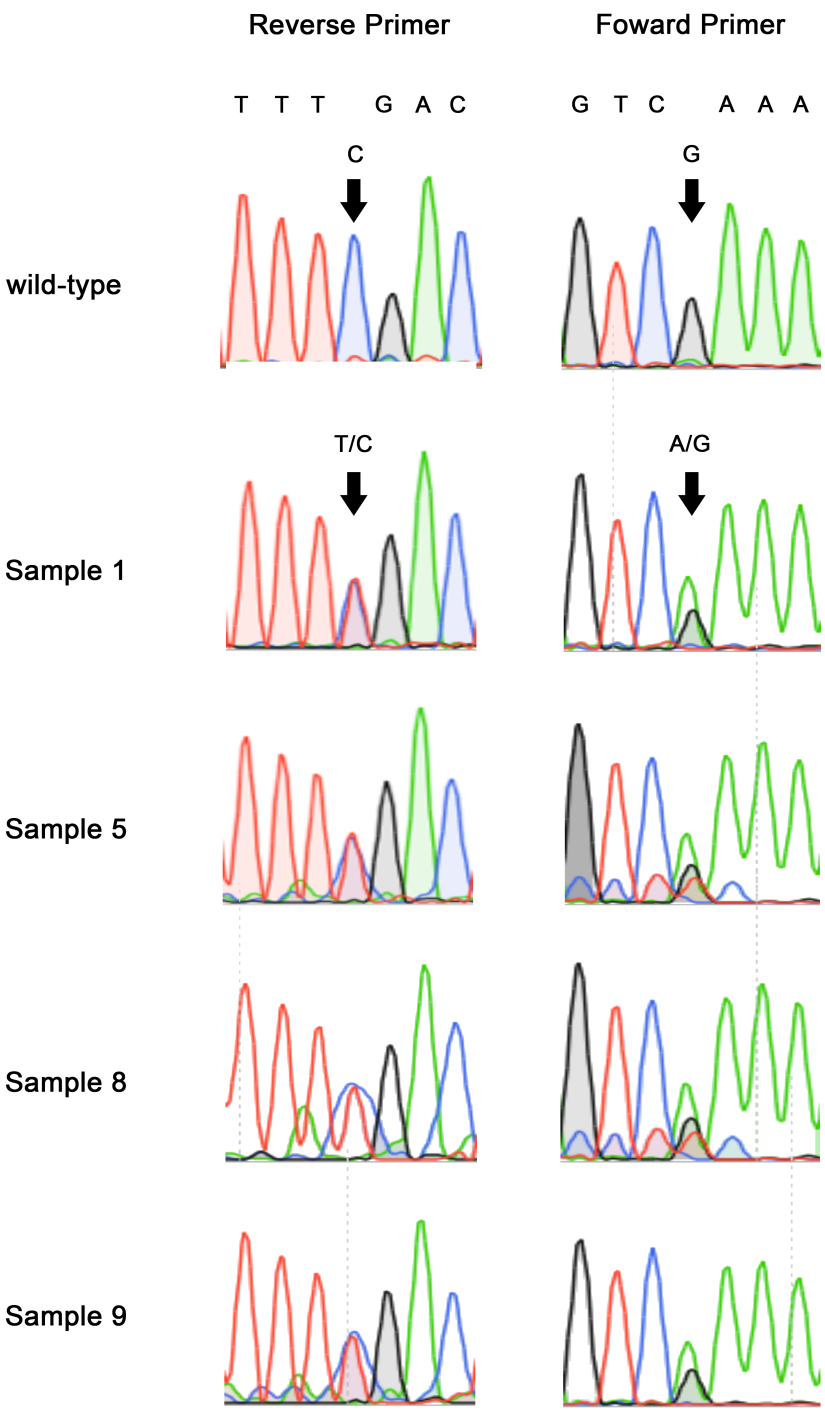

Figure 4.5: **Chromatograms of positive samples.** More samples are shown in Figure 4.4.

Table 4.3: **PCR primers used in the amplification of hPNPO coding exons**

Target	Sequence
Exon 1	Forward: 5'-TGGGTTCCTCTGTTCTCGCTCTAATTGG-3' Reverse: 5'- GTTATTTGACCTTGCTACCTGTCGAGT-3'
Exon 2	Forward: 5'-GGGACAGCAAGTTGTCTGAGTTTTTATGC-3' Reverse: 5'- GAGAAGTAGCTCCTGAATGGCAGACG-3'
Exon 3	Forward: 5'-TCCATAAGGTCCCCTTCTCCAAATACAGT-3' Reverse: 5'- TCAAGGAAGGCCTTCCTGAGGAGGTAA-3'
Exons 4 & 5	Forward: 5'-TCAAGACTCACAAATCTGTGGGTGAGAG-3' Reverse: 5'- CTTTTCACTGTTTTCACTGGGCTGGAGA-3'
Exons 6 & 7	Forward: 5'-TGAAAAGGACAGTGAGAAGGGAAAGTGG-3' Reverse: 5'- TACGCCACCATTACACTCCACTTGACCA-3'

Table 4.4: **Summary of the relationship between residual PNPO activities and seizure onsets**

Residual Activity	Mutation	N_{cases} (Homozygote only)	Age of Seizure Onset	Time Scale
$\sim$ 10 %	R225H/C/L	19	30 min; 2 h; 3 h; 3 h; 10 h; 10 h; 11 h; 24 h; 24 h; 1 d; 2 d; 2 d; 6 d; n/a; n/a; n/a; n/a; n/a; n/a	Hour - **Day**
$\sim$ 20 %	R95H/C	5	Birth; 1 h; 2 h; $>$ 24 h; 48 h	Hour - **Day**
$\sim$ 40 %	D33V	3	6 h; 3 wk; 3 wk	Hour - **Week**
$\sim$ 80 %	R116Q[a]	7	3 h; 5 mo; 8 mo; 20 mo; 3y 2 mo; None[b]; n/a	Hour - **Year**

[a]A total of nine homozygous cases were reported. Two of them also are homozygous for R225H, which are not included here;
[b]Mother received multivitamin during pregancy.
Abbreviation: min-minute; wk-week; h-hour; d-day; mo-month; y-year; n/a-not available
Data is from Hoffmann *et al.* [102], Veerapandiyan *et al.* [157], Ware *et al.* [111], Khayat *et al.* [104], Mills *et al.* [98], Plecko *et al.* [108], Levtova *et al.* [114], Xue *et al.* [100], Guerriero *et al.* [123], and Wilson *et al.* [158].
See Table 4.5 for all published PNPO mutations so far and for ages of seizure onset in corresponding patients.

Table 4.5: **Summary of PNPO mutations and seizure onsets from published cases**

Seizure onset	Mutation	State	Reference
5 mo	c.445_448del/R138H	Compound heterozygote	Xue *et al.* [100]
24 h	c.445_448del/R161C	Compound heterozygote	Xue *et al.* [100]
24 h	c.445_448del/R161C	Compound heterozygote	Xue *et al.* [100]
2 wk	D33V/R225C,R116Q	Compound heterozygote	Mills *et al.* [98]
36 h	D33V/c.246delT	Compound heterozygote	Hoffmann *et al.* [102]; Schmitt *et al.* [109]; Hatch *et al.* [130]
2 mo	D33V/E120K	Compound heterozygote	Mills *et al.* [98]
3 h	D33V/c.264-21_264-1delinsC	Compound heterozygote	Mills *et al.* [98]
40 d	E50K/R116Q	Compound heterozygote	Xue *et al.* [100]
<12 h	E50K/VS3-1G>A	Compound heterozygote	Mills *et al.* [91]
6 h	IVS5+1 G>A/c.620delG	Compound heterozygote	Veeravigrom *et al.* [107]
6 d	R141C/c.279_290del	Compound heterozygote	Plecko *et al.* [108]
< 1 mo	R161G/P150Rfs*27	Compound heterozygote	Fung *et al.* [159]
5 d	D33V/Y157*	Compound heterozygote	Wilson *et al.* [158]

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Seizure onset	Mutation	State	Reference
30 min	R95H/E50K	Compound heterozygote	Mills *et al.* [98]
5 h	Q214fs/?	Heterozygote	Mills *et al.* [98]
3 d	A161C	Homozygote	Jaeger *et al.* [115]
Birth	A174X	Homozygote	Ruiz *et al.* [103]
$\sim$ 8 mo	c.363+5G>A	Homozygote	Kvarnung *et al.* [160]
3 wk	D33V	Homozygote	Goyal *et al.* [113]
6 h	D33V	Homozygote	Mills *et al.* [98]; Hatch *et al.* [130]
3 wk	D33V	Homozygote	Mills *et al.* [98]
12 h	G118R	Homozygote	Pearl *et al.* [105]
12 h	IVS+2T>C	Homozygote	Porri *et al.* [106]
90 min	P213S	Homozygote	Mills *et al.* [98]; Hatch *et al.* [130]
None[a]	P213S	Homozygote	Mills *et al.* [98]; Hatch *et al.* [130]
5 mo	R116Q	Homozygote	Mills *et al.* [98]
3 h	R116Q	Homozygote	Mills *et al.* [98]
20 mo	R116Q	Homozygote	di Salvo *et al.* [116]

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Seizure onset	Mutation	State	Reference
3 y 2 mo	R116Q	Homozygote	di Salvo *et al.* [116]
8 mo	R116Q	Homozygote	di Salvo *et al.* [116]
10 h	R225C	Homozygote	Veerapandiyan *et al.* [157]
1 d	R225H	Homozygote	Ware *et al.* [111]
24 h	R225H	Homozygote	Mills *et al.* [98]
24 h	R225H	Homozygote	Plecko *et al.* [108]
3 h	R225H	Homozygote	Plecko *et al.* [108]
2 d	R225H	Homozygote	Plecko *et al.* [108]
n/a	R225H	Homozygote	Plecko *et al.* [108]
11 h	R225H	Homozygote	Plecko *et al.* [108]
6 d	R225H	Homozygote	Plecko *et al.* [108]
2 d	R225H	Homozygote	Plecko *et al.* [108]
3 h	R225H	Homozygote	Plecko *et al.* [108]
2 h	R225H	Homozygote	Levtova *et al.* [114]
10 h	R225H,R116Q⋆	Homozygote	Mills *et al.* [98]

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Seizure onset	Mutation	State	Reference
30 min	R225H,R116Q⋆	Homozygote	Mills *et al.* [98]
n/a	R225L	Homozygote	Guerriero *et al.* [123]
n/a	R225L	Homozygote	Guerriero *et al.* [123]
n/a	R225L	Homozygote	Guerriero *et al.* [123]
n/a	R225L	Homozygote	Guerriero *et al.* [123]
1 d	R229Q	Homozygote	Ware *et al.* [111]
n/a	R229Q	Homozygote	Guerriero *et al.* [123]
<1 h	R229W	Homozygote	Mills *et al.* [91]
<1 h	R229W	Homozygote	Mills *et al.* [91]
>24 h	R95C	Homozygote	Hoffmann *et al.* [102]
48 h	R95C	Homozygote	Hoffmann *et al.* [102]; Bagci *et al.* [110]
Birth	R95C	Homozygote	Hoffmann *et al.* [102]; Bagci *et al.* [110]
2 h	R95C	Homozygote	Mills *et al.* [98]
1 h	R95H	Homozygote	Khayat *et al.* [104]
30 min	X262Q	Homozygote	Mills *et al.* [91]

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Seizure onset	Mutation	State	Reference
30 min	X262Q	Homozygote	Mills *et al.* [91]
None[a]	c.364-1G>C	Homozygote	Wilson *et al.* [158]
None[b]	R116Q	Homozygote	Wilson *et al.* [158]
n/a	c.363+5G>A	Homozygote	Wilson *et al.* [158]
n/a	c.363+5G>A	Homozygote	Wilson *et al.* [158]
n/a	R225C	Homozygote	Wilson *et al.* [158]
n/a	R116Q	Homozygote	Wilson *et al.* [158]
3 wk	W65L	Homozygote	Wilson *et al.* [158]
n/a	c.263+2T>C	Homozygote	Wilson *et al.* [158]

[a]Due to prophylactic treatment; [b]Mother received multivitamin during pregancy;?: the 2nd mutation is unknown; $\star$ Homozygote for both mutations

Abbreviation: min-minute; wk-week; h-hour; d-day; mo-month; y-year; n/a-not available

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The advance of DNA sequencing technologies have unprecedentedly expedited the molecular diagnosis of various diseases. In Online Mendelian Inheritance In Man (OMIM, https://omim.org), a primary database that curates information on both diseases/phenotypes and disease-causing genes, the number of phenotypes with known molecular basis has been dramatically increased since 2010 when the whole-genome sequencing and whole-exome sequencing were introduced [161]. From 2010-2016, a total of 990 causal genes were identified, which has significantly improved our understanding of previously undiagnosed diseases, especially rare diseases [162]. The identification of these genes, however, most of time was not directly translated into disease treatment due to our limited knowledge on the biology of these genes. Thus, to improve treatment and to allow patients to fully benefit from these gene discoveries, we need to study the fundamental biology of these genes and to study the pathophysiology associated with disease-causing mutations. While some studies can be performed in human patients, most studies will have to rely on animal models. Therefore, establishing valid animal models will be an essential step in the process of expanding our knowledge on disease-causing genes/mutations and of finding treatments for patients.

My thesis work on fly studies represents a concrete example of using *Drosophila* models to study the fundamental biology of *hPNPO* gene and the molecular and cellular mechanisms of disease causing-mutations in *hPNPO*. These studies expand our knowledge on *hPNPO* gene, hPNPO mutations and hPNPO deficiency, and provide experimental evidence that may lead to an effective treatment for adult epilepsy patients in the future. Furthermore, validated *Drosophila* models can be used for exploring other biological questions related to PNPO deficiency and for exploring other treatment options of PNPO deficient patients.

In Chapter 2, I report the establishment of *Drosophila* models of PNPO deficiency. Using

both behavioral and electrophysiological approaches, we show that PNPO deficiency causes seizures in *Drosophila* as it does in humans. Moreover, seizures and conditional lethality are correlated with low internal PLP levels and can be completely rescued by the ubiquitous expression of *hPNPO*. Furthermore, analysis of the spike pattern from electrophysiological recordings reveals a potential involvement of GABA dysfunction in seizures caused by PNPO deficiency. Lastly, cell type-specific *sgll* KD indicates an important role of brain-expressed *sgll* in survival and in maintaining normal brain functions.

It will be interesting to dissect the role of PNPO in peripheral tissues in maintaining normal brain functions and it will also be interesting to further determine whether PNPO in the brain acts in a cell autonomous or a cell non-autonomous manner. Lastly, it is important to confirm the role of GABA pathway in PNPO-deficiency-induced seizures with other approaches such as measuring GABA levels in the brain using HPLC.

In Chapter 3, I report the functional and molecular characterization of three human seizure-causing hPNPO mutations in *Drosophila* models. In this study, three PNPO deficient mutant KI lines and one reference WT KI line were generated, which were further studied at different levels. We found that severe hPNPO deficiency led to lethality during development, intermediate hPNPO deficiency caused conditional lethality and seizures, and mild hPNPO deficiency shortened lifespan. In addition, we found that different hPNPO mutations decreased protein stability, altered protein localization, or reduced its transcription. Lastly, genetic studies demonstrated that R95H mutation had a dominant-negative effect. Based on our fly studies and previously published structural studies, we predicted that a number of hPNPO mutation would have dominant-negative effects.

Each finding in this study is exciting and worth to be followed in the future. For example, 1) the developmental role of PNPO in different larval stages can be further determined, which can be achieved by a genetic approach (e.g. Temporal and Regional Gene Expression Targeting system [163]) or a diet-treatment approach (e.g. chemically-defined diet [164]); 2) while we have shown that seizures precede lethality in PNPO deficient flies (see Chapter 2) and

we used lethality to represent the seizure conditions, it would be better to examine seizures using electrophysiology, a more sensitive approach that may lead to findings of subtle changes in KI flies; 3) it is important to examine the immediate consequences of the mislocalization of R116Q mutant protein, which can be done in primary neuron cultures by expressing tagged R116Q mutant protein and examining the morphology and electrophysiological properties of neurons. This approach can also be used to study the interaction of R116Q mutant protein with WT hPNPO protein; 4) it is necessary to provide direct biochemical evidence that the dominant-negative effect of R95H is mediated through the formation of the heterodimer. One feasible approach will be to express two hPNPO variants that are tagged with different epitopes in a cell line followed by examination of the interaction with co-immunoprecipitation; 5) it is worth to study the dominant-negative effect of those predicted mutations.

In Chapter 4, I report a human study to test the hypothesis that mild PNPO deficiency contributes to adult epilepsy. To date, we have collected a total of 36 samples from adult generalized epilepsy patients. Sequencing results show that there are three variants in these samples: two of them are synonymous and the third one is R116Q. Among these samples, six are heterozygous for R116Q, so the allele frequency of R116Q is 8.33 %, which is higher than that in any races in the general population. Power analysis, based on the current effect size and the averaged allele frequency, showed that a total of ~ 300 samples will be needed to reach a power level of 0.8. While more samples will be needed, our fly studies and published human cases has already indicated that R116Q is a deleterious allele and has a long-term effect.

5.2 Future Directions

1) Study other symptoms manifested by epilepsy patients. These symptoms include those specifically related to PNPO deficiency and those generally manifested by epilepsy patients such as sudden unexpected death in epilepsy (SUDEP). SUDEP is defined as "sudden, unexpected, witnessed or unwitnessed, non-traumatic, and non-drowning death in patients

with epilepsy with or without evidence for a seizure, and excluding documented status epilepticus, in which postmortem examination does not reveal a structural or toxicological cause of death" [165]. It is the main cause of death in epilepsy patients with the incidence of 1.2 in 1,000 adults and 0.2 in 1,000 children with epilepsy per year [166]. The pathophysiology of SUDEP is still unknown. The premature death of *sgll* mutant flies mimics SUDEP in epilepsy patients, indicating that it is possible to explore SUDEP in these flies.

2) Identify molecules that are related to VB6 through suppressor/enhancer screens. Although the key enzymes that are involved in the VB6 metabolism have been identified, other players that can potentially affect the biological function of VB6 remains unknown. These players include transporters that transport PL from outside to inside brain cells and chaperone proteins that deliver PLP to apoenzymes. The hypomorphic $sgll^{95}$ allele is suitable for both suppressor and enhancer screen. Given the overall conservation of the VB6 pathway between humans and flies, it is also possible to perform suppressor/enhancer screen using KI alleles. Different modifiers may be identified for different KI alleles, not only because these KI alleles have different mutation sites but also because they lead to different phenotypes.

3) Examine gene-gene interactions between *sgll* and other seizure genes in *Drosophila*. It has been reported that seizures in some patients with channelopathies respond to VB6 treatments [134, 135]. However, the underlying neurobiological mechanism(s) is not understood. The establishment of *Drosophila* PNPO deficiency models paved the path for studying genetic/functional interactions between *sgll* and other seizure genes.

4) Study PNPO deficiency in hPNPO mutation KI mouse models. In alignment with fly studies, I have recently generated KI mouse lines using CRISPR/Cas9. These mouse models will be used to validate key findings in fly studies and to perform anatomical studies as mice and humans share similar brain structure and neurocircuitry.

5) Explore the contribution of mild deficiency in other essential metabolic genes to human diseases. Essential genes are genes that are indispensable for survival and/or reproduction [167]. While it is well known that complete loss of essential genes leads to embryonic

lethality or sterility, it is less studied how mild deficiency in essential genes causes diseases by interacting with other genes or environmental factors. My thesis work provides a concrete example that mild deficiency in an essential gene interacts with environmental conditions to cause diseases.

This concept can be further applied to other essential genes, especially essential metabolic genes. We can start with disease-causing essential metabolic genes because findings in these studies can be directly translated to disease treatment and the treatment can be as simple as a dietary change. There are about 1200 such genes in OMIM, predicted by their catalytic activities (https://david.ncifcrf.gov). The potential contribution of mild deficiency of these genes can be studied using *Drosophila* models in combination with available human genetic data (www.gnomad.broadinstitute.org).

CHAPTER 6

APPENDIX

6.1 Mild PNPO Deficiency Affects PLP in an Environment-Dependent Manner

Previously we examined PLP as well as other B6 vitamers in $sgll^{95}$ and w^{1118} flies reared on the sugar-only diet with or without PN supplementation. We found that PLP level was decreased in $sgll^{95}$ flies compared to that in w^{1118} flies and the reduced PLP level was rescued by PN supplementation (Figure 2.4), and that PMP level was unchanged in $sgll^{95}$ flies and it did not respond to PN supplementation. To expand this study, we measured levels of B6 vitamers in $sgll^{95}$, ubiquitous *sgll* KD, and w^{1118} flies that were collected from breeding bottles, i.e., under the normal diet condition. Based on the survival studies on the normal diet, on the sugar-only diet, and on the sugar-only diet with PN or PLP supplementation, we know that ubiquitous *sgll* KD flies have much more severe PNPO deficiency than $sgll^{95}$ flies.

As shown in Figure 6.1, there is no significant difference in PLP between $sgll^{95}$ and w^{1118}, which is consistent with the survival study showing that $sgll^{95}$ flies survive well on the normal diet. However, PLP level is significantly reduced in ubiquitous *sgll* KD flies, indicating a severe PNPO deficiency in these flies, which is also consistent with the survival study showing that ubiquitous *sgll* KD flies do not survival well on the normal diet. Since PNPO converts PMP to PLP, PMP level is accordingly increased in *sgll* KD flies. Therefore, severe PNPO deficiency leads to a reduction in PLP and an increase in PMP in a dietary VB6-independent manner. In contrast, mild PNPO deficiency does not lead to changes in PLP in response to dietary VB6 supply.

Studies in mammals and humans show that PL and PA level in the body represents VB6 storage and metabolism status, respectively [73]. Consistent with that, we found both levels were decreased in *sgll* mutants, compared to w^{1118} flies, demonstrating that PNPO deficiency affects anabolism and catabolism of VB6, even with additional dietary VB6. Since there is

no difference in PL or PA between two *sgll* mutants, it is likely that these two B6 vitamers are less sensitive to the severity of PNPO deficiency.

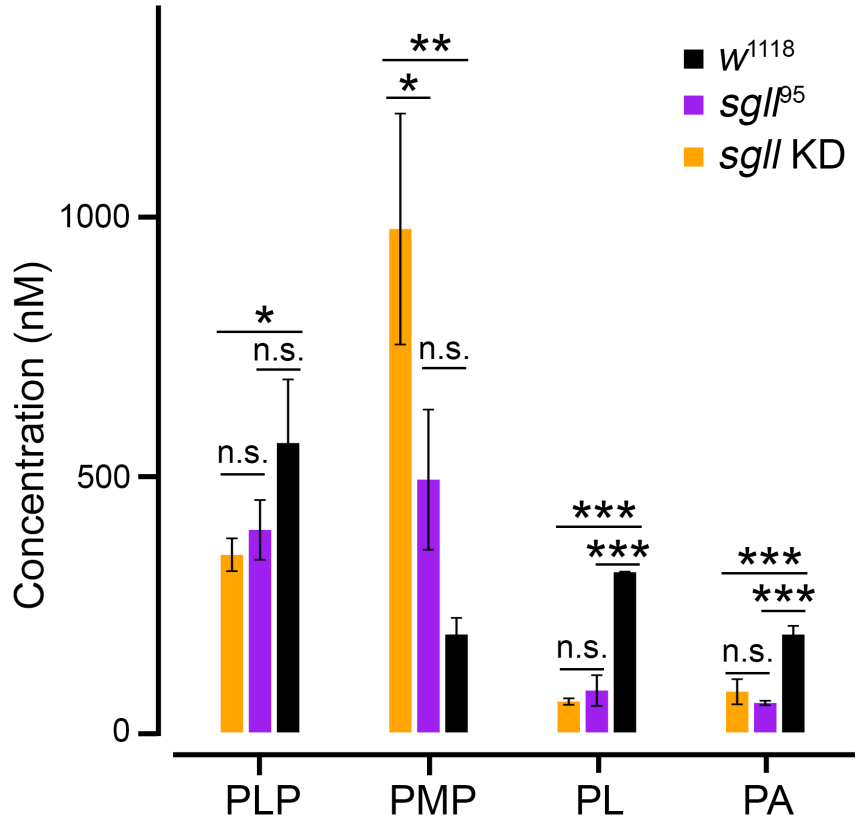

Figure 6.1: **B6 vitamers in WT and *sgll* mutant flies reared on the normal diet.** Six B6 vitamers and PA were measured in whole flies. PN, PNP, and PM were undetectable. Error bar = Mean ± SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s.: $P > 0.05$; One-way ANOVA with Tukey's *post hoc*, n = 3 biological replicates per genotype per condition.

6.2 *sgll*^{95} Flies Have a Shorter Lifespan

The missense mutation in *sgll*^{95} flies reduces the internal PLP level and induces lethality when flies were reared on the sugar-only diet. However, it does not affect the internal PLP level and survival on the normal diet within 10 days when flies were reared on the normal diet.

To study the long-term effect of this mutation, we examined the lifespan of $sgll^{95}$ flies and its controls, w^{1118}. As shown in Figure 6.2, $sgll^{95}$ flies have a dramatically reduced lifespan (median: 39 vs. 102 days for $sgll^{95}$ and w^{1118}, respectively), suggesting that this mutation does have a long-term effect on fitness.

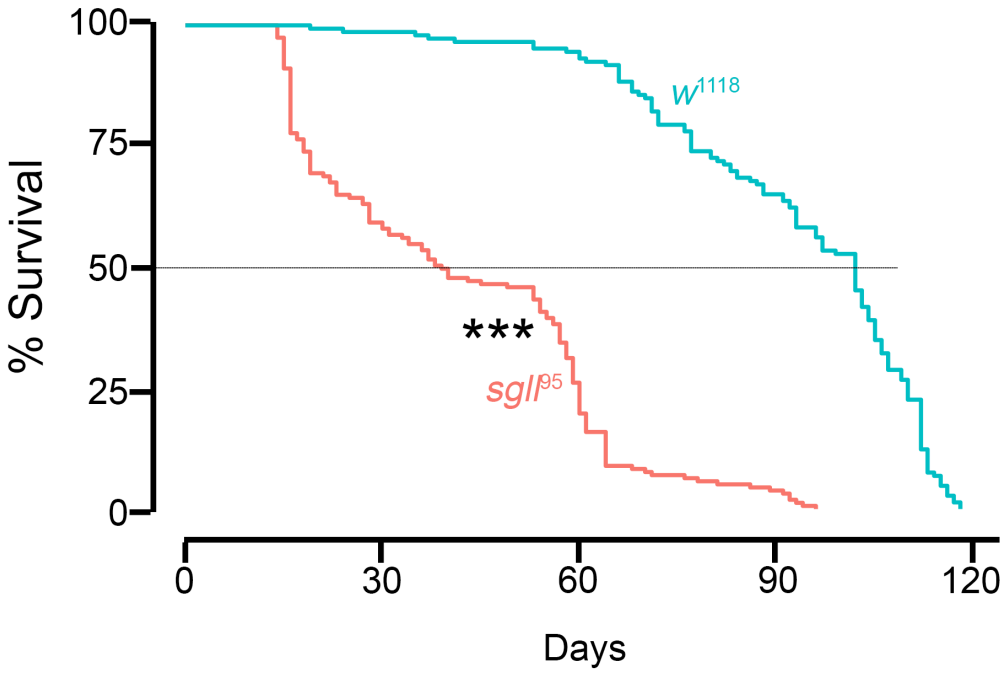

Figure 6.2: ***sgll*^{95} flies have a shorter lifespan.** Homozygous flies from each genotype were tested on the normal diet. *** $P < 0.001$, Log-rank test, n = 147-156.

6.3 Seizures in PNPO deficiency flies resemble that in WT flies caused by GABA blockade

Previous studies show that DLM spiking can be elicited by a number of activities such as flight [121, 168, 169], grooming [64], courtship [170], the giant fiber-mediated escape reflex [64, 171, 63], and electroconvulsive seizures (ECS) [49, 51]. Recently, Lee *et al.* developed a new data analysis method to analyze the DLM spike pattern [53]. In this analysis, they

examined the regularity of spikes using two parameters: instantaneous firing frequency (ISI^{-1}) and instantaneous coefficient of variation (CV_2). The instantaneous firing frequency was calculated from the inter-spike interval (ISI) between successive spikes and it took the value of 1/ISI. The instantaneous coefficient of variation is the variability between adjacent spike intervals and it was calculated as 2 times the result of the absolute value of the difference between two adjacent spike intervals divided by their sum. The factor 2 was included in the calculation so that the instantaneous coefficient of variation will have a mean of 1 for a Poisson process [172]. The spike pattern of each spiking train was then plotted on a ISI^{-1} - CV_2 plot. In the plot, each trajectory has a directionality pointing from the start point of the spike train to the end point of it.

Using this method, Lee *et al.* [53] analyzed the DLM spikes associated with ECS, flight, and grooming and they found that these DLM spikes have distinguishable trajectories, i.e., different spike patterns in the ISI^{-1} - CV_2 plots, suggesting that spike pattern may represent the intrinsic characteristic of DLM firing. Lee and colleagues further studied the spike patterns of the DLM spikes elicited by acute picrotoxin (PTX) treatment. PTX is a non-competitive antagonist of the $GABA_A$ receptor and it is well known that PTX treatment can induce seizures [173]. Interestingly, they found that the seizure spike patterns from these flies evolved from flight-like firing to bursting discharges. The bursting discharge was unique because it was only associated with GABA blockade and not associated with ECS in different genetic mutants or natural behaviors such as glooming or flight.

Given that PLP, the product for PNPO, is involved in the synthesis of GABA, it is likely that GABA dysfunction is implicated in the seizures in the *sgll* mutants. To explore this possibility, we performed the spike-pattern analysis on DLM spike trains recorded from both WT and *sgll* mutant flies reared on either the sugar-only diet or the normal diet. We found that, indeed, *sgll* mutants show similar bursting discharge patterns in our analysis, which were only observed when they were reared on the sugar-only diet (Figure 2.3 C). The similarity suggests a possibility of GABA dysfunction in *sgll* mutants. Other approaches will

be needed to confirm it.

Other than the GABA measurement mentioned in the Conclusions section, GABA treatment is another approach. However, there are two factors that may affect the final results and interpretations: 1) the half-life of GABA is very short; it is about 17 min in mice [174]; 2) it is still controversial whether GABA can cross the blood-brain barrier (BBB) [175]. Therefore, constant GABA treatments and measuring GABA levels in the brain after treatment should be included in the experimental design.

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