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Hippocampal distribution of IL-1β and IL-1RI following lithium-pilocarpine-induced status epilepticus in the developing rat

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ABSTRACT

The contribution of Interleukin-1β (IL-1β) to neuronal injury induced by status epilepticus (SE) in the immature brain remains unclear. The goal of this study was to determine the hippocampal expression of IL-1β and its type 1 receptor (IL-1RI) following SE induced by the lithium-pilocarpine model in fourteen-days-old rat pups; control animals were given an equal volume of saline instead of the convulsant. IL-1β and IL-1RI mRNA hippocampal levels were assessed by qRT-PCR 6 and 24 h after SE or control conditions. IL-1β mRNA expression was increased 6 h following SE, but not at 24 h; however IL-1RI mRNA expression was unaffected when comparing with the control group. IL-1β and IL-1RI immunoreactivity was not detected in control animals. IL-1β and IL-1RI were expressed in the CA1 pyramidal layer, the dentate gyrus granular layer and the hilus 6 h after SE, whereas injured cells were detected 24 h following seizures. Early expression of IL-1β and IL-1RI in the hippocampus could be associated with SE-induced neuronal cell death mechanisms in the developing rat.

Key words: developing rat, IL-1β, IL-1RI, hippocampus, status epilepticus.

INTRODUCTION

Epidemiologic evidence indicates that Status epilepticus (SE) occurs more frequently in children than in adults (DeLorenzo et al. 1995, 1996). Actually, there is an increased incidence of SE in individuals <1-4 years old (Lowenstein et al. 1998). It has been proposed that two-weeks-old rats are comparable with the first years of human life, when children are highly susceptible to seizures (Moshé et al. 1983, Shinnar et al. 1997, Haut et al. 2004). SE produces neuronal cell death in the developing rat brain; however, the intensity of neuronal damage and the brain regions affected follow an age-
dependent pattern (Sankar et al. 1998). In 2 weeks old rats, the CA1 field and dentate gyrus (GD) are the most vulnerable hippocampal regions to the neuronal damage produced by lithium-pilocarpine-induced SE (Sankar et al. 1998, Niquet et al. 2007, López-Meraz et al. 2010), displaying necrotic and apoptotic neuronal cell death morphology, respectively (López-Meraz et al. 2010).

SE causes a neuroinflammatory response in the hippocampus when induced in the immature brain. That response includes an overexpression of astrocytes and activated microglia (Rizzi et al. 2003, Ravizza et al. 2005), as well as an acute gene expression increase of inflammatory cytokines, such as IL-1β, interleukin-1 receptor antagonist (IL-1Ra), tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) (Rizzi et al. 2003, Ravizza et al. 2005, Järvelä et al. 2011). In fifteen-days-old rat pups, SE promotes an increase in IL-1β mRNA levels in hippocampus 4 h after seizures, an effect that disappears at 18 h; whereas gene expression of IL-1Ra, IL-6 or TNFα remain unchanged (Rizzi et al. 2003, Ravizza et al. 2005). Omran and collaborators showed that hippocampal IL-1β is upregulated (mRNA and protein) in 11 days-old rats 2 h after lithium-pilocarpine-induced SE (acute stage), as well as during the chronic stage, when spontaneous seizure occurred (Omran et al. 2012). Similarly, hippocampal tissue from children with medial temporal lobe epilepsy displayed higher expression of IL-1β than control patients (Omran et al. 2012).

In the adult brain, it has been shown that SE also upregulates IL-1β mRNA in hippocampus 2 h after SE (De Simoni et al. 2000, Ravizza and Vezzani 2006); whereas the IL-1β type 1 receptor (IL-1RI) is overexpressed in damaged CA3 field 18 h and 24 h after seizures (Ravizza et al. 2008). Recently, it has been shown that hippocampal concentration of IL-1β is augmented in adult rats after pentylentetrazole-induced chemical kindling; this effect is associated with an increase in the number of IL-1RI immunoreactive cells in dentate gyrus (Kolosowska et al. 2014).

Evidence indicates that seizures increase IL-1β and that its actions are mediated primarily through IL-1RI. Thus, neuronal damage induced by SE may involve the participation of the IL-1β/IL-1RI system. In this study, we investigated whether SE induced in fourteen-days-old (P14) rat pups increases the expression of IL-1β and IL-1RI in injured hippocampal areas. To achieve this goal, IL-1β and IL-1RI were identified in the dorsal hippocampus by immunohistochemical procedures and quantitative RT-PCR, whereas cell death was assessed by Fluoro-Jade B staining.

MATERIALS AND METHODS

ANIMALS

The animals used in this study were Wistar rats derived from animals obtained from Rismart Mexico. The animals were born in our colony (Centro de Investigaciones Cerebrales, Universidad Veracruzana, Mexico). In this study, pups of both sexes were used. The day of birth was considered day zero. Pups were housed with their dams in cages containing aspen chip bedding (Rismart Mexico), with 12 h light-dark cycles (lights on at 0800) and had free access to food (Rismart Mexico) and water. All experiments were conducted during the light period. Experiments were approved by a Committee of Graduate Program in Neuroethology, Instituto de Neuroetología, Universidad Veracruzana and conducted in accordance with Mexican guidelines on the care and use of laboratory animals (NOM-062-ZOO-1999) and Guide for the care and use of laboratory animals (eighth edition, National Research Council 2011).

INDUCTION OF SE

Postnatal-day 13 rat pups were given intraperitoneal injections of lithium chloride (3 mEq/kg; #L-0505 Sigma), and 20 h later, during the P14, SE was
induced with subcutaneous injection of pilocarpine hydrochloride (n=32 rats) (100 mg/kg; #P6503 Sigma) as described previously (López-Meraz et al. Control rats were given an equal volume of lithium chloride and saline instead of the convulsant drug (n=13 rats). Behavioral motor seizures were carefully monitored by an experienced analyst and scored according to the scale proposed by Haas et al. (1990): 0= Behavioral arrest, 1= Mouth clonus, 2=Head bobbing, 3=Unilateral forelimb clonus, 3.5=Alternating forelimb clonus, 4=Bilateral forelimb clonus, 5=Bilateral forelimb clonus with rearing and falling over, 6=Wild running and jumping with vocalizations, 7=Tonus. Only animals reaching SE, defined as near continuous seizure activity lasting over 30 min (Wasterlain and Chen 2006), were included in the study. After SE, pups received 1 ml isotonic 5% dextrose in saline solution subcutaneously to avoid dehydration without stressing the cardiovascular system. After the cessation of seizures, pups were placed back with their mothers (approximately 6 h to avoid cannibalism) or euthanized 6 h following SE onset as described later on. Time of separation from the mother was controlled and similar in control and SE groups. P14 rats tolerated well the pilocarpine dose given, which was effective to induce convulsive SE and neuronal damage as reported previously in literature without mortality (Sankar et al. 1998, Niquet et al. 2007, López-Meraz et al. 2010); no anticonvulsant drug was applied to the rat pups.

IL-1β AND IL-1RI mRNA EXPRESSION BY qRT-PCR

Rats were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 6 h (n=7) or 24 h (n=9) after induction of SE and the whole hippocampus was ice-cold dissected. Matched control animals (n=7) were subjected to a similar procedure. This time points were selected considering that in P14 rat pups, hippocampal neuronal cell death has been detected from 7 to 24 h after SE (Sankar et al. 1998, Niquet et al. 2007, López-Meraz et al. 2010).

RNA was isolated from rat hippocampus with RNA MirVana Paris (AM1556) following the manufacturer’s recommendations (Applied Biosystems, USA). RNA concentration was determined from optical density values at 260 nm obtained with a NanoDrop 1000 (ThermoScientific, USA), and RNA integrity was determined by agarose gel electrophoresis. Reverse transcription reactions were carried out with 1 μg of total RNA in a final volume of 20 μL, using M-MLV reverse transcriptase, according to the manufacturer’s recommendations (Invitrogen, USA). Real time PCR reactions were performed in triplicate, each containing 1 μL of cDNA, 5 pmoles of each primer (forward and reverse), 10 μL of Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, ThermoScientific, USA), and nuclease-free water up to 20 μL. Reactions were run in a 7500 thermal cycler (Applied Biosystems, USA), according to following protocol: 2 min at 50°C, 10 min at 95°C, 40 cycles of 30 sec at 95°C and 1 min at 60°C, followed by a melting curve analysis. Primers were designed with the Primer 3 software (Rozen and Skaletsky 2000), and sequences are shown in Table I. Triplicates for each sample containing primers for β-actin were also included to normalize the data. Amplification efficiencies were obtained with the LinReg program, and changes in relative gene expression were calculated as reported by Pfaffl (2001), using β-actin as internal control.

TISSUE PROCESSING FOR HISTOLOGICAL ANALYSIS

Brain tissue was processed as described previously (López-Meraz et al. 2010). Briefly, rats were anesthetized with an overdose of pentobarbital (100 mg/kg i.p.) 6 h (n=7) or 24 h (n=9) after induction of SE. Matched control animals were subjected to a similar procedure (n=7). Then, pups underwent transcardiac perfusion with 4% phosphate-buffered (PB) paraformaldehyde (#P-6148 Sigma). Brains were kept in situ at 4 °C overnight, after which they
were removed and postfixed in the same perfusate for 2 h. Subsequently, brains were dehydrated, embedded in paraffin and cut into 10-μm-thick coronal sections at the level of dorsal hippocampus. Brain sections were deparaffinized, rehydrated in a graded series of ethanol and dH₂O with a final wash in 0.1 M PB before any procedure.

**FLUORO-JADE B STAINING**

Neuronal injury after SE was addressed by Fluoro-Jade B (F-JB; #AG310, Chemicon) staining. Sections were incubated with 0.06% KMnO₄ (#P-9810, Sigma) followed by 0.001% F-JB and evaluated under fluorescent light. Counting of F-JB-positive cells was performed bilaterally in the CA1-subiculum pyramidal layer, dentate gyrus (DG) granule cell layer and hilus from the dorsal hippocampus on four adjacent sections from 7-9 animals per group as previously described. Brain sections were examined using an Olympus AX70 fluorescent microscope equipped with selective excitation and emission filters.

**LOCALIZATION OF IL-1β AND IL-1RI BY IMMUNOHISTOCHEMISTRY**

Coronal brain sections were subjected to an antigen retrieval procedure by heating slides in a microwave oven in a 10 mM citrate solution (pH 6.0). Then, sections were cooled to room temperature. Slides were rinsed in distilled water, washed in 0.1 M PB for 10 min and incubated in a blocking buffer (0.5% goat serum S-1000, Vector Labs, in 0.1 M PB) at room temperature for 1 h. They were then incubated overnight at 4 °C in a humidified chamber with primary antibody [rabbit polyclonal anti-IL-1β (ab9787 Abcam) or rabbit polyclonal anti-IL-1RI (sc 25775 Santa Cruz)] diluted 1:500 or 1:200, respectively, with blocking buffer (goat serum, S-1000, Vector Labs.). Sections were washed in PB three times and were exposed to biotinylated goat anti-rabbit (BA 1000, Vector Labs) diluted 1:1000 with the blocking solution for 2 h at room temperature, followed by three washes in PB for 10 min, and incubated with ABC-peroxidase complex 1:1000 (Vectastain ABC kit elite DK-6100 standard, Vector Labs) during 90 min. Staining was revealed after incubation with DAB Peroxidase Substrate Kit, 3,3’-diaminobenzidine (SK-4100 Vector Labs). Finally, sections were washed, dehydrated and mounted with non-aqueous mounting medium (Permoun, Fisher) to perform cell counts. A negative control, sections were incubated without primary antibodies. Counts of IL-1β and IL-1RI immunoreactive (IR) cells were performed similarly to injured cell analysis. Brain sections were examined using an Olympus AX70 microscope.

**STATISTICAL ANALYSIS**

Data displaying different variances were analyzed with a Kruskal–Wallis one-way ANOVA (non parametric statistical method) followed by a Dunn’s test; in such cases, results are reported as medians and interquartile range. Those data that followed a normal distribution were analyzed using one-way

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**Table I**

Sequence of primers used to amplify IL-1-b, IL-1RI, and β-actin.

<table>
<thead>
<tr>
<th>Primer sequence from 5’ to 3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1b</strong></td>
<td></td>
</tr>
<tr>
<td>F TGAAGCAGCTATGGCAACTG</td>
<td>200</td>
</tr>
<tr>
<td>R CTGCCCTTCTGAAGCCTCTTG</td>
<td></td>
</tr>
<tr>
<td><strong>IL-1RI</strong></td>
<td></td>
</tr>
<tr>
<td>F TGTGCGCTGAAGACACAGAG</td>
<td>199</td>
</tr>
<tr>
<td>R CGTGACGTTGCAGATCGTT</td>
<td></td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td></td>
</tr>
<tr>
<td>F AGGCTGTGGCTGTCCCTGTAT</td>
<td>201</td>
</tr>
<tr>
<td>R GCTGTGGTGTTGAAGCCTGTA</td>
<td></td>
</tr>
</tbody>
</table>

F=Forward, R=Reverse
ANOVA (parametric statistical method) followed by a Tukey test; in these cases, data are shown as mean ± S.E.M. Statistical significance for all comparisons was considered when p < 0.05. Sigma Stat 3.5 (Systat Software Inc.) or Prisma Graphpad 5 (GraphPad Software Inc.) were used to perform statistical analysis or graph editing.

RESULTS

CONVULSIONS

All animals injected with pilocarpine developed generalized motor seizures scored as stage 5.1 ± 0.2 and reached SE. Latency to SE was 10.7 ± 0.5 min, and duration of behavioral SE was 5.4 ± 0.1 h. Thus, all the rats from the SE group were included in the study.

IL-1β AND IL-1RI mRNA EXPRESSION IN RAT HIPPOCAMPUS

One way ANOVA analysis showed differences in IL-1β mRNA expression after SE [F(2,20)=5.227, p=0.01]. IL-1β mRNA expression was increased 6 h following SE when comparing with the control group and 24 h after seizures group (1455% and 245% fold higher than control group, respectively; p<0.05). Levels of IL-1RI mRNA showed a similar pattern; however there was not a statistically significant difference between groups [F(2,18)=2.705, p=0.23] (Figure 1).

IL-1β AND IL-1RI CELL EXPRESSION AND NEURONAL CELL DEATH IN HIPPOCAMPUS

In this section, results are described by hippocampal region, comparing the immunolocalization of IL-1β and IL-1RI and the presence of injured cells. IL-1β or IL-1RI IR cells were not detected in control animals at any hippocampal area. Neither neuronal damage nor IL-1β/IL-1RI immunoreactivity was observed in CA2 or CA3 subfields in P14 rat pups following SE.

In CA1 pyramidal layer, one way ANOVA analysis showed that SE promoted IL-1β [H(2)=16.34, p<0.001] and IL-1RI [H(2)=16.70, p<0.001] cellular expression. IL-1β and IL-1RI IR cells were detected 6 h after SE (p<0.01), but not 24 h after seizures, when expression returned to control levels. F-JB positive injured cells were detected [H(2)=18.76, p<0.001] at 24 h (p<0.01) but not 6 h after SE onset in the CA1-subiculum field (Figure 2 and 3).

DG showed a similar pattern of IL-1β and IL-1RI expression and cellular injury than the CA1 area. IL-1β [F(2,20)=75.32, p<0.001] and IL-1RI [F(2,20)=21.33, p<0.001] cellular expression was...
increased 6 h after SE onset (p<0.01) in the granule cell layer. Twenty-four h after SE, the number of cells immunoreactive to IL-1β and IL-1RI in DG returned to control levels. F-JB positive injured cells were detected in DG [H(2)=20.05, p<0.001] only 24 h (p<0.01) but not 6 h after SE onset (Figure 2 and 3).

In hilus IL-1β [F(2,20)=85.48, p<0.001] and IL-1RI [F(2,20)=66.36, p<0.001] cellular expression was increased 6 h after SE (p<0.01). Then, 24 h
following SE the number of cells that expressed IL-1β or IL-1RI returned to control levels. By contrast, in the hilus there was an increase in the number of injured cells \([F(2,20)=22.20, p<0.001]\) 6 h after SE onset \((p<0.01)\), effect that persisted 24 h after SE \((p<0.01)\) (Figures 2 and 3).

**Figure 3** - Images of Interleukine-1β (IL-1β), type 1 IL-1 receptor (IL-1RI) and Fluoro-Jade B (F-JB) positive cells in the CA1 pyramidal layer, dentate gyrus (DG) granule cell layer and hilus from dorsal hippocampus in control conditions and 6 h following status epilepticus (SE). Arrows point immunoreactive (dark cells) or F-JB positive cells (white cells) in every hippocampal area 6 h following SE. Control hippocampus does not show immunoreactive or injured cells. Abbreviations: pl, pyramidal layer, gl, granule cell layer. Scale bars = 50 µm.
DISCUSSION

The main finding of our study is to demonstrate that SE in the immature rat brain induces an early IL-1β and IL-1RI expression in CA1 area, DG and hilus, the same hippocampal subfields where neuronal injury was detected. Our data suggest that the localization of these neuroinflammatory markers differ depending on the hippocampal area. We found an increase in the number of IL-1β and IL-1RI IR cells 6 h after SE in CA1, hilus and DG, but not at 24 h after seizures, when injured neurons were detected. These data support the contribution of IL-1β on neuronal cell death mechanisms promoted by SE.

Prior studies in developing animals have investigated the pattern of mRNA expression of the IL-1 family cytokines in the hippocampus after SE. However information about the hippocampal distribution of these cytokines after SE at early ages is limited. Experimental findings suggest that the inflammatory process activated in the hippocampus can be associated with cell death mechanism triggered in this brain area after SE (Rizzi et al. 2003, Ravizza et al. 2005). Our first results demonstrated that SE promotes an increase of IL-1β mRNA expression in the hippocampus, effect that is observed as an acute episode, since it is found 6 h but not at 24 h after seizures. No changes in IL-1RI mRNA level were observed after SE. Our results are in concordance with previous studies displaying that mRNA of IL-1β is augmented 2-4 h after kainic acid, lithium-pilocarpine or soman-induced SE in rat pups (Rizzi et al. 2003, Ravizza et al. 2005, Järvelä et al. 2011, Omran et al. 2012). Thus, it seems that under our experimental conditions, 6 h after SE could be a time point where hippocampal IL-1β gene expression was already increased to promote the synthesis of new protein to be expressed in specific hippocampal fields. Additionally, the regulation of IL-1RI gene and protein expression could have a different time course, so that it was possible to detect an increase in the protein but not in the mRNA. A previous report showed that IL-1RI mRNA was increased in the cortex, amygdala and hippocampus from kindled adult rats 2 h following convulsive seizures (Plata-Salamán et al. 2000). Thus, this information suggests that SE activates an inflammatory response in the hippocampus where the IL-1β/IL-1RI system could be required at initial stages of damaging processes such as neuronal cell death.

Previously it was shown that IL-1β mRNA expression is increased in hippocampus displaying gliosis and active microglia after SE in developing rats (Rizzi et al. 2003; Ravizza et al. 2005). Our results also show that depending on the hippocampal area, IL-1β or IL-1RI IR cells seems to have a distinct pattern of expression. In the CA1-subiculum pyramidal layer, cells express IL-1β and IL-1RI, which could imply a paracrine or autocrine signaling. In fact, it has been shown that CA3 hippocampal neurons are capable of expressing IL-1β and IL-1RI 18 h after SE induced in adult rats (Ravizza et al. 2008). A different pattern occurs in DG and hilus, where there was a smaller number of IL-1β and IL-1RI IR cells. In these areas, it seems that IL-1β was expressed by microglia and IL-1RI by neurons. However, additional double-labelling experiments are necessary to confirm the phenotype of this IR cells. A similar increase in the number of IL-1RI IR cells was observed in DG granule layer after fully kindled seizures in adult rats (Kołosowska et al. 2014). Then, this evidence shows that hippocampal expression of IL-1RI is susceptible to be modified by seizures in both immature and adult brains, which in turn can mediate the physiological effects of IL-1β.

Results from our immunohistochemical analysis evidenced the hippocampal subfields where IL-1β and IL-1RI proteins were found following SE in P14 rat pups. IL-1β and IL-1RI IR cells were detected in the CA1-subiculum pyramidal layer, DG inner granular layer and hilus, hippocampal...
areas where injured cells were also observed, as previously reported (Sankar et al. 1998, Niquet et al. 2007, López-Meraz et al. 2010). These data supports that SE induced in the developing rat promotes a microenvironment that might favor neuronal cell death in the hippocampus and that may includes the acute and transient expression of IL-1β and IL-1RI, as shown here and by others (Rizzi et al. 2003, Ravizza et al. 2005, Järvelä et al. 2011, Omran et al. 2012, Kołosowska, et al. 2014), glia activation (Rizzi et al. 2003), augmented expression of other inflammatory cytokines (Rizzi et al. 2003, Ravizza et al. 2005, Järvelä et al. 2011) and increased hippocampal excitability (Naylor et al. 2013). IL-1β could interact with IL-1RI and trigger the extrinsic pathway of cell death resulting in necrotic neuronal cell death in the CA1 area. Previously, it was showed that Caspase-8 is overexpressed in CA1 area after SE in P14 rat pups (López-Meraz et al. 2010) and this caspase participates in the maturation of IL-1β (Maelfait et al. 2008), suggesting that SE may implicates per se a facilitated sustained inflammatory response in this hippocampal area. A recent study shows that i.c.v. injection of IL-1β increases necrotic neuronal cell death in the CA1 hippocampal area following SE in P14 rat pups, an effect that was mediated through the activation of IL-1RI (Medel-Matus et al. 2014). Nevertheless, it has not been demonstrated yet if the exogenous administration of IL-1β induces a similar effect in DG or hilus. Therefore, most of the evidence shows that a transient increase of IL-1β and IL-1RI does occur in areas destined to die or present injury, but there is not a clear mechanisms explaining why there is a long delay from the time the cytokine goes up to the time the injury first appears. Additional functional studies are needed to probe whether the activation of the IL-1β/IL-1RI system has a causative role in cell death due to seizures in rat pups.

In conclusion, results from this study demonstrated a transient increase in IL-1β and IL-1RI that precedes the neuronal injury observed in hippocampal areas (CA1, dentate gyrus and hilus) after SE in the developing rat. This findings support the importance of the IL-1β/IL-1RI system as a possible therapeutic target to prevent or reduce neuronal damage after SE in the immature brain, similar to what has been illustrated before by using different models of epilepsy and rats of different ages (Vezzani et al. 2011, Omran et al. 2012, Noe et al. 2013).

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The authors declare no conflict of interests.

RESUMO

A contribuição de Interleucina-1β (IL-1β) para a lesão neuronal induzida pelo estado epiléptico (SE) no cérebro imaturo ainda permanece obscuro. O objetivo deste estudo foi determinar a expressão do hipocampo de IL-1β e seu receptor tipo 1 (IL-1RI) após SE induzido pelo modelo de lítio-pilocarpina em ratos com quatorze dias de idade; animais controle receberam um volume igual de soro fisiotélico em vez do agente convulsivo. Níveis de hipocampo de IL-1β e IL-1RI foram avaliados por qRT-PCR 6 e 24 h após a condições SE ou de controlo. A expressão IL-1β mRNA e expressão de IL-1RI foi detectada no hipocampo dorsal, através de procedimentos de imunohistoquímica. A expressão IL-1β de mRNA foi aumentada 6 h após a SE, mas não para as 24 h; no entanto a expressão de IL-1RI de ARNm não foi afetada quando comparada com o grupo controle. A imunorreatividade de IL-1β e IL-1RI não foi detectada em animais usados como controle. IL-1β e IL-1RI foram expressos na camada piramidal CA1, a camada
granular após 6 h do estado epiléptico, enquanto que as células lesadas eram detectadas 24 h após as convulsões. A expressão precoce de IL-1β e IL-1RI no hipocampo pode ser associada com os mecanismos de morte celular neuronal induzida pelo SE com ratos em desenvolvimento.

**Palavras-chave:** rato em desenvolvimento, IL-1β, IL-1RI, hipocampo, estado epiléptico.

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