Animal models of brain dysfunction in phenylketonuria

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Abstract

Phenylketonuria (PKU) is a metabolic disorder that results in significant brain dysfunction if untreated. Although phenylalanine restricted diets instituted at birth have clearly improved PKU outcomes, neuropsychological deficits and neurological changes still represent substantial problems. The specific mechanisms by which Phe affects the brains of individuals with PKU are yet fully determined. The use of animal models in PKU research significantly broadens the possibilities for investigating these mechanisms. This report presents an overview of findings from animal studies on the mechanisms of Phe action in the PKU brain, discussing the importance of changes in protein synthesis, transport of large neutral amino acids across the blood–brain barrier, synthesis of monoamine neurotransmitters, activity of glutamate receptors, animal behavior, and translation of animal behavioral data to patients with PKU. This report shows that great progress has been made in past years and demonstrates the importance of further animal research to understand the neuropathological mechanisms underlying brain dysfunction in PKU. A better understanding of these mechanisms will guide the development of optimal treatment strategies for PKU.

Introduction

Phenylketonuria (PKU; OMIM 261600 and 261630) is a metabolic disorder that results in significant cognitive and behavioral difficulties [1]. Although there is now a relatively large body of research on the disorder, we still do not have a thorough understanding of the brain pathology underlying the psychological impairments associated with PKU [2]. With this in mind, it is important to be aware of the contributions that animal studies have made to our current knowledge and may make to our future knowledge. In this report, an overview of research with animal models is presented.

Before discussing animal models of PKU, it is important to understand the neurochemistry of the disorder. PKU is based on a deficiency in the activity of phenylalanine hydroxylase (PAH; EC 1.14.16.1), resulting in increased phenylalanine (Phe) and decreased tyrosine (Tyr) concentrations in the blood and brain [1,3]. The most relevant biochemical abnormalities are decreases in the activity of Tyr and tryptophan (Trp) hydroxylases, which lead to deficiencies in the neurotransmitters dopamine and serotonin. From a clinical perspective, these deficiencies may result in abnormal muscle tone and movement, irritability and lethargy, seizures, poor temperature control, progressive developmental delay, and microcephaly [4].

A deficiency in the cofactor tetrahydrobiopterin (BH4) also occurs in approximately 2% of the PKU population. BH4 deficiency results in decreased activity of PAH and other enzymes, including Tyr hydroxylase, Trp hydroxylase, and nitric oxide synthase. This deficiency leads to a mild elevation in Phe, although high elevations in Phe are rarely seen.

Findings from studies before 2000

Until 2000, most animal studies to mimic human PKU were performed in rats [5,6], although some were performed in mice [7]. These models were all based on an increase in Phe concentration that was accomplished by either supplementation of Phe, administration of enzyme inhibitors (para-chloro-Phe; alpha-methyl-Phe), or a combination of both.

Abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; BH4, tetrahydrobiopterin; IQ, intelligence quotient; BBB, blood–brain barrier; LNNA, large neutral amino acid; CE/LIF, capillary electrophoresis and laser-induced fluorescence; AGS, audiogenic seizures.


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All of these models had major drawbacks. Supplementation of Phe resulted in increased Tyr rather than the decrease in Tyr that is associated with PKU [8]. Glycine also increased with Phe supplementation [9], and because it is not clear why this would be associated with PAH deficiency, the validity of this model was further diminished. In addition, administration of enzyme inhibitors resulted in inhibition of other hydroxylating enzymes [5], and as such this model probably resembled BH4 deficiency more closely than PKU [5]. Nonetheless, studies using these models were a beginning in unraveling the black box of the brain pathology that underlies psychological impairment in patients with either untreated PKU (severe mental retardation and behavioral problems) or early- and continuously-treated PKU (mild decreases in intelligence quotient (IQ) [10]), problems in school achievement [11], and impairments in specific neuropsychological functions [6,12–14].

Findings from in vitro studies in the rat also provided evidence of abnormal myelin, protein, and neurotransmitter synthesis, as well as the possible role of the blood–brain barrier (BBB) [15–23]. With regard to myelin, although studies initially suggested impaired synthesis, later studies indicated myelin breakdown with normal synthesis, resulting in a net decrement in myelin. These observations were supported by findings from Hommes [15–17], demonstrating that Phe supplementation inhibits ATP sulphurylase, which results in an unstable myelin structure. Hommes attempted to repeat his previous studies in the rat using one of the first “PKU mouse” models developed by McDonald [19], but unfortunately it was later found that this mouse model resembled BH4 deficiency rather than PAH deficiency. It also remained unclear whether the effects of PKU on myelin were partially explained by findings from studies using enzyme inhibitors [18]. In addition, it should be acknowledged that PKU does not resemble the leukodystrophies in which abnormal (lipid) myelin metabolism determines clinical outcome.

In addition to myelin abnormalities, research findings have pointed to a problem in protein synthesis. Both acute and chronic supplementations of Phe have been shown to result in a decrease in net protein synthesis in rats and mice [7,20,21]. The research of Binek-Singer et al. [7], however, suggested that Phe supplementation resulted in low concentrations of other amino acids and that these low concentrations caused decreased protein synthesis. This decrease in protein synthesis was at least in part counteracted through the supplemental administration of specific amino acids.

The idea that administering specific amino acids could reverse the brain pathology associated with PKU had already emerged [23]. Andersen et al. [23] showed that giving large neutral amino acids (LNAAs) prevented the influx of Phe into the brain. It was, however, unclear whether the effect of amino acid supplementation was related to action at specific membranes such as the BBB or to other cellular or metabolic processes in the body (including general anabolism).

As stated previously, results from studies using rat models and early mouse models of PKU should be considered with caution because they may reflect the consequences of BH4 deficiency rather than PAH deficiency. Nonetheless, in PAH deficiency, there is clear evidence that neurotransmitter metabolism is affected, especially the metabolism of Tyr to dopamine and Trp to serotonin [24–26].

**BTBR-Pahenu2 mouse model of PKU**

*Phe and other LNAAs*

The development of a genetic mouse model for PKU, the BTBR-Pahenu2 mouse, gave new impulse to research aimed at elucidating the mechanisms of brain pathology in PKU [27,28]. The Pahenu2 mouse was derived from chemically induced mutagenesis of male BTBR mice [27]. The Pahenu2 mouse carries a point missense mutation in the PAH gene [28] and closely mimics the genetics, biochemistry, and neurobiology of human PKU.

Mice that are homozygous for the mutated gene (Pahenu2 mouse) are characterized by a light fur coat caused by inhibition of melanin biosynthesis, indicating the deficiency in PAH activity and high body Phe levels. In confirmation of this, different research groups reported blood Phe concentrations in Pahenu2 mice 8–25 times higher than in heterozygous carriers or wild-type BTBR mice [29–33]. The total brain Phe concentration in Pahenu2 mice may approach a millimolar level [29,31,32]. It appears that the Phe concentration in these mice may differ in different brain structures with the highest concentration in posterior cortex and the lowest in striatum [31]. Overall, these findings demonstrate that Pahenu2 mice have a significantly greater level of Phe in the brain in comparison with their wild-type counterparts, which is indirect evidence supporting a direct neurotoxic effect of Phe in the brain.

Interestingly, extracellular concentration of unbound Phe measured in the brains of live Pahenu2 mice by using microdialysis with capillary electrophoresis and laser-induced fluorescence (CE/LIF) detection was about 3–4% of the total brain Phe concentration reported for these animals [29,31,32, Martynyuk et al., unpublished observations]. Also, the CE/LIF measurements showed that extracellular brain levels of unbound Phe were more than 30 times higher in Pahenu2 mice than in wild-type mice, an increase that is significantly greater than that of total Phe in the Pahenu2 mouse brain compared to the wild-type mouse brain [29,31,32]. Considering relatively low absolute values of unbound Phe concentration in the Pahenu2 mouse brain, the pathophysiologic effects produced by Phe may be caused by higher local concentrations of Phe that result from the dynamic exchange between pools of bound and unbound Phe, which are not accessible during sampling with microdialysis.

Phe and other LNAAs such as Tyr, Trp, leucine, isoleucine, histidine, methionine, threonine, and valine are transported into the brain via the BBB by the common L-type amino acid carrier [34–36]. Therefore, it is obvious that in PKU, when blood Phe concentrations are high, Phe should outcompete other LNAAs for the L-type amino acid transporter and thus limit their influx to the brain. Indeed, both animal and human studies demonstrated elevated levels of Phe and reduced concentrations of other LNAAs in the PKU brain [30,37].

The direct neurotoxic effects of elevated Phe and reduced levels of LNAAs in the brain are thought to be the major causes of brain abnormalities in PKU [37]. The metabolic pathways leading to the synthesis of the vital catecholamine neurotransmitters dopamine and norepinephrine, as well as the indolamine neurotransmitter serotonin, involve the aromatic LNAAs Tyr and Trp as precursors of the catecholamines and serotonin, respectively. The deficiency of Tyr and Trp in PKU brain caused by competition of these amino acids with Phe for the L-type amino acid transporter at the BBB may be one of the reasons for the decreased production of dopamine, norepinephrine, and serotonin observed in PKU [31,38–41]. In addition, significant reduction in these and other LNAAs may form the basis for reduced protein synthesis in the PKU brain [30]. These possibilities argue for dietary supplementation with LNAAs as a method to improve brain function in PKU [37,42]. The Pahenu2 mouse data, however, indicate that a reduction in the influx of LNAAs to the brain may have limited direct effect on monoamine neurotransmitter synthesis in particular, and protein synthesis in general.

Thus, as discussed in detail below, it appears that reduced brain levels of both Tyr and Trp are not the causative factors in the deficient production of dopamine and serotonin in the Pahenu2 mouse brain [31,38–41]. Also, the reduction in cerebral protein synthesis observed in the Pahenu2 mouse brain [30] is not likely due to-
duced influx of LNAAs to the brain as a result of their competition with high levels of Phe for the L-type amino acid transporter at the BBB. This deficit of LNAAs is compensated for by amino acids derived from protein breakdown within the brain [30]. The dietary supplementation with LNAAs may nonetheless be beneficial in PKU [37,42], most likely by decreasing brain influx of Phe [37]. However, potential side effects of supraphysiological concentrations of LNAAs should be carefully assessed.

**Monoamine neurotransmitters**

Puglisi-Allegra et al. [38] demonstrated that the Pahenu2 mouse brain was characterized by a marked decrease in serotonin levels in the prefrontal cortex, cingulate cortex, nucleus accumbens, caudate, putamen, hippocampus, and amygdala. Norepinephrine, dopamine, and their metabolites were also reduced in many of these areas. In support of a deficiency of monoamine neurotransmitter in these mice, the major metabolites of serotonin and dopamine, 5-hydroxyindoleacetic acid (5-HIAA), and 3,4-dihydroxy-phenylacetic acid (DOPAC) were also reduced in the nigrostriatal region of the brain [43].

The observed deficit in monoamine neurotransmitters, especially the marked decrease in serotonin levels, is difficult to reconcile with the reduced concentrations of their aromatic amino acid precursors. Reduced Trp levels in the brain were non-significant [39,41]. On the other hand, the brain level of an immediate precursor of serotonin, 5-hydroxytryptophan, which is a hydroxylation product of Trp catalyzed by Trp hydroxylase, was found to be significantly reduced in Pahenu2 mice [40,41]. The authors suggested that inhibition of Trp hydroxylase by high levels of Phe is a crucial factor in serotonin deficiency in Pahenu2 mice. In support of their hypothesis, Pascucci et al. [41] reported that the 5-hydroxytryptophan-treated hyperphenylalaninemic Pahenu2 mice responded to stress with an increase of serotonin release in prefrontal cortex, a response that was not observed in untreated Pahenu2 mice.

Similar to the role of Trp in serotonin production in the Pahenu2 mouse brain [38–41], it appears that a decrease in brain Tyr level is also not a limiting factor in the biosynthesis of dopamine in Pahenu2 mice. Joseph and Dyer [31] found that brain dopamine levels almost normalized in Pahenu2 mice that were fed a low Phe diet supplemented with Tyr, despite the fact that brain Tyr concentrations at this time were still significantly reduced. Interestingly, in another study performed in these mice, a low Phe diet without Tyr supplementation did not cause normalization of brain dopamine [41]. Further investigation is required to determine whether high Phe levels, comparable to their inhibitory role in the Trp-serotonin pathway, directly inhibit the Tyr hydroxylase-mediated conversion of Tyr to DOPA, or, as Joseph and Dyer [31] favored whether Phe at high concentrations impairs the myelination necessary for normal Tyr hydroxylase activity. Reduced synthesis of the Tyr hydroxylase protein itself is another possibility for diminished production of dopamine, which is suggested by these authors based on findings by Smith and Kang [30] that protein synthesis was reduced in the Pahenu2 mouse brain. The possibility that Phe may serve as a substrate for Tyr hydroxylase at certain concentrations is also not excluded [31,41].

**Glutamate**

The Pahenu2 mouse data indicate that, in addition to alterations in the monoamine neurotransmitters, abnormalities in the glutamatergic system may contribute to the brain pathology of PKU. Glutamate is a major excitatory neurotransmitter in the central nervous system and plays a crucial role in neuronal physiology by activating ionotropic glutamate receptors of N-methyl-D-aspartate (NMDA), (RS)-amino-3-hydroxy-5-methyl-4-isoxazolopyrioxic acid (AMPA), and kainate subtypes. Disturbances in glutamatergic activity contribute to the pathophysiology of many neurological and cognitive disorders [44]. The in vitro measurements showed that Phe at high concentrations significantly depressed excitatory glutamatergic synaptic transmission by a combination of pre- and postsynaptic actions, including attenuation of neurotransmitter release, competition for the glutamate-binding site of AMPA/kainate receptors, and competition for the glycine-binding site of NMDA receptors [45,46].

Consistent with the antiglutamatergic effects of Phe in neuronal cultures, the expression of NMDA receptor NR2A and AMPA receptor Glu1 and Glu2/3 subunits in the brain of hyperphenylalaninemic Pahenu2 mice was significantly increased, but not expression of the NMDA receptor NR2B subunit, which was decreased [32]. In the adult brain the NR2B/NR2A ratio decreases with age. Higher expression of NR2B is associated with a greater plasticity in the younger brain. The increase in NR2A/NR2B proportion may thus represent a premature “aging” of the PKU brain that leads to impaired synaptic plasticity and memory formation. For example, Tang et al. [48] demonstrated that over-expression of NMDA receptor NR2B in the brains of mutant mice leads to enhanced learning ability and memory. Therefore, the findings reported by Glushakov et al. [32] allow speculation that a decrease in NMDA NR2B receptor expression in the PKU brain may contribute to the PKU-specific impairment in learning and memory.

Both in vivo and in vitro data [32,45,46] suggest that variation in Phe levels and inhibition by Phe of excitatory glutamatergic activity with subsequent facilitation of glutamatergic activity during a transient decreases of Phe levels may underlie audiogenic seizures (AGS) in PKU mice. Susceptibility to AGS in PKU mice increased with decreasing Phe concentration, but within the hyperphenylalaninemic range. Normalization of plasma Phe levels resulted in a termination of AGS; susceptibility to AGS gradually returned after return of hyperphenylalaninemia [33]. The impaired glutamatergic system may also contribute to abnormal grooming in these mice. Although, all wild-type, heterozygous, and homozygous Pahenu2 BTBR mice exhibited an incomplete sequence of grooming primarily devoted to Phase 1, hyperphenylalaninemia reversibly prolonged the time spent grooming in the Pahenu2 mice [Martynyuk et al., unpublished observations]. The contribution of impaired monoamine neurotransmitter systems to AGS and abnormal grooming in the Pahenu2 mice cannot be excluded at this time.

The finding in Pahenu2 mice of epileptic seizures [33] and abnormal grooming patterns, which have distinctive characteristics that are easy to compare across studies, not only further validates the Pahenu2 mouse as a neurobiological model for human PKU, but may also expedite investigation of the mechanisms of neurotoxic effects of Phe. This is especially true when considering the discrepancies in the results of the behavioral studies (see below). Given the known genetic mutation and the well defined metabolic outcome in the Pahenu2 mouse, this animal model may also help to elucidate the mechanisms of brain function impairment in other neuropsychiatric disorders that share similar symptoms with PKU but often have more complex genetic and cellular mechanisms, such as epilepsy and autism. For example, of relevance to autism, Pahenu2 mice exhibit hypoactivity, decreased exploratory behavior, cognitive deficits, and increased susceptibility to epileptic seizures.

**Behavioral research**

Studies examining behavior in Pahenu2 mice are described below in chronological order. In the first such study [48], two tasks were used to assess learning. In one task, mice had to discriminate
between a baited and non-baited cap using scent (either cinnamon or nutmeg) as a cue to locate a food rewards. After this task was mastered, a reversal task was administered in which the scent previously associated with the non-baited cap was now linked to the food reward. Re-reversals were then performed. Results showed that the rate and accuracy of food acquisition during the first reversal did not differ between Pah<sup>enu2</sup> mice and control (BTBR wild type or BTBR heterozygous) mice. The subsequent re-reversal task, however, revealed a significant impairment in the Pah<sup>enu2</sup> mice. The other task of this study was to assess latent learning. Mice were allowed to freely explore a novel area in which a water bottle was placed. The mice were then water-deprived and the speed at which they could find the water bottle was assessed. Results showed that the latent learning of Pah<sup>enu2</sup> mice was impaired in comparison with control mice. Together these results make a strong argument for impaired learning in Pah<sup>enu2</sup> mice.

In another learning study, Sarkissian et al. [49] used a T-maze alternation task and an eight-arm radial maze task. In the T-maze task, food-deprived mice had to find a food reward at the end of a single baited arm of the maze. Pah<sup>enu2</sup> mice took significantly longer than control mice to locate the baited arm. In the eight-arm radial maze task, food rewards could be obtained in four randomly selected arms, which were indicated by a light directly above a food cup. After the food reward was collected, the arm was no longer lit. Optimal foraging behavior required that animals minimize reentries into arms visited previously. Results showed that Pah<sup>enu2</sup> mice made more entries to previously visited arms and required more time to reach food rewards than control mice. The authors concluded that Pah<sup>enu2</sup> mice were impaired in simple discrimination, short-term memory, reference memory, habit learning, and memory for a visual stimulus.

In a third study, Mibalick et al. [50] used an olfactory learning task that was similar to that used by Zagreda et al. [47]. They found that learning rates did not differ between Pah<sup>enu2</sup> and control mice, but in the reversal task a more subtle impairment was observed in the Pah<sup>enu2</sup> mice than was found by Zagreda et al. [47]. Nevertheless, the Pah<sup>enu2</sup> mice required more sessions to meet the criterion for discrimination acquisition than control mice, corroborating the findings of Zagreda et al. [47].

In a fourth study, Cabib et al. [51] investigated spatial novelty and object discrimination. In these tasks, either a change in the location of an object (spatial novelty) or a new object (object recognition test) occurred within a circular field. Pah<sup>enu2</sup> mice, in contrast with controls, did not show enhanced exploration of objects that were either novel or displaced. This indicates that Pah<sup>enu2</sup> mice were impaired in recognizing environmental changes. In this same study, Cabib et al. [51] also studied emotional reactivity. They found that Pah<sup>enu2</sup> mice were not emotionally different from control mice, although moderate motor disturbances were identified that can interfere with the measurement of cognition (e.g., time to master a task).

Taken together, the behavioral data suggest the presence of mild behavioral impairments in Pah<sup>enu2</sup> mice, particularly with regard to behavioral flexibility (i.e., altering previous behaviors in response to changing environmental demands). Reversal procedures are particularly likely to reveal deficits in behavioral flexibility, and even when not explicitly mentioned many of the previously described tasks had elements of reversal. For example, the T-maze task may be viewed as requiring rapid and serial reversal. In the spatial novelty and object discrimination tasks, behavioral flexibility was a critical element because the animal had to respond to a sudden environmental change and alter a previously acquired behavioral response. Therefore, the subtle behavioral impairment of Pah<sup>enu2</sup> mice appears to be associated with a loss of behavioral flexibility.

**Anatomical findings related to behavior**

A failure in the procedural learning and memory system, subserved by the striatum of the brain [52], is a possible underpinning of the loss of behavioral flexibility during learning tasks in Pah<sup>enu2</sup> mice. The procedural system depends on dopaminergic input to the striatum from the substantia nigra, and to a lesser degree the ventral tegmental area. As mentioned earlier, dopamine is reduced in the brains of Pah<sup>enu2</sup> mice [39,40,43], and neurodegenerative changes have also been reported in the substantia nigra and ventral tegmental area [43]. In the substantia nigra, the number of Tyr hydroxylase-positive neurons is dramatically reduced, and the presence of infiltrating macrophages in this area may be in response to dying dopaminergic neurons [53]. Because the striatum receives dopaminergic projections from the substantia nigra, reduced dopaminergic signal transduction leads to reduced striatal function that is critical for behavioral flexibility.

More general aspects of PKU brain anatomy may account for behavioral impairments that affect both declarative (i.e., hippocampus-dependent) and procedural memory systems. First, PKU brains are characterized by hypomyelination and demyelination [54,55]. Myelination deficits occur in specific forebrain tracts. Hypomyelination was observed in the corpus callosum and anterior commissure, two critical fiber tracts for interhemispheric communication and information processing. In contrast, the commissure of the ventral fornix (input/output pathway of the hippocampus) and the internal capsule appeared to be intact [55]. Nevertheless, this specific loss of fiber tracts will result in impaired information processing, leading to reduced behavioral performance. Second, glutamatergic signaling, critical for synaptic plasticity underlying behavioral processes, is abnormal in Pah<sup>enu2</sup> mice [45,46,56]. Third, elevated Phe causes reduced synaptic density in mixed cortical cultures from normal mice [57]. Although the Phe concentrations used were beyond the physiologic range, high Phe may have caused morphologic alteration in synaptogenesis. Taken together, these cumulative effects likely contribute to a global behavioral decline.

**Translation of behavioral data to patients**

Mental retardation is severe in untreated patients with PKU [1]. In comparison, the behavioral impairments in untreated Pah<sup>enu2</sup> mice are mild. Nevertheless, striking similarities are found between Pah<sup>enu2</sup> mice and untreated PKU patients. In humans, untreated PKU leads to microcephaly, impaired development of cognitive skills, motor impairment, and seizures [4]. Many of these features have been reported in the Pah<sup>enu2</sup> mouse, which validates this mouse as a PKU model.

The behavioral tests performed with Pah<sup>enu2</sup> mice address basic and relevant features of underlying memory systems. However, they do not address overall cognitive function. Regarding mental retardation, it is not possible to equate human intelligence tests with tasks suitable for administration to mice. A better approach may be to develop animal-based tasks for administration to humans, as has been successfully done to examine cognitive dysfunction in individuals with Down syndrome [58]. For PKU research, it would be useful to focus on behavioral flexibility. For example, more demanding reversal and re-reversal paradigms such as the Y-maze reference memory task may be useful because interactions between the hippocampus and striatum are critical [59,60]. Reversal learning impairments have been repeatedly reported in humans with mental retardation [61,62]. From this perspective, it would be interesting to examine behavioral flexibility scores from the Behavioral Flexibility Scale-Revisited [61] in untreated patients with PKU.
Finally, although the PaH1enu2 mouse model has demonstrated its value and clear potential to contribute to our understanding of the genetics, biochemistry, and neurobiology of PKU in humans, it should be realized that there are no available mouse models of mental retardation. It has been claimed that certain mouse strains provide models of mental retardation, but the behavioral differences between impaired and unimpaired mice is by no means comparable to those observed in humans. As such, one may wonder whether the mouse is a particularly appropriate species in which to model mental retardation in humans with PKU.

Conclusions
Notwithstanding the vast amount of data on the pathogenesis of PKU, neuropsychologic deficits and neurologic changes remain an important issue. The results of animal studies related to the cellular mechanisms of brain dysfunction in human PKU have been reviewed. Progress in animal studies has been made, especially investigating the PaH1enu2 mouse, showing that transport of LNAAs across the BBB, protein synthesis, synthesis of monoamine neurotransmitters, and the activity of glutamate receptors may be involved in the cascade that begins with high plasma Phe levels and ultimately results in brain dysfunction. This review also shows the importance of animal behavior studies and the translation of the behavioral data to patients with PKU. Further exploration of the cellular mechanisms of Phe-induced changes in the PaH1enu2 mouse brain may provide important insights into the neurobiological basis of more challenging studies involving neurologic, cognitive, neuropsychologic, and psychiatric disorders in human PKU.

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