Tryptophan and Kynurenine Pathway Metabolites in Animal Models of Retinal and Optic Nerve Damage: Different Dynamics of Changes

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Kynurenines, products of tryptophan (TRP) metabolism, display neurotoxic (e.g., 3-hydroxykynurenine; 3-HK), or neuroprotective (e.g., kynurenic acid; KYNA) properties. Imbalance between the enzymes constituting the kynurenine pathway (KP) plays a role in several disease, including neurodegeneration. In this study, we track changes in concentrations of tryptophan and its selected metabolites after damage to retinal ganglion cells and link this data with expression of KP enzymes. Brown-Norway rats were subjected to intravitreal N-methyl-D-aspartate (NMDA) injection or partial optic nerve crush (PONC). Retinas were collected 2 and 7 days after the completion of PONC or NMDA injection. Concentrations of TRP, kynurenine (KYN), and KYNA were determined by high performance liquid chromatography (HPLC). Data on gene expression in the rat retina were extracted from GEO, public microarray experiments database. Two days after NMDA injection concentration of TRP decreased, while KYN and KYNA increased. At day 7 compared to day 2 decrease of KYN, KYNA and further reduction of TRP concentration were observed, but on day 7 KYN concentration was still elevated when compared to controls. At day seven concentration of TRP, 3-HK, and KYN was higher, whereas concentration of KYNA declined. In vivo experiments showed that retinal damage or optic nerve lesion affect TRP metabolism via KP. However, the pattern of changes in metabolite concentrations was different depending on the model. In particular, in PONC KYNA and KYN levels were decreased and 3-HK elevated. These observations correspond with data on expression of genes encoding KP enzymes assessed after optic nerve crush or transection. After intraorbital optic nerve crush downregulation of KyatI and KyatIII between 24 h and
INTRODUCTION

Kynurenine pathway (KP) is the major route for tryptophan (TRP) metabolism in most mammalian tissues. Through this pathway 95% of dietary TRP is transformed to metabolites known as kynurenines (Fujigaki et al., 2017; Sas et al., 2018). Imbalance in KP has been postulated to contribute to pathological mechanisms in several neurological and neurodegenerative diseases like brain ischemia, epilepsy, major depression, Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, multiple sclerosis, HIV associated neurocognitive disorders, or schizophrenia (Maddison and Giorgini, 2015; Fujigaki et al., 2017; Lovelace et al., 2017).

Kynurenine pathway starts with the oxidative cleavage of TRP catalyzed by one of the rate-limiting enzymes: tryptophan 2,3-dioxygenase (TDO), present mostly in liver or indoleamine 2,3-dioxygenase (IDO), present in other tissues including neuronal cells and glial cells, converting TRP to N-formylkynurenine (Maddison and Giorgini, 2015). N-formylkynurenine is further metabolized by kynurenine formamidase to kynurenine (KYN). KYN is a central metabolite of the KP and its concentration reflects TRP metabolism along the whole KP. It can be metabolized in one of three branches of KP in reactions primarily catalyzed by one of three different enzymes (Fujigaki et al., 2017):

1. by kynurenine 3-monooxygenase (KMO) to produce 3-hydroxykynurenine (3-HK), subsequently by kynureninase (KYNU) to 3-hydroxyanthranilic acid (3-HAA), then converted to quinolinic acid (QUIN), which is finally converted to NAD⁺;
2. by one of four kynurenine aminotransferase isoenzymes (KAT I, II, III, IV) to produce kynurenic acid (KYNA) by irreversible transamination of KYN;
3. or by KYNU to produce anthranilic acid.

Importantly, KP is crucial for cellular energy metabolism through production of nicotinamide adenine dinucleotide (NAD⁺). It is also postulated that KP may modulate immune response through regulation of NAD⁺ availability for energy-demanding immunological reactions (Savitz, 2019).

Several kynurenines display neuroprotective or neurotoxic properties. 3-HK induces oxidative damage and cell death by promoting free radicals production (Okuda et al., 1998; Wei et al., 2000; Ramírez-Ortega et al., 2017). Quinolinic acid (QUIN) is also neurotoxic, possibly acting via activation of glutamatergic N-methyl-D-aspartate (NMDA) receptor (Guillemin, 2012). In contrast, KYNA is considered to be neuroprotective. KYNA is the only known endogenous antagonist of the NMDA receptor and non-competitive antagonist of the alpha-7 nicotinic acetylcholine receptor, i.e., KYNA acts as an endogenous modulator of glutamatergic and cholinergic neurotransmission (Sas et al., 2018). It was shown that KYNA can protect neurons against excitotoxic damage caused by QUIN (Ferreira et al., 2018; Pierozan et al., 2018). KYNA is also a ligand for the orphan G protein-coupled receptor (GPR35), which is predominantly located in immune cells, suggesting its role in inflammatory pathogenesis of neurological disorders (Wang et al., 2006). KYNA is an agonist of aryl hydrocarbon receptor (AhR) and functions as a ROS scavenger (Wirthgen et al., 2017).

Presence of KP enzymes and kynurenines was demonstrated in retinas of several vertebrate species (Rejdak et al., 2001, 2003a,b, 2004b). KP was shown to play a role in the retina ontogenesis (Rejdak et al., 2003b). Increase in retinal KYNA content in response to excitotoxic damage was postulated as a part of endogenous anti-excitotoxic defense mechanisms (Rejdak et al., 2003a). KP was also demonstrated to be affected in the course of spontaneous glaucomatous retinal degeneration in DBA/2J mice (Rejdak et al., 2004a; Schuettauf et al., 2007). However, only few TRP metabolites were studied in the retina under pathological conditions.

It has been proposed that increase in retinal KYNA might result either from an enhanced influx of blood-borne KYNA after compromise of the blood-retina barrier or in situ increased biosynthesis of KYNA at the lesion site, release from the damaged neurons or from activated microglia and infiltrating macrophages (Lovelace et al., 2017). KP enzymes were shown to be present in the retina: KAT I is preferentially localized on Müller cell endfeet, while KAT II and KAT III are expressed in retinal ganglion cells (Perkins and Stone, 1982; Rejdak et al., 2001, 2003b, 2011). Increased expression of IDO has been shown in retina in the course of diabetic retinopathy, as a result of microglia activation (Hu et al., 2017).

The aim of this study was to track changes in concentrations of tryptophan metabolites in two animal models or retinal degeneration and to link this data with available data on expression of KYNA pathway enzymes.

MATERIALS AND METHODS

Animals

Adult female Brown-Norway rats (Charles River, Sulzfeld, Germany)1 with a body weight (BW) of 150–200 g were used.

1https://www.criver.com/
Brown-Norway rats are a pigmented strain that is widely used in experimental ophthalmology. Some recent data show that the outcome of glaucoma-like insult may significantly differ in pigmented and non-pigmented strains (Gurdita et al., 2017). Moreover, albino rats display spontaneous ocular lesions that may interfere with the experimental lesions (Shibuya et al., 2015).

The animals were kept under a 12 h–12 h light–dark cycle with food and water ad libitum. All experiments were performed in compliance with the Institute for Laboratory Animal Research [Guide for the Care and Use of Laboratory Animals. Association for Research in Vision and Ophthalmology (ARVO)]. Statement for the Use of Animals in Ophthalmic and Vision Research was also followed. The procedures were approved by respective local ethics committee. Numbers of the tested eyes (N) for each experiment are given in the figure captions.

**Intravitreal NMDA Injection**

Retinal ganglion cell (RGC) damage was induced by intravitreal NMDA injection as we previously described (Thaler et al., 2010a; Fiedorowicz et al., 2014). Intravitreal NMDA injection induces inner retina damage by overstimulation of NMDA receptors for glutamate (i.e., excitotoxicity), the main excitatory neurotransmitter of the retinal neurons. Briefly, animals were anesthetized by intraperitoneal injection of 7% chloral hydrate solution (6 ml/kg body weight) and local anesthesia in the form of eye drops (oxybuprocaine, Alcaine, Alcon) was also applied. Two microliters of NMDA solution (10 mM in 0.2 phosphate buffered saline, PBS; pH 7.2; reagents obtained from Sigma-Aldrich, Steinheim, Germany) were intravitreally injected into the posterior side of the globe, 1 mm behind the limbus, with a heat-pulled glass capillary connected to a microsyringe (Drummond Scientific, Broomall, PA, United States) under direct observation through the microscope. Antibiotic eye drops (ofloxacin, Floxal, Bausch & Lomb, Rochester, NY, United States) were applied after the injection. Any rat that exhibited lens damage, retinal hemorrhage, retinal detachment, vitreous hemorrhage or other postoperative complications was excluded from the study. Contralateral eyes served as control eyes and were injected with PBS.

**Partial Optic Nerve Crush**

Partial optic nerve crush (PONC) was performed as we previously described (Thaler et al., 2010b). Briefly, the animals were anesthetized by intraperitoneal injection of 7% chloral hydrate solution (6 ml/kg body weight). Local anesthesia in the form of eye drops (oxybuprocaine, Alcaine, Alcon, Fort Worth, TX, United States) was also applied. Optic nerves were exposed by incising the conjunctiva of the eye followed by separation of the retractor bulbi muscle, then piercing and dissecting the meninges with blunt forceps. A cross-action calibrated crush forceps (jaws 0.4 mm apart) was placed approximately 2 mm behind the globe and the nerve was partially crushed for 15 s.

**Sample Collection**

Animals were placed in a transparent euthanasia chamber (40 × 30 × 25 cm). CO₂ (>99.9%) was provided with flow rate at 8 l/min until animals death (CO₂ gas was maintained for another 2 min after no obvious sigh of breath was observed). Retinas were collected 2 and 7 days after the completion of PONC or NMDA injection. Following hemisection of the eyes along the ora serrata, the cornea, lens and vitreous body were removed. Samples were immediately frozen in liquid nitrogen and stored at −80°C until the biochemical determinations were performed.

**Determination of Tryptophan and Its Metabolites**

Tryptophan, KYN, and KYNA concentrations were measured according to Zhao et al. (2010). In brief, studied substances were analyzed by high-performance liquid chromatography (HPLC) system [The UltiMate 3000 Analytical systems (Thermo Fisher Scientific, Waltham, MA, United States)]. The samples were separated on analytical column (Agilent HC-C18; 250 × 4.6 mm, i.d.). The mobile phase was composed of 20 mmol/L NaAc, 3 mmol/L ZnAc₂, and 7% acetonitrile. It was pumped at a flow-rate of 1 mL/min and the volume per injection was 100 µL. The wavelength of UV detector was set at 365 nm for KYN and at 250 nm for TRP determination. KYNA was quantified fluorometrically (excitation 344 nm, emission 398 nm), 3-hydroxykynurenine (3-HK) was analyzed with the use of an electrochemical detector (The Thermo Scientific Dionex UltiMate 3000 ECD-3000RS), connected to an analytical cell with the oxidation voltage set at 0.6 V, according to the method described by Heyes and Querry (1988). Waters Spherisorb S3 ODS2 150 × 2.1 mm column (United States) was perfused with a mobile phase consisting of 2% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM sodium EDTA, and 8.9 mM heptane sulfonic acid (flow 0.3 ml/min; the volume per injection was 10 µL). Chromelone software was used to control HPLC systems and record of chromatographic date. The limit of detection (LOD) was determined on the basis of the calibration curve. LOD of 3-HK was 0.00000248 µg/10 µL. The coefficient variation (CV) in all cases studies did not exceeded threshold of 15–20%.

**Visualization of Retinal Ganglion Cells**

Visualization of RGC cells was performed in a separate group of animals as we described previously (Thaler et al., 2012). Briefly, 5 days after PONC/NMDA injection, the animals were anesthetized and 7 µL of (hydroxyxylbatimidine methanesulfonate, Molecular Probes, Eugene, OR, United States) was applied to both superior colliculi. Two days later, the animals were sacrificed and retinal flat mounts were prepared and evaluated under the microscope.
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Gene Expression Analysis

Data on gene expression in the retina was extracted from Gene Expression Omnibus (GEO)6 an international public repository of high-throughput functional genomic data sets, with Genevestigator v5.11.05 (Nebion AG, Zurich, Switzerland) using single experiment analysis tool (Hruz et al., 2008). Search phrases were: "Ido," "Kat," "Kynu," "Kmo," and "Rattus norvegicus." Within found results keywords: "retina" and "RGC" were searched. Only data sets with at least three data points available were included in the analysis. Absolute expression differences in between groups were assessed statistically using standard deviation.

Statistics

Differences in between groups were assessed statistically using the Kruskal–Wallis test followed by Dunn’s multiple comparisons test. P < 0.05 was considered statistically significant. Sample sizes (N) for each group are provided in the figure captions. Data are presented as mean ± standard deviation.

RESULTS

Both NMDA injection and PONC resulted in a remarkable reduction of backlabelled RGCs 7 days after the injury (Figure 1). However, RGC reduction was more pronounced in retinas from NMDA-treated eyes. Concentration of TRP, KYN, 3-HK, and KYNA did not differ significantly in eyes injected with PBS 2 or 7 days before the retinas collection (p > 0.05 for all the metabolites, Figure 2). Two days after NMDA injection TRP concentration was lower than in vehicle treated group (281.4 ± 6.5 vs. 367.9 ± 9.7 nmol/g; p < 0.0001) but KYNA and KYN levels were higher (KYNA 13.13 ± 0.87 vs. 6.32 ± 0.90 nmol/g; p < 0.0001; KYN 27.57 ± 0.91 vs. 18.06 ± 1.50 pmol/g; p < 0.0001). Seven days after NMDA injection, concentration of TRP was lower than 2 days after the injection (237.3 ± 6.8 nmol/g, p < 0.001; 2 vs. 7 days). Seven days after NMDA injection KYN and KYNA were lower than 2 days after the procedure (KYNA 8.55 ± 0.88 nmol/g, p < 0.001 vs. 7 days; KYN 14.50 ± 1.21 pmol/g, p < 0.0001; 2 vs. 7 days). However, at day 7 KYN concentration was higher than in vehicle-treated eyes (18.34 ± 1.08 vs. 8.55 ± 0.88 nmol/g; p < 0.0001). 3-HK concentration 2 or 7 days after NMDA injection was not significantly different than in PBS-injected eyes (p > 0.05).

Similarly, concentration of TRP, KYN, 3-HK, and KYNA did not differ significantly in animals that underwent sham surgery 2 or 7 days before the retinas collection (p > 0.05 for all the metabolites, Figure 3). However, 2 days after PONC procedure TRP and 3-HK concentrations were higher than in sham group (TRP 396.6 ± 16.2 vs. 223.1 ± 6.0 nmol/g, p < 0.0001; 3-HK 1.38 ± 0.28 vs. 3.84 ± 0.91 nmol/g, p < 0.05) and concentrations of KYN and KYNA were lower than in sham operated animals (KYN 3.17 ± 0.44 vs. 19.76 ± 0.73 nmol/g, p < 0.0001; KYNA 12.47 ± 0.58 vs. 34.36 ± 2.07 pmol/g, p < 0.0001). Seven days after PONC, concentration of TRP, 3-HK, and KYNA was higher than 2 days after the surgery (TRP 457.2 ± 17.8 nmol/g, p < 0.05, 2 vs. 7 days; KYN 6.19 ± 0.40 nmol/g, p < 0.0001, 2 vs. 7 days; 3-HK 20.06 ± 2.92 nmol/g, p < 0.001), whereas concentration of KYNA declined compared to day 2 6.57 ± 0.51 pmol/g, p < 0.001, 2 vs. 7 days).

According to data extracted from GEO, IONC resulted in downregulation of KyatI after 24 h (p < 0.05), 48 h (p < 0.05), and 3 days (p < 0.01), and KyatIII after 24 h (p < 0.05), 48 h (p < 0.05), and 3 days (p < 0.01). Kmo was upregulated only in one time point (12 h, p < 0.05). There were no significant changes in expression of Ido1, Kynu, and Kmo (Figure 4).

Kyat was downregulated after 12 h (p < 0.05), 48 h (p < 0.01), and 7 days (p < 0.01) after IONT. KyatIII was downregulated 12 h (p < 0.05), 48 h (p < 0.01), and 7 days after IONT (p < 0.01) and upregulated 15 days after IONT (p < 0.01). Ido1 expression was reduced only 15 days after IONT (p < 0.05, Figure 5).

In rat Müller cells subjected to stretching forces neither after one or 24 h no changes in expression of Ido1, KyatI, KyatIII, Kynu, and Kmo in the retinal glial cells exposed to mechanical stretching were observed (Figure 6).

DISCUSSION

Our experiments demonstrate that concentrations of various TRP metabolites are affected in animal models of retinal damage.
However, the pattern of changes in metabolite concentrations was different depending on the model of retinal damage. After NMDA injection TRP concentration declined while KYN level was elevated. KYNA concentration raised transiently 2 days after the insult and then decreased. After PONC TRP and 3-HK concentration was elevated and both KYN and KYNA concentrations decreased. These two models differ in exact mechanisms of damage, its dynamics and severity (Thaler et al., 2010a, 2011).

Mammalian retina seems to be highly susceptible to excitotoxic neurodegeneration. Glutamate neurotoxicity (or excitotoxicity) seems to be an important pathogenetic factor in numerous retinal diseases, e.g., retinal ischemia, glaucoma, retinal detachment, traumatic injuries, diabetic, and high blood pressure retinopathy (Niwa et al., 2016). Excitotoxicity is mediated by excessive activation of glutamate receptors and subsequent calcium flux to the neuronal cells. This phenomenon results in numerous potentially neurotoxic effects: nitric oxide synthase activation, generation of nitric oxide (\(\text{NO}^{\cdot}\)), activation of phospholipase A2 and excessive production of superoxide radical (\(\text{O}_2^{\cdot-}\)). \(\text{NO}^{\cdot}\) and \(\text{O}_2^{\cdot-}\) react and form peroxynitrite (\(\text{ONOO}^{\cdot}\)), a highly nitrating agent that is toxic to neurons (Lipton et al., 1993). Intravitreal administration of NMDA results in a selective RGC death in a dose-dependent manner (Nakazawa et al., 2005). After application of a dose corresponding to our experimental setting, RGC number starts to decrease after 6 h. After 24 h RGC count decrease by over 80% and after 7 days by over 90% (Manabe and Lipton, 2003; Fiedorowicz et al., 2014). After NMDA injection TUNEL-positive cells are observed both in the ganglion cell layer and the inner...
FIGURE 3 | Concentrations of tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA), and 3-hydroxykynurenine (3-HK) in retinas of animals that underwent partial optic nerve crush (PONC) (right side of the graph) or sham surgery (left side). Kynurenine metabolite concentrations were measured 2 and 7 days after PONC or sham surgery. $N = 9$ (sham 2 days and PONC 2 days), $N = 16$ (sham 7 days), or $N = 18$ (PONC 7 days). Mean ± SD. *$p < 0.05$, ***$p < 0.001$. 

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nuclear layer that contains both neurons (amacrine, horizontal, and bipolar cells) and glial cells (Müller cells) (Manabe and Lipton, 2003). Number of microglia/macrophages in the inner retinal layer was shown to increase 1 day after the NMDA injection and reached its peak 3 days after the procedure (Wada et al., 2013).

Elevation of KYNA after NMDA injection in the current set of experiments correspond to the previously published results (Rejdak et al., 2003a). It seems that elevated KYN and KYNA levels could reflect endogenous anti-excitotoxic defense mechanisms (Rejdak et al., 2003a; Schuettauf et al., 2007; Zarnowski et al., 2007). These changes in KP might correspond to the increase in number of microglia/macrophages cells (Wada et al., 2013). Moreover, a decrease in TRP levels also supports the hypothesis that enhanced in situ KYNA synthesis is responsible for its elevation in this model. Unfortunately, there were no available data on KP enzymes gene expression after intravitreous NMDA administration.
Partial optic nerve crush is more complex model of retinal damage than NMDA injection. The injury and the cascade of secondary events reflects the pathological changes occurring in traumatic optic neuropathy and mimics some features of glaucoma (Yoles and Schwartz, 1998). Extent of RGC loss after the optic nerve crush depends on duration of the procedure (Tan et al., 2012). In our setting RGC count is reduced by over 50% 7 days after PONC as we shown in our previous works (Schuettauf et al., 2000; Thaler et al., 2011).

Despite the increased TRP levels after PONC, we noted a decrease in both KYN and KYNA levels. These observations correspond with gene expression data after both after optic nerve crush and transection, where \textit{Ido1} and \textit{Kynu} expression is unchanged, \textit{Kmo} elevated, \textit{KyatI} and \textit{KyatIII} reduced. It suggests that in this case the TRP metabolism through KP is shifted toward production of toxic downstream KYN metabolites that could contribute to the retinal damage in this model (Figure 7). This supposition is supported with the observation that a neurotoxic 3-HK metabolite was significantly elevated in this model. KMO, an enzyme catalyzing KYN conversion to 3-HK is primarily produced in microglia in the nervous system (Parrott and O’Connor, 2015). Therefore, a shift toward “neurotoxic” 3-HK branch of KP may indicate microglia activation in PONC. This is in agreement with previous results showing that in optic nerve crush opening of the blood-brain/retina barrier and subsequent massive microglia/macrophages flux occurs.
Microglia/macrophages were detected by immunohistochemistry at the lesion site 2 days after crush and the number of these cells peaked 6 days after crush (Thaler et al., 2011). Data obtained in ocular tissue correspond with that derived from central nervous system cells, where glial origin KAT presented on proliferating (astro)glial cells in lesion site is responsible for increased KYNA production (Wu et al., 1991).

An interesting new direction for the evaluation of the role on KP imbalance in retinal neurodegeneration would be identification of cells involved in the observed phenomena in two models of retinal and optic nerve damage. Another open important question is whether KP modulation in NMDA-induced retinal toxicity or PONC will enable inhibition of retinal damage. Our study on effects of acetoacetate and
FIGURE 7 | A simplified scheme illustrating metabolic fates of tryptophan and potential consequences of imbalance in its turnover. Kynurenine pathway is the major route for tryptophan metabolism in most mammalian tissues (the metabolites within the scope of this paper are inside a blue box). KYNA is an agonist of aryl hydrocarbon receptor (AhR) and functions as an ROS scavenger and suppresses inflammatory response. A shift toward 3-HK and NAD⁺ production enhances inflammation.

β-hydroxybutyrate administration in NMDA-induced toxicity model suggest that KP modulation may be involved in the neuroprotective mechanism of this approach (Thaler et al., 2010a). Another work showed that systemic administration of KYNA precursor KYN prevents NMDA-induced retinal damage (Vorwerk et al., 1996). It is therefore probable that other neuroprotective approaches are also at least partially mediated by KP normalization that could be an attractive target for neuroprotection of the retina.

A limitation of our study is mRNA analysis based on public GEO database and not on our samples. However, this approach is recently quite frequently used (Yang and Mei, 2015; Cui et al., 2018; Giummarra et al., 2018). Another limitation of our study design is a lack of parallel analysis of inflammatory markers in the current study. Since the amount of retinal tissue is very limited, we decided to perform extensive chromatographic determinations of kynurenines to meet the main goal of the study. It is noteworthy that the inflammatory aspects of retinal and optic nerve damage in both utilized models were described previously (Thaler et al., 2011).

CONCLUSION

Our results demonstrate imbalance in KP in the insulted retina. The KP response to retinal/optic nerve damage depended on the nature of the insult. In particular mechanical damage to the optic nerve resulted in remarkable decline in retinal KYNA concentration and this response could be explained by differences in the expression of KP enzymes. Our results support the view that development of different strategies targeting the KP and leading to increased KYNA concentration could be beneficial in diseases involving retinal neurodegeneration. Future studies are needed to fully elucidate contribution of KP enzymes to retinal degeneration and its potential for future neuroprotective therapies.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Medical University of Lublin.

AUTHOR CONTRIBUTIONS

MT and RR conceived and designed the study and administered the project as Principal Investigators. MF and TC wrote the first draft of the manuscript. MF, TC, ST, and FS performed the animal experiments. TK and WT performed the biochemical determinations. DN, KW, and MR curated and analyzed the data. MT, AK, WT, PG, EZ, and TA revised and validated the manuscript. All authors gave final approval of the submitted version.

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