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## Molecular Defects of Vitamin B<sub>6</sub> Metabolism Associated with Neonatal Epileptic Encephalopathy

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### 1. Introduction

Neonatal epileptic encephalopathy (NEE) is a seizure disorder that occurs within hours from birth and arises from central nervous system (CNS) dysfunctions of various origins, including metabolic or inflammatory conditions, abnormalities of brain structure and cerebrovascular diseases. In some rare circumstances, NEE is refractory to conventional antiepileptic drugs (AEDs) but responds very well to treatment with vitamin B<sub>6</sub> in the form of either pyridoxine (PN) or pyridoxal 5'-phosphate (PLP) [1-7]. Vitamin B<sub>6</sub> exists in all organisms in the form of six vitamers but only PLP is the biologically active form acting as a cofactor for many enzymes (**Figure 1.**).

Vitamin B<sub>6</sub>-dependent NEE derives from a deficiency of cellular PLP; either due to inborn errors in enzymes, such as pyridoxine 5'-phosphate oxidase (PNPOx) and pyridoxal kinase (PL kinase), involved in the PLP salvage pathway or from inherited mutations in other enzymes, such as  $\alpha$ -aminoacidic semialdehyde dehydrogenase (also known as antiquitin), involved in other metabolic pathways, which lead to the accumulation of intermediates that react with PLP, reducing its availability.

Clinical phenotypes observed in vitamin B<sub>6</sub>-dependent NEE patients may include fetal distress, hypoglycemia, acidosis, anemia, and asphyxia [2,5-7]. The health state of untreated patients may undergo progressive deterioration, which can lead to death within weeks. Surviving children are usually mentally retarded and are dependent on vitamin B<sub>6</sub> to

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control the disease. Several known cases of B<sub>6</sub>-dependent NEE, however do not or only mildly manifest some of the above clinical features, and are characterized by mild to moderate developmental delay.

This chapter will review the molecular mechanisms of how in-born errors in PNPOx or antiquitin affect PLP levels in the cell and lead to NEE. We will also review important clinical and general features associated with PLP dependent NEE, and provide some directions for clinicians to diagnose and treat or manage the disease.

## 2. Vitamin B<sub>6</sub>

There are six B<sub>6</sub> vitamers, including the primary forms pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM), and their phosphorylated derivatives PLP, pyridoxine 5'-phosphate (PNP) and pyridoxamine 5'-phosphate (PMP) (**Figure 1**). Pyridoxal 5'-phosphate, the active form of vitamin B<sub>6</sub> serves as a cofactor for over 160 vitamin B<sub>6</sub> dependent enzymes (or PLP-dependent enzymes) involved in amino acid metabolism (e.g. degradation of threonine and glycine), neurotransmitter biosynthesis, biologically active amine biosynthesis, lipid metabolism, carbohydrate breakdown, heme synthesis, nucleic acid synthesis and several other metabolic pathways [8,9].

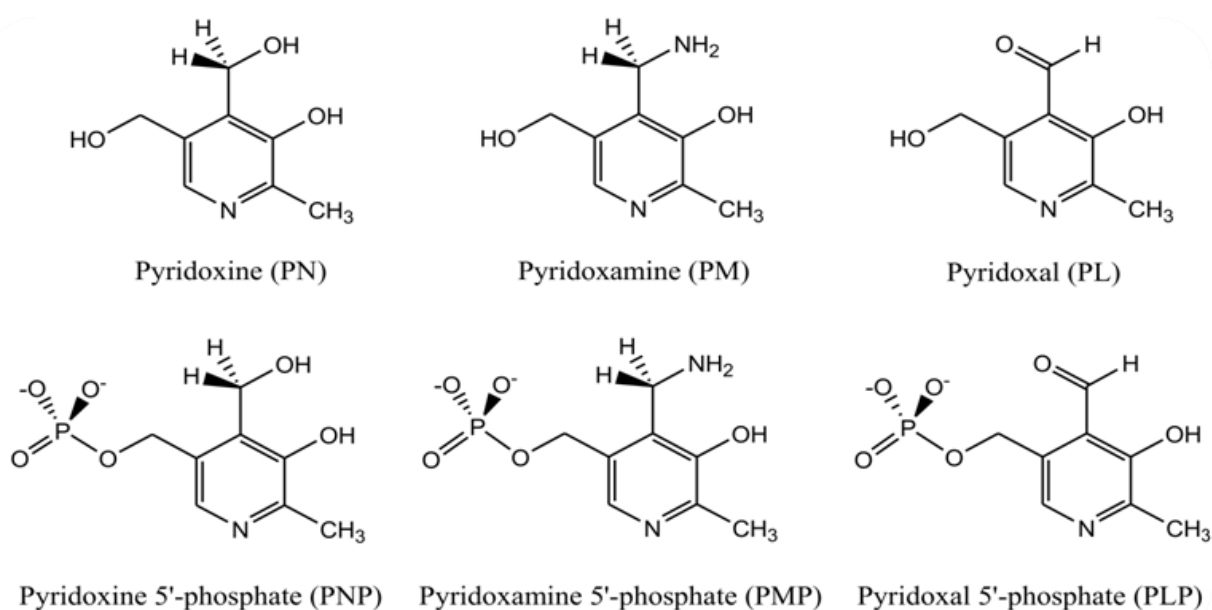


Fig. 1. Structures of B<sub>6</sub> vitamers

### 2.1 Vitamin B<sub>6</sub> metabolism

Unlike microorganisms and plants, animals cannot synthesize PLP from small metabolites by a *de novo* pathway and require the dietary B<sub>6</sub> vitamers, pyridoxal, pyridoxine, or pyridoxamine (**Figure 1**), for the synthesis of PLP via a so-called salvage pathway [10]. Enzymes which are critical for this salvage pathway are PL kinase, PNPOx and several different phosphatases (**Figure 2**).

PL kinase catalyzes the phosphorylation of the 5'-alcohol group of PN, PM, and PL to form PNP, PMP, and PLP, respectively (**Figures 1 and 2**). Both PNP and PMP are subsequently converted to PLP in an FMN-dependent oxidative process catalyzed by PNPOx (**Figures 2 and 3**). PLP adds to newly synthesized apo-B<sub>6</sub> enzymes converting them to the catalytically active holo-B<sub>6</sub> enzymes. Typically, PLP is then released during protein turnover of B<sub>6</sub> enzymes and is converted to PL by cellular phosphatases, followed by re-phosphorylation to PLP by PL kinase [10,11].

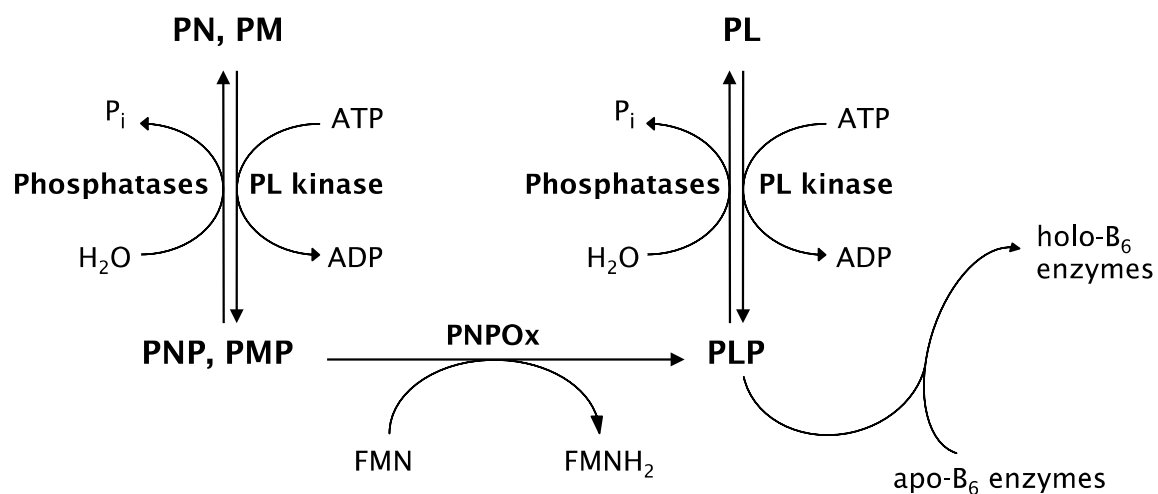


Fig. 2. The PLP salvage pathway

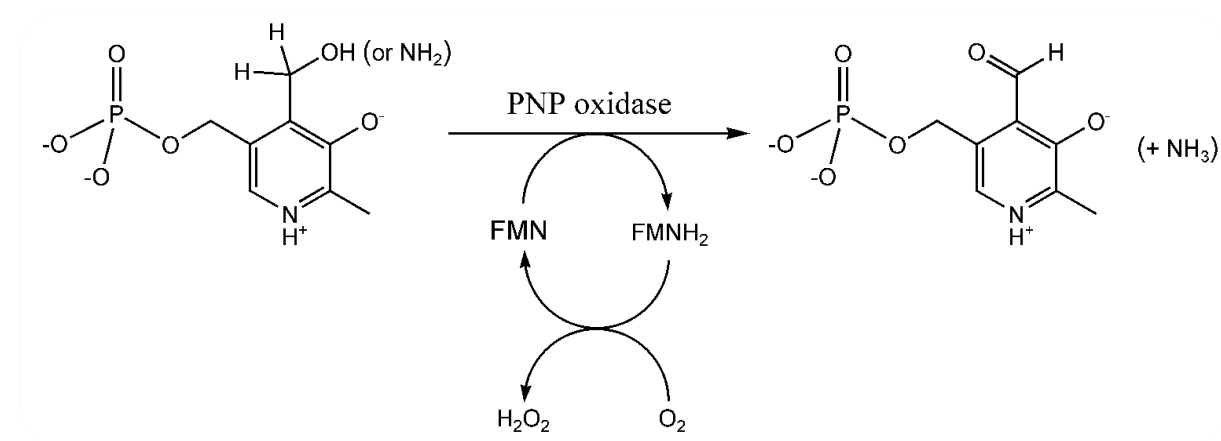


Fig. 3. Conversion of PNP (or PMP) to PLP by PNPOx

Mammals acquire B<sub>6</sub> vitamins via intestinal absorption (**Figure 4**) and convert them into PLP through the salvage pathway (**Figure 2**). B<sub>6</sub> vitamins are absorbed from food and from the microflora, which normally colonizes the large intestine. In animal-derived foods, vitamin B<sub>6</sub> is mainly present as PLP, associated to glycogen phosphorylase, and in smaller amounts as PMP, while in plants it is present as PN and PN-5'-beta-D-glucoside [12]. PN-5'-beta-D-glucoside appears to have reduced availability because of the need for hydrolysis by an intestinal glucosidase [13]. In human breast milk, vitamin B<sub>6</sub> is present as PLP and PL [14].

The form of vitamin B<sub>6</sub> that is usually employed to fortify foods is pyridoxine hydrochloride, since this is the most commercially convenient form.

Once ingested (**Figure 4**), PLP, PNP and PMP are dephosphorylated by the ecto-enzyme tissue-specific intestinal phosphatase, prior to absorption from the upper small intestine by a carrier-mediated system [15]. The portal circulation delivers PM, PN and PL to the liver, where they are phosphorylated by PL kinase. PNP and PMP are then both oxidized to PLP by PNPOx. PLP must return to the blood stream in order to reach all tissues. Albumin-bound PLP represents about 60% of circulating vitamin B<sub>6</sub>, with PN, PM and PL making up the remaining part [16]. While dephosphorylated B<sub>6</sub> vitamers can enter the cells and pass the blood-brain barrier, PLP needs to be dephosphorylated again. Tissue-specific phosphatases, expressed in placenta and germ cells [17,18], and the tissue-nonspecific alkaline phosphatase (TNAP) are membrane-associated enzymes responsible for this step. PL is the vitamer form that is mainly acquired by neurons; once formed by TNAP at the choroid plexus, PL is transported into the cerebrospinal fluid by an active transport mechanism and crosses the neuron plasma membranes to be re-phosphorylated in the cells [16]. In the cytoplasm, PL, PN and PM are converted into the 5'-phosphorylated vitamers by PL kinase, while PNPOx converts PNP and PMP into PLP.

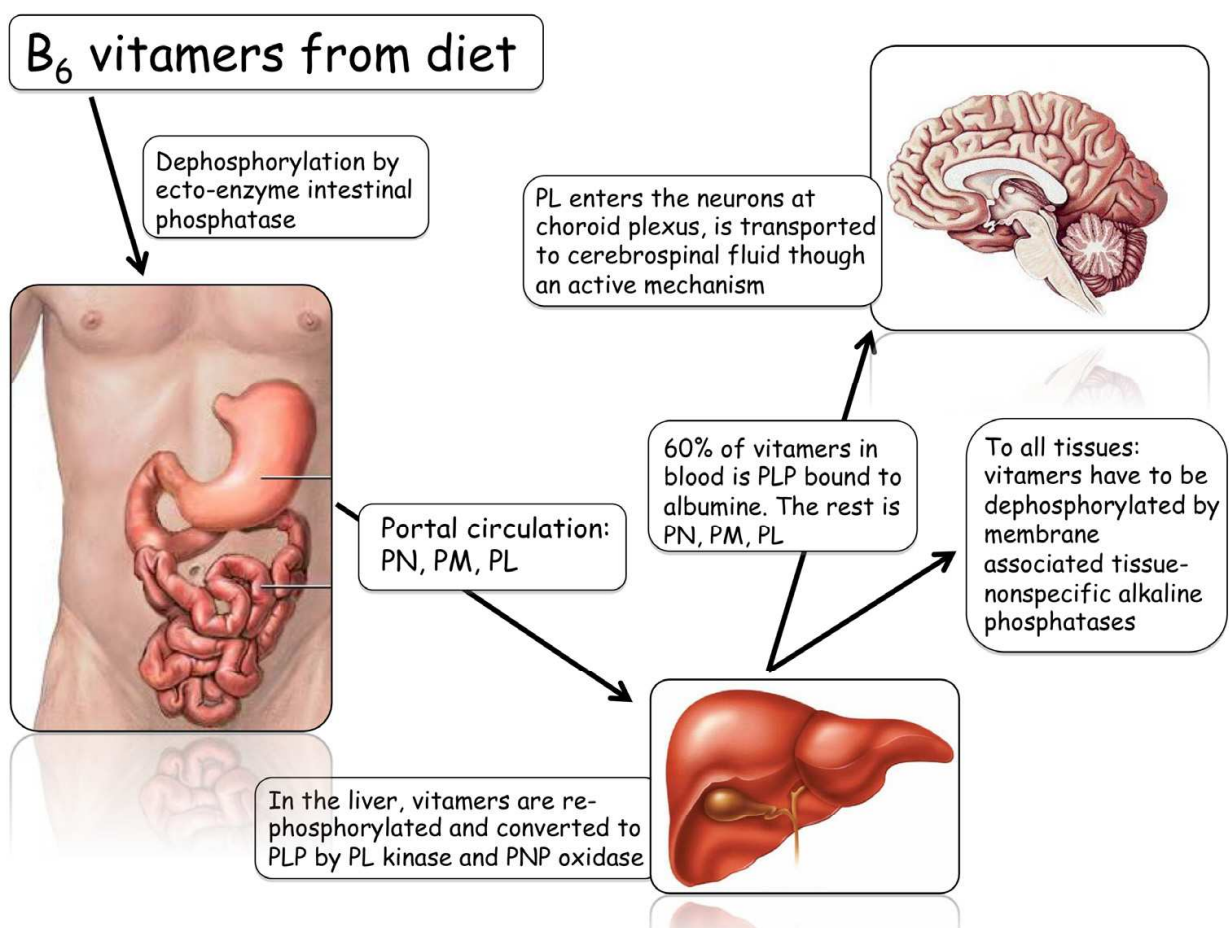


Fig. 4. Vitamin B<sub>6</sub> transport in human organs.

The expression of mRNA of all enzymes of the salvage pathway is ubiquitous, but is highly regulated at the level of transcription in a tissue-specific manner. While PL kinase is expressed in all tissues, although with the presence of isoforms [19], the major sites of PNPOx expression are liver, skeletal muscle and kidneys; PNPOx is also present, to a lesser extent, in the brain, in particular in the cerebral cortex [20]. Remarkably, PNPOx activity seems to be absent in liver and in neurally derived tumors [21,22]. It should be emphasized that the pool of free PLP *in vivo* must be maintained at a very low level in the body, to prevent toxic buildup. In fact, PLP being a very reactive aldehyde, easily combines with amines and thiols in the cell [23]. This characteristic has been related to the neurotoxic effect of excess consumption of vitamin B<sub>6</sub> [24], even at a dosage of 200 mg/day. The current recommended dietary allowance for B<sub>6</sub> is 1-2 mg/day for adult individuals. People taking high doses of B<sub>6</sub> are known to develop acute, profound and permanent sensory neuropathy, and in some instances motor neuropathy and damage of the central nervous system [25]. High doses of PLP are also found to induce convulsion in animal studies; suggested to be caused by formation of a Schiff base between PLP and a crucial lysine residue located in an extracellular loop of the GABA receptor [26]. PLP has also been reported to inhibit phenol sulfotransferase, an important detoxification enzyme [27].

One mechanism involved in PLP homeostasis is the regulation of its rate of production by PL kinase and PNPOx. Interestingly, it has been shown that both enzymes are able to bind their reaction product, PLP, and that their activity is finely regulated by it. Inhibition of PNPOx activity by the PLP product has been observed in the *E. coli* enzyme, with a  $K_i$  of 8  $\mu\text{M}$  [28]. Also, substrate inhibition of *E. coli* PL kinase by MgATP has been observed in the presence of either PNP or PLP [29]. A concurring mechanism for maintaining low levels of free PLP is expected to be its dephosphorylation by phosphatases. Catalytic conversion of pyridoxal to 4-pyridoxic acid by aldehyde oxidases and NAD-dependent dehydrogenases also keeps the level of PLP low [30]. These regulatory mechanisms keep the concentration of free PLP as low as 1  $\mu\text{M}$  in eukaryotic cells [31].

The low free PLP concentration in the cell is not believed to be sufficient to allow for spontaneous formation of holo-B<sub>6</sub> enzymes [31,32] and raises the intriguing question of how PLP is actually delivered to the apoenzymes that use it as a cofactor. The importance of vitamin B<sub>6</sub> in several cellular processes and in the onset of different pathologies should also be looked at from this new perspective. Studies on PNPOx and PL kinase suggest that once PLP is formed by these enzymes, it is unlikely to be released into solution, where it could be sequestered and/or dephosphorylated by phosphatases, and that it could be rather directly channeled to apo-B<sub>6</sub> enzymes [32-34]. Failure to maintain a correct tuning between PLP biosynthesis and degradation, transport to peripheral tissues and delivery to newly formed B<sub>6</sub> enzymes might end up in vitamin B<sub>6</sub>-associated pathogenesis. The salvage pathway is very efficient, resulting in only small amounts of vitamin B<sub>6</sub> being required in the diet. Vitamin B<sub>6</sub> deficiency is rare in humans receiving a normal diet.

## 2.2 PLP-dependent enzymes and brain function

The role of PLP-dependent enzymes in brain function is vital since the synthesis of several neurotransmitters, including D-serine, D-aspartate, L-glutamate, glycine,  $\gamma$ -aminobutyric



acid (GABA), serotonin, epinephrine, norepinephrine, histamine and dopamine is dependent on these enzymes. For example, branched-chain amino acid aminotransferase (BCAT), a PLP-dependent enzyme, catalyzes the transamination of essential branched-chain amino acids, such as leucine and valine, to form L-glutamate. L-Glutamate is considered to be one of the major excitatory signals in the mammalian central nervous system and is important for cognition, learning and memory. Low or abnormal levels have been implicated in Alzheimer's, Parkinson's, Huntington's, and Tourette's diseases [35-39]. L-Glutamate also serves as a precursor for the formation of GABA, catalyzed by the PLP-dependent enzyme glutamate decarboxylase (GAD). GABA is a major inhibitory neurotransmitter in the mammalian CNS. Low levels of GABA have been implicated in symptoms associated with epilepsy, convulsions, Parkinson's, Alzheimer's and Huntington's diseases [40-43]. The PLP-dependent enzyme DOPA decarboxylase (also known as aromatic L-amino acid decarboxylase (AAAD)) catalyzes the synthesis of dopamine via decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA). Dopamine serves as a precursor for norepinephrine and epinephrine. AAAD also catalyzes the synthesis of serotonin via decarboxylation of L-5-hydroxytryptophan. Low levels of dopamine and serotonin have been involved in clinical disorders, including Parkinson's disease and Attention Deficit Hyperactivity Disorder (ADHD) [44,45]. The PLP-dependent enzyme serine racemase catalyzes the direct racemization of L-serine to D-serine. Endogenous D-serine is required for physiological NMDA (N-methyl D-aspartate) neurotransmission, neuronal plasticity, memory and learning [46]. Histidine decarboxylase (HDC), another PLP-dependent enzyme, is responsible for the catalytic conversion of L-histidine to histamine. Histamine plays important roles in a number of physiological processes, among them as a neurotransmitter involved in memory [47,48]. Glycine is formed from L-serine by the PLP-dependent serine hydroxymethyltransferase and is degraded by the glycine cleavage system, of which one of the four protein components is glycine decarboxylase, another PLP-dependent enzyme. Glycine is also a neurotransmitter, acting as both inhibitory and excitatory functions within the CNS [49]. PLP-dependent enzymes also take part in the kynurenine oxidative pathway of tryptophan degradation, whose catabolic intermediates (kynurenic acid, 3-hydroxykynurenine and quinolinic acid) are involved in the physiological tuning of the CNS and in the etiogenesis and progression of several human neurodegenerative disorders [50,51].

It is obvious that inadequate PLP levels will result in loss of activity of B<sub>6</sub> enzymes, leading to a significant decrease of some or several neurotransmitters. As mentioned above, PLP, as well as the other phosphorylated forms of vitamin B<sub>6</sub>, do not easily cross the blood-brain barrier; therefore PLP must be synthesized within the brain tissues from the non-phosphorylated vitamers. Thus, the potential for PLP depletion in the brain is likely to occur with inactivation of the B<sub>6</sub> salvage pathway enzymes [31]. Moreover, because apo-B<sub>6</sub> enzymes in the brain compete for the available PLP, even a modest decrease in PLP availability could have more dire consequences on brain function than would be expected. Those apo-B<sub>6</sub> enzymes that bind PLP with higher affinity may get most of the available PLP. As an example, DOPA decarboxylase is known to be drastically affected by PLP deficiency [52]. Consistently, inactivation of DOPA decarboxylase has been used in the past as a model for determining B<sub>6</sub> status.

### 2.3 Pyridoxine 5'-phosphate oxidase (PNPOx)

PNPOx is a flavin mononucleotide (FMN)-dependent enzyme, catalyzing the terminal step in the *de novo* vitamin B<sub>6</sub> biosynthesis pathway in *E. coli*. The enzyme is also part of the salvage pathway in *E. coli* and mammalian cells [10,11,28,53-61]. FMN is a phosphorylated form of vitamin B<sub>2</sub> (riboflavin). The catalytic mechanism of PNPOx involves direct hydride transfer from the 4' hydroxyl group of PNP or the 4' amino group of PMP to FMN to form PLP and FMNH<sub>2</sub>, respectively (**Figure 3**). Oxidized FMN is then regenerated by the transfer of two electrons to oxygen, forming hydrogen peroxide [57]. The enzyme is sluggish with a low catalytic rate constant ranging from 0.2 sec<sup>-1</sup> to 0.8 sec<sup>-1</sup> and K<sub>m</sub> values in the low micromolar range for both the substrates PNP and PMP [28,55,56]. The product PLP has a greater affinity than either of the substrates, suggesting that it may function as a product inhibitor of the enzyme [28,58,59].

The crystal structure of the human enzyme, as well as those from other species have been determined, all of which show a similar two-fold related dimer with two FMN molecules bound at the dimer interface making non-covalent interactions with both subunits (**Figure 5**) [55-57,60,61]. Mutations of key amino acid residues can affect folding of the enzyme into a form with diminished activity or no activity, can strongly influence the binding affinity of substrates, can reduce the affinity for the cofactor FMN and can affect catalytic activity. Knowing the residues involved in substrate and cofactor binding and in the catalytic mechanism is of paramount importance for understanding the function of PNPOx.

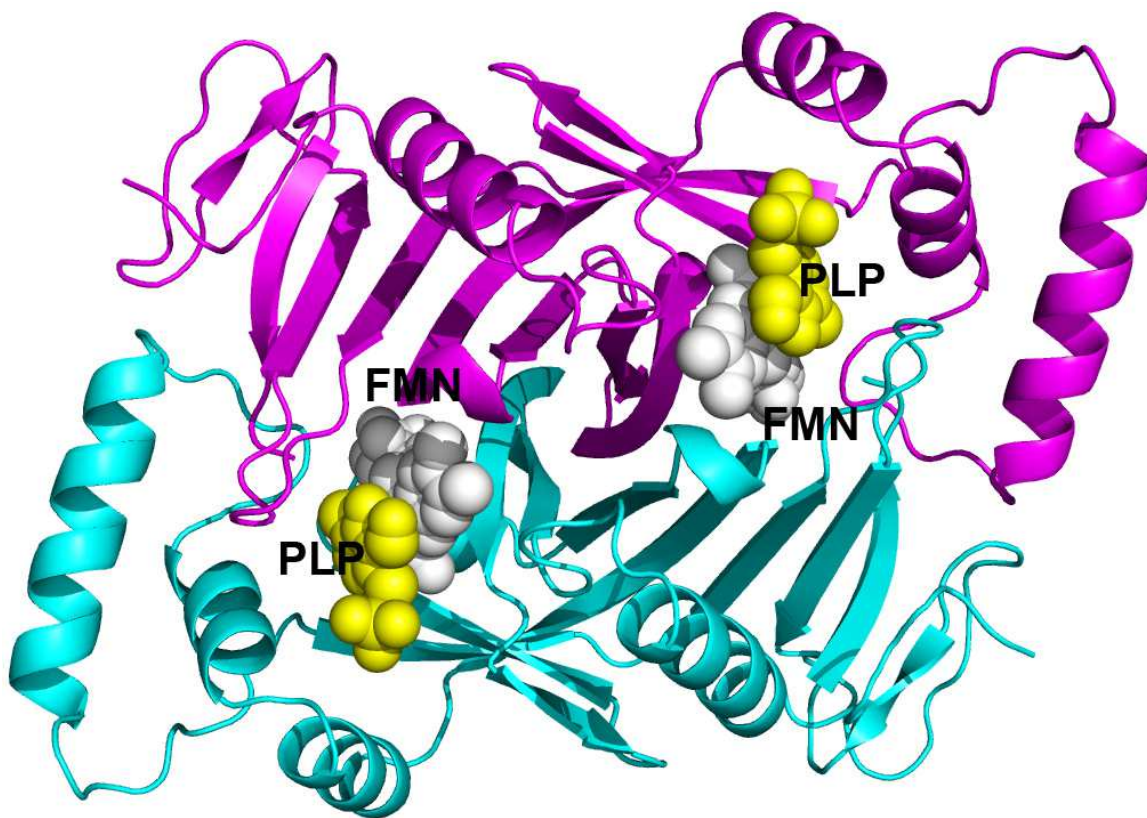


Fig. 5. Three-dimensional structure of human PNPOx complexed with the cofactor (FMN, in grey) and the product (PLP, in yellow). The two monomers forming the homodimeric structure of the protein are colored magenta and cyan.



In the structure of human PNPOx, the isoalloxazine ring of FMN is involved in both hydrophobic and hydrogen-bond interactions with both subunits (monomers) of the protein, including Thr111, Glu174, Phe110, Leu98 and Met96 (Figure 6) [56]. The FMN ribityl group makes hydrogen-bond interactions with Met96, Gln139, Trp219, Pro261, Trp219, Lys117, Glu217, Gln174, Arg229, Arg138 and Arg141. The FMN phosphate moiety, which is located deeper into the protein structure, makes hydrogen-bond/salt-bridge interactions to Arg95, Arg141, Arg161, Ser175, Arg116, Lys117 and Arg229. The substrate PNP, as well as the product PLP, binds at the *re*-face of the bound FMN, where the PLP pyridine ring stacks parallel against the pyrazine and pyrimidine portions of the FMN isoalloxazine ring with extensive van der Waals contacts between the two, with the C4' of PLP and N5 of FMN separated by about 3.4 Å (Figure 6). The phosphate moiety of PLP, which extends toward the surface of the binding pocket, makes salt bridges and hydrogen bond interactions to Lys100, Arg161, Arg225, Tyr157 and Ser165. The O3'-hydroxyl group of PLP makes a hydrogen bond interaction to His227, while the carbonyl oxygen on C4' makes a water-mediated interaction to Glu77. Lastly, the pyridine nitrogen makes a water-mediated hydrogen bond interaction with Trp206. Not only do the interactions described above help stabilize FMN and substrate binding, but they also ensure correct orientation of FMN and PNP for substrate oxidation. Additionally, the interactions with both FMN and the substrate/product, which are at the interface of the two subunits, help stabilize the homodimeric structure.

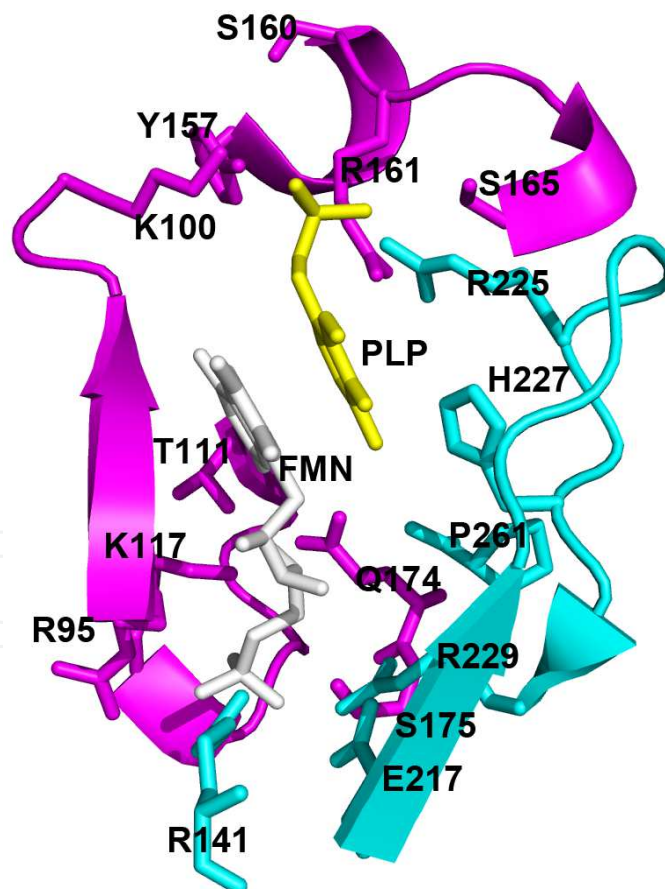


Fig. 6. The active site of human PNPOx, showing the bound PLP (yellow) at the *re*-face of the FMN (grey). The two monomers, forming the homodimeric structure of the protein, are colored magenta and cyan, respectively. For clarity, not all the residues interacting with the ligands are shown.

### 3. PLP deficiency and NEE

PLP deficiency due to dietary insufficiency is rare, since most dietary sources contain vitamin B<sub>6</sub>. Nonetheless, pathogenic mutations in the PNPO gene encoding for PNPOx are known to cause a reduced or null activity of PNPOx, resulting in PLP deficiency. Disruption of the salvage pathway can also occur by pathogenic mutations in the PL kinase gene or by drug induced inhibition of PNPOx and PL kinase enzymatic activity [5,6,62-67]. Metabolites that accumulate as a consequence of inherited defects in enzymes of other metabolic pathways and drugs are also known to form complexes with PLP and reduce its availability [1,67-70]. Since PLP must be metabolized by brain cells to meet the demand of newly synthesized apo-B<sub>6</sub> enzymes disruption of the PLP salvage pathway could lead to improper functioning of PLP-dependent enzymes in the brain, resulting in neurological disorders, such as NEE.

#### 3.1 PNPOx deficiency-related NEE

The most severe form of NEE has been found to be due to mutations in PNPOx, which lead to null or decreased activity of the enzyme. Observed mainly in Turkish and Asian populations, this syndrome, called PNPOx deficiency, shows symptoms such as fetal distress, hypoglycemia, anemia, acidosis and asphyxia [2,5-7,71]. This disorder is sometimes referred to as PLP-dependent NEE as individuals with PNPO deficiency respond only to PLP or PL therapy, and not to the other forms of vitamin B<sub>6</sub>, such as PN and PM. At least seven mutations in the PNPO gene, including homozygous missense, stop codon, nonsense, splice site and frameshift mutations have been identified to cause PLP-dependent NEE [5,6,62-67]. Most patients with these mutations are born prematurely and the disease can be fatal. Surviving children are usually mentally retarded and show an abnormal dependence on PLP/PL. The fact that these patients are unresponsive to treatment with PN but responsive to PL demonstrates that the defect is in PNPOx, which is required for PN conversion to PLP. Conversion of PL to PLP is achieved by PL kinase and does not require PNPOx activity (**Figure 2**). However, PLP from the diet has to be converted to PL by phosphatases, and is then reconverted to PLP in the cells by PL kinase.

#### 3.2 Pathogenic mutations in PNPOx

The human PNPOx gene is situated on chromosome 17q21.2 [72], and several mutations in the gene have been implicated in NEE. In a study by Mills *et al.*, the authors sequenced the PNPOx gene in patients with NEE and identified homozygous missense (R229W, in which the arginine residue in position 229 is substituted with a tryptophan residue), splice site (IVS3-1g > a) and stop codon (X262Q) mutations [5]. The stop codon and splice site mutations result in null PNPOx activity. The missense mutation (in genetics, defined as a point mutation in which a single nucleotide is changed, resulting in a codon that codes for a different amino acid) was reported to lead to ~70% reduction in PNPOx activity compared to the wild-type enzyme. Although significant, a 70% reduction in activity does not explain the observed drastic pathological effect in patients [5]. A later study showed the activity of the mutant enzyme to be almost null [66].

Other PNPOx, including two novel homozygous missense mutations (R95H and R95C, in which arginine residue 95 is substituted by either a histidine or a cysteine) and a nonsense

mutation (A174X; a nonsense mutation is a point mutation in a sequence of DNA that results in a premature stop codon, or a nonsense codon in the transcribed mRNA, and in a truncated, incomplete, and usually nonfunctional protein product) have also been identified in several patients with classical symptoms of PLP-dependent NEE [5,6,62-67]. As will be discussed later, the elucidation of the crystal structure of PNPOx and the mechanism of PNP or PMP oxidation [57] have now contributed to the precise mechanism governing how some of these mutations lead to partial or complete loss of enzymatic function.

### 3.3 Molecular basis of reduced enzymatic activity of PNPOx R229W and R95C mutants

Our research group has carried out studies using site-directed mutagenesis, enzyme kinetics, X-ray crystallography and other functional studies aimed at understanding on a molecular level why PNPOx R229W and R95C mutants lead to reduced oxidase activity with the concomitant onset of NEE. The results from the R229W mutant have been published quite recently [66]. Arg229 is strictly conserved, and in the wild-type enzyme, this residue makes a short hydrogen-bond interaction with the FMN phosphate moiety (**Figures 6 and 7**). Arg229 also makes both intra- and inter-subunit hydrogen-bond interactions with Arg141, Ser175, Glu217 and Ala260. Another interesting observation is that the side chain of Arg229 is sandwiched tightly between the residues Arg141, Glu217, Ala260, Trp219, Gln139, and Pro261.

The R229W variant shows ~850-fold less catalytic efficiency than the wild-type enzyme due to a ~192-fold decrease in PNP affinity and ~4.5-fold decrease in the catalytic rate constant  $k_{cat}$ . This shows that the *in vitro* activity of the R229 mutant enzyme is less than 0.2% of the wild-type enzyme, effectively making the activity so low that it will not function in the production of PLP from PNP. Fluorescence titration experiments also indicate an ~50-fold reduction in FMN affinity for the R229W mutant. Normally, wild-type PNPOx does not require the additional FMN to the assay because of its very tight binding. However, the mutant PNPOx loses even more activity unless FMN is added to the assay. Crystallographic studies showed that the substitution of the charged Arg229 residue with the larger tryptophan residue at the FMN binding site (**Figure 7**) has led to loss of hydrogen-bond and salt-bridge interactions with FMN. Additionally, the substitution with a bigger amino side chain has led to an alteration of the active site structure of PNPOx, resulting in loss of two critical hydrogen-bond interactions between PNP and the residues His227 and Arg225 (**Figure 7**). These two hydrogen-bond interactions have previously been shown to be important for substrate binding and proper orientation for catalysis by allowing the PNP ring to orient parallel to the FMN ring [57]. Expectedly, in the ternary three-dimensional structure of the mutant enzyme, the PLP and FMN ring moieties have moved significantly apart and are no longer parallel to each other (**Figure 7**) [66]. The C4' of the substrate and N5 of FMN are now separated by ~4.5 Å compared to the ~3.4 Å catalytic optimal position observed in the wild-type enzyme. In effect, the inability of Arg225 and His227 to make contact with the substrate has led to the destabilization of the substrate, preventing its proper orientation relative to FMN for efficient hydride transfer.

In yet another study by our group (unpublished data) with the PNPOx R95C mutant, we have also observed a 4.8 fold decrease in  $k_{cat}$  compared to the wild-type enzyme, and a ~70-fold increase in  $K_m$  relative to the substrate PNP resulting in an ~341-fold decrease in the catalytic efficiency of the mutant enzyme compared to the wild-type enzyme. The FMN

affinity is also reduced by ~15% compared to the wild-type enzyme. Arg95 forms salt bridge/hydrogen bond interactions with the phosphate side chain of the FMN molecule utilizing two of the guanidinium group nitrogens (**Figure 6**), and the diminished binding of FMN to the enzyme may be a result of the loss of this interaction. In the absence of a crystal structure, we can only speculate that this mutation also leads to loss of several important interactions between the protein and PNP as evidenced by a decrease in PNP affinity, resulting in improper positioning of the substrate for optimal catalysis.

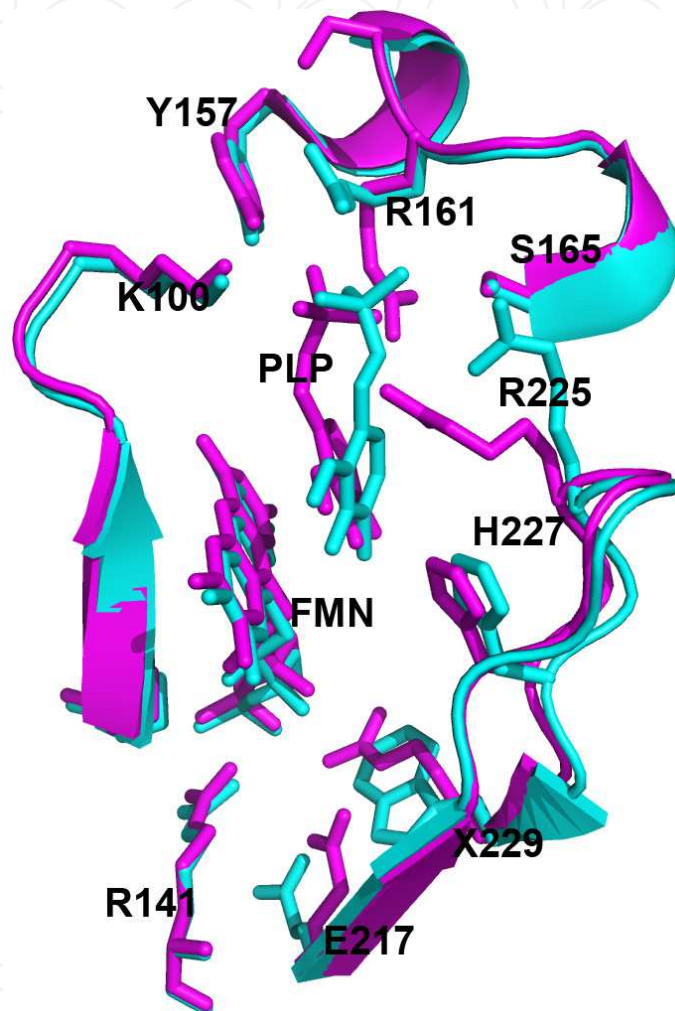


Fig. 7. Superposition of the active site of R229W PNPOx mutant (cyan) with that of wild-type PNPOx (magenta). Note the significant movements of the residues His227 and Arg225 in the R229W mutant form, compared to the wild-type, resulting in the ligand PLP moving away from its optimal catalytic position.

Neither of these mutations seems to have any significant effect on the secondary structure or thermal stability of the enzyme, even though affinity of the enzyme towards its cofactor FMN is diminished. These results provide molecular insight into how the R229W and R95C missense mutations affect PNPOx catalytic activity, and clearly explain why the treatment of NEE with PN is unsuccessful in patients with PNPOx mutants because of the inability of the enzyme to oxidize PNP (or PMP) to form PLP. The current treatment with PLP or PL is appropriate since the other enzyme involved in PLP metabolism, PL kinase, can convert PL



to PLP. Nevertheless, it's clear that PLP made in the cell from PL is not enough, indicating that PL kinase alone and most likely PNPOx alone is not sufficient to meet the cell's requirements and both the salvage pathway enzymes are necessary to provide adequate PLP to activate the many different apo-B<sub>6</sub> dependent enzymes. It is possible, although yet to be proven, that the use of riboflavin (vitamin B<sub>2</sub>) and PN, in conjunction with PL, may offer some improvement over the current treatment protocol of PL or PLP alone due to the apparent loss of FMN from the PNPOx pathogenic variants.

### 3.4 Predicting the molecular basis of reduced enzymatic activity of other PNPOx pathogenic mutants

The precise mechanisms governing how the splice site (IVS3-1g > a), stop codon (X262Q), nonsense (A174X), and R95H missense mutations lead to partial or complete loss of PNPOx function are not known. Nevertheless, based on the crystal structure of PNPOx and the studies with the R95C and R229W mutants, several inferences can be made. In particular, it's easy to predict how the mutant R95H may also affect the enzymatic activity based on the study with R95C. Although His95 may still be capable of forming hydrogen-bond interactions with the FMN phosphate, most likely the interaction would be weaker. Unlike the wild-type Arg, which makes a bidentate interaction with the FMN phosphate moiety, His can only utilize one of its nitrogens for interacting with the phosphate moiety. This mutation could also result in a decreased binding of PNP, as observed in the R229W and R95C mutants.

In the splice site (IVS3-1g > a) mutation, the residues 122-139 are absent from the enzyme. Some of these residues are involved in the formation of the FMN binding cavity and interact with the cofactor. Others contribute to either a hydrophobic patch or a network of inter-dimer hydrogen-bond interactions that stabilize the tertiary and quaternary structure of the wild-type protein. Notable among these residues are Asn124, Ser128, Tyr132, Glu134, Glu139, Asn137, Trp133, Phe131 and Leu129. Therefore, these amino acids are expected to be important for a proper tertiary fold, dimer stabilization, and binding of the cofactor for consequent catalysis. It is perhaps no surprise that the splice site mutation leads to total enzyme dysfunction.

The abolition of the stop codon (X262Q) causes a C-terminal 28 amino acid extension (residues 262-289). The last residue at the C-terminus of the wild-type protein is the proline 261, and is strictly conserved in all PNP oxidases. Pro261 is tucked at the dimer interface, and is located at the *re*-face of the FMN and directly underneath PNP (**Figure 6**). It's likely that the C-terminus extension would result in at least the first five extended residues (Gln262, Leu263, Trp264, Asp265, Leu266) being accommodated at the dimer interface and more significantly occupy the substrate/product binding site permanently to inhibit substrate binding for catalysis. The extra 23 residues that extend from the active site may fold outside the dimer interface. On the other hand, if the 28-amino acid peptide extension does not fold as suggested above, it might disturb the dimer interface and result in the dissociation of the subunits into non-functional monomers. This mutation also results in a PNPOx with null activity.

### 3.5 Clinical and general biochemical features of patients with PNPOx deficiency-related NEE and management of the disorder

There are several clinical features that manifest in patients with PNPOx deficiency-related NEE or PLP-dependent NEE. These include, but are not limited to, fetal distress, convulsion,



hypoglycemia, lactic acidosis, intractable seizures, hypoglycemia, acidosis, anemia, increased blood lactate, electroencephalogram with burst suppression pattern, and asphyxia [5,6,62-67]. Due to the severe deficiency of PLP, people with this disorder show reduced activity of several PLP-dependent enzymes, e.g. aromatic L-amino acid decarboxylase, ornithine aminotransferase, serine hydroxymethyltransferase, etc. As a result, cerebrospinal fluid (CSF), plasma and urine of these patients show deficient flux through PLP-dependent pathways [5,72], including lowered levels of homovanillic acid, 5-hydroxyindoleacetic acid and arginine; raised levels of 3-methoxytyrosine, L-DOPA, vanillacetate, glycine, threonine, histidine, ornithine and taurine. Most children with this disorder are born premature, progressively deteriorate and die within weeks if untreated. Surviving children are usually mentally retarded, and non-responsive to AEDs or pyridoxine, but dependent on a daily consumption of PLP to control the disease. This underscores the importance of promptly and properly diagnosing PNPOx deficiency-related NEE for correct pharmacological intervention.

In a study by Wang *et al.* [73], the investigators gave 94 patients who were on AEDs, but still had intractable epilepsy, intravenous PLP, that was continued for another 3 days if seizures were controlled. Eleven of the patients responded to the intravenous PLP with complete control of the seizures. PLP was then replaced with oral pyridoxine in these patients and six relapsed. For patients who had a seizure relapse, the intravenous PLP was again administered followed by a daily oral PLP. AEDs were sequentially withdrawn and the patients remained on oral PLP. Three of the patients relapsed while three remained seizure free with withdrawal of the AEDs. Clinicians can use a similar protocol to diagnose PNPOx deficiency-related NEE. It's also very important that after initial diagnosis of PLP dependency, there should be a follow-up to further confirm the diagnosis, i.e. whether the disorder is PNPOx deficiency related by carrying out a metabolic investigation, including screening the CSF for threonine and glycine levels, urine for vanillacetic acid levels and the CSF for neurotransmitters. Diagnosis should also be confirmed by mutational analysis of PNPOx genes.

Hoffmann *et al.*, have suggested an oral PLP dose of 30 mg/kg per day in three doses plus 100 mg i.v. pyridoxine (in a single dose), plus 3-5 mg/kg folinic acid for treating PNPOx deficiency-related NEE [6]. The investigators found no therapeutic response with pyridoxine and folinic acid when used without PLP (the latter compound discussed in some detail later). In some instances, the above recommended PLP dose may have to be increased to effectively treat the disease [6]. As noted by Wang *et al.*, [73], it may also require concomitant use of PLP and AEDs to effectively treat the disorder. Although not yet proven, it might also be useful to administer PLP/PL in conjunction with riboflavin (vitamin B<sub>2</sub>), the metabolic precursor of FMN since studies showed that mutations could drastically decrease the affinity of PNPOx for the FMN cofactor [66]. Against this backdrop is the potential of neurotoxic side-effects of too much PLP.

### 3.6 Pyridoxine-dependent NEE and management of the disorder

There are several cases of NEE where patients responded to treatment with pyridoxine. These PN-dependent NEE patients rarely show the severe fetal distress, elevated threonine and glycine, PLP-dependent enzyme deficiency, and premature birth that characterize

PNPOx deficiency-related NEE. PN-dependent NEE is an autosomal recessive disease of probably underestimated incidence, first described in 1954 [74], that is usually presented with neonatal epileptic encephalopathy. It is believed that lack of biological markers has contributed to the under diagnosis of several cases of PN-dependent NEE disorder [1].

Children with PN-dependent seizures have mutations in the ALDH7A1 gene (maps to 5q31), which encodes for  $\alpha$ -amino adipic semialdehyde dehydrogenase (also called antiquitin). Sequencing of ALDH7A1 gene from individuals with pyridoxine-dependent seizures revealed homozygous as well as compound heterozygous mutations [75]. A comprehensive review of these mutations (missense, nonsense, splice-site and small deletions) has recently been provided by Schärer *et al.* [70]. One of these (E399Q, a missense mutation) is of particularly high frequency, and seems to alter cofactor binding and catalysis, having a significant impact on enzyme activity.

Antiquitin functions as an aldehyde dehydrogenase within the cerebral lysine catabolism pathway by catalyzing the oxidation of  $\alpha$ -amino adipic semialdehyde, derived from pipercolic acid, to  $\alpha$ -amino adipic acid [70,75]. The abolished antiquitin activity thus results in accumulation of pipercolic acid,  $\alpha$ -amino adipic semialdehyde ( $\alpha$ -AASA) and piperidine-6-carboxylic acid (P6C) (**Figure 8**). P6C reacts with free PLP (**Figure 8**) forming a Knoevenagel condensation product depleting PLP, leading to an imbalance between the excitatory neurotransmitter glutamic acid and the inhibitory neurotransmitter GABA, thus providing an explanation for the observed seizures.

As noted by Gospe, *et al.*, patients with PN-dependent NEE are usually diagnosed by administration from one to five 100 mg doses of pyridoxine intravenously (under electroencephalography and vital-sign monitoring) during a clinical seizure, and noting prompt resolution of the clinical and electrographic features [4]. Alternatively, patients may be given 15 mg/Kg per day and noting complete control within a week [69,76]. For pyridoxine-deficient NEE, seizure relapse would occur after withdrawal of pyridoxine, but promptly controlled when re-administered. It's important that AEDs are sequentially withdrawn to confirm the diagnosis. It should be noted that, if there is no improvement with pyridoxine administration, the patient should be promptly treated with PLP. Screening for PN-dependent seizures is also possible via determination of plasma pipercolic acid and by measuring urinary or plasma  $\alpha$ -AASA and P6C levels [77,78]. Diagnosis is also confirmed by mutational analysis of the antiquitin gene. Typically, onset of such seizures is in neonatal period or shortly thereafter with irritability. Those infants need to be treated using pharmacologic doses of pyridoxine and a life-long therapy is needed. Seizures recur upon pyridoxine withdrawal, and if untreated die from status epilepticus. The children with this disorder show mild to moderate developmental delay, and do very well even with a single intravenous dose of PN to prevent seizures [69].

It's quite possible that treatment with PL/PLP in the pyridoxine deficient NEE patients could also work, and physicians should consider combining both PN and PLP/PL therapy. A lysine-free diet has also been recommended for treating this disorder. While the rationale for a lysine restricted diet is clear (**Figure 8**), the clinical effect on the outcome in pyridoxine-dependent seizures still has to be documented before this can be universally recommended. Pyridoxine treatment does not address the potential toxicity of accumulating pipercolic acid,  $\alpha$ -AASA and P6C.

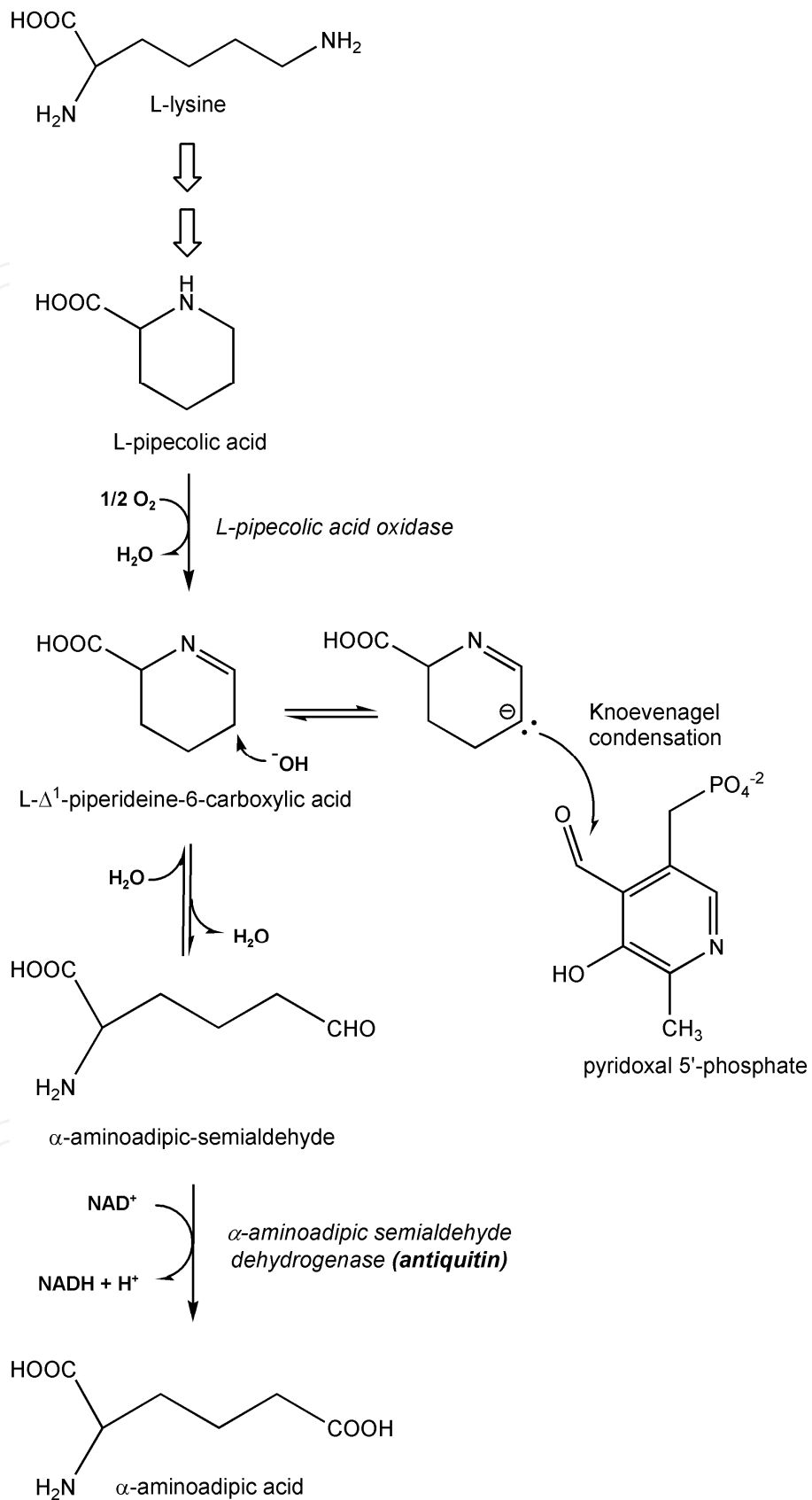


Fig. 8. The interaction of lysine degradation intermediates with pyridoxal 5'-phosphate. Pyridoxine-dependent NEE is due to a deficient activity of antiquitin.

A few cases of neonatal epileptic encephalopathy have been reported to respond to treatment with folinic acid (5-formyl tetrahydrofolate, also known as leucovorin) [79,80]. These cases have been attributed to a distinct disorder with respect to pyridoxine-dependent epilepsy, until it was demonstrated that folinic acid-responsive patients also respond to pyridoxine, are antiquitin deficient and have mutations in the ALDH7A1 gene [80]. The response to folinic acid, which has been serendipitously discovered, is variable and is accompanied by a variable degree of responsiveness to pyridoxine. In our opinion, this observation, together with the fact that all folinic acid-responsive subjects showed the presence of two unidentified compounds in the cerebrospinal fluid, suggest that folinic acid-responsive seizures, although related to pyridoxine-dependent epilepsy, might be considered a different condition.

Another rare neonatal encephalopathy condition, the so-called hyperprolinemia type II, usually presented in childhood with fits and encephalopathy, is caused by the deficiency of the enzyme  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase, involved in proline degradation and encoded by the ALDH4A1 gene. This deficiency results in the accumulation of proline and L- $\Delta^1$ -pyrroline-5-carboxylic acid in plasma and the consequent excessive excretion of the latter compound in urine [81]. L- $\Delta^1$ -Pyrroline-5-carboxylic acid reacts in a Knoevenagel condensation with the aldehyde carbon of PLP, in a reaction similar to the one shown in **Figure 8** for L- $\Delta^1$ -piperidine-6-carboxylic acid [82,83]. The molecular mechanism of PLP depletion is indeed related to the one described before for the antiquitin-dependent epileptic encephalopathy. Seizures, which manifest in approximately half of the affected individuals, also respond well to pyridoxine treatment.

#### 4. Conclusions

Neonatal epileptic encephalopathies are rare neurological disorders that may originate from PLP deficiency, owing to defects in the PNPOx enzyme of the vitamin B<sub>6</sub> salvage pathway or in the antiquitin enzyme of the lysine degradation pathway. The basic metabolic faults of these two distinct causes of the disease are completely different. PNPOx defects slow down or block the conversion of PNP and PMP into PLP. Patients respond to treatment with PL or PLP but are insensitive to PN, and the disorder is usually referred to as PNPOx deficiency-related or PLP-dependent NEE. On the other hand, antiquitin mutations result in the accumulation of metabolic intermediates that react with PLP to form complexes that do not permit activation of apo-B<sub>6</sub> enzymes into their active holo-forms. Patients affected by antiquitin defects can be treated with any of the B<sub>6</sub> vitamers (PN is the most used vitamer), since their salvage pathway is functioning correctly to produce PLP. Several different pathological mutations of PNPOx and antiquitin have been characterized in detail. In particular, our research group has characterized the missense mutations R95C and R229W of PNPOx. Patients affected by this disorder present several clinical and general features that are useful for diagnosing and managing the disease.

Like NEE, several other neurological disorders, including seizures, autism, attention deficit hyperactive disorder, Alzheimer's, Down Syndrome, schizophrenia, Parkinson's, learning disability, anxiety disorders have also been associated with PLP deficiency [45,84-89]. This has inspired considerable interest in treating the symptoms of some of these disorders with vitamin B<sub>6</sub>. However, studies on the effect of B<sub>6</sub> supplementation show conflicting results; some of the diseases have been responsive to PN or PLP/PL. For example PLP has been

successfully used in Japan in the treatment of a range of epilepsies from infantile spasms to status epilepticus [89,90]. PN has also been shown to improve drug-induced parkinsonism and psychosis in a schizophrenic patient [88]. Other studies have shown no such beneficial effect, although most of these studies were conducted with PN and not PL or PLP. It is quite clear that therapy with PN or PM in patients with a defective PNPOx will not be successful, because this enzyme is needed to oxidize PNP and PMP into PLP after the kinase has changed them into PNP and PMP. Also, therapy with PL, PN or PM in patients with a dysfunctional PL kinase will not work, because these vitamers would have to be phosphorylated by the kinase.

It is also apparent from this review that neurological disorders, including epileptic episodes that are not responsive to conventional anticonvulsants could be due to mutations in either PNPOx, its salvage partner PL kinase or antiquitin. The genes coding for these enzymes are clearly candidates for mutational analysis in affected patients. If such an error is identified, studies could be performed to investigate the role of the mutations in the enzymatic activity and the associated phenotypes. This perhaps would point to the correct pharmacologic intervention.

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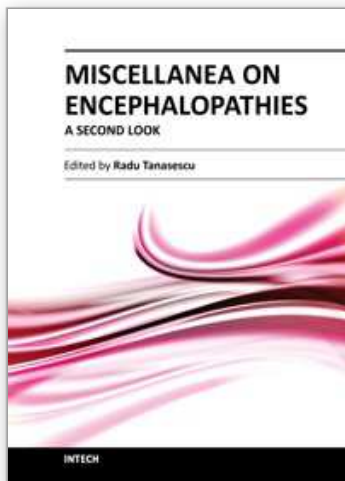


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