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The Activation of Excitatory Amino Acid Receptors Is Involved in tau Phosphorylation Induced by Cold Water Stress*

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Abstract In order to investigate whether excitatory neurotransmission system takes roles in tau phosphorylation caused by cold water stress, mice were treated with cold water stress (CWS), which were forced to swim at 4℃ for 5 min. The tau phosphorylation in mice brains was analyzed by immunobloting and immunohistochemistry with c-fos and phosphorylation-dependent tau antibodies. To evaluate the imbalance of excitatory or inhibitory neurotransmitters system, HPLC was used to detect amino acid neurotransmitters in brain after CWS. And the phosphorylated tau in brains of CWS mice, which were pre-treated with different antagonists for excitatory amino acid receptors and L-type calcium channel, was analyzed. The phosphorylated tau in hippocampus was significantly increased accompanied by an increase of c-fos expression at 1 h after CWS. HPLC showed that the content of all detected excitatory and inhibitory amino acid neurotransmitters appeared an acute increase then decrease pattern. At 15 min after CWS, aspartate and glycine appeared a significant increase, and aspartate, glutamate, taurine and GABA significantly decreased at 1 h. When the animals were pre-treated with NMDA receptor antagonist MK-801 (5 mg/kg) and AMPA receptor antagonist DNQX (0.5, 5 mg/kg), tau phosphorylation caused by CWS were significantly suppressed. Whereas, metabolic glutamate receptor antagonist, MAP-4, had no significant effect on tau phosphorylation. In addition, L-type calcium channel blocker, nimodipine (0.05, 0.5 mg/kg), also could inhibit CWS caused tau phosphorylation. These results indicated that CWS affects tau phosphorylation by mediating excitatory neurotransmission system through ionic excitatory amino acid receptors. The activation of excitatory neurotransmission system takes roles in CWS induced tau phosphorylation in hippocampus.

Key words cold water stress, tau phosphorylation, excitatory amino acid neurotransmitter, HPLC
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Tau is a mainly but not exclusively neuronal microtubule-associated protein. Tau takes roles in stabilization of axonal microtubules, signal transduction, interaction with actin cytoskeleton, neurite outgrowth and regulation of intracellular vesicle transport[1–5]. In Alzheimer′s disease (AD), tau in brain is phosphorylated at a number of sites resulting in reduced ability to bind microtubules [6]. Moreover, aberrantly hyperphosphorylated tau aggregates into neurofibrillary tangles (NFT) in AD brains[7–8].

Although, the toxic effect of β-amyloid is considered as an important reason for tau protein phosphorylation in AD brains[9], discovery of mutations in tau gene as causes of a form of dementia (FTDP-17) has leaded increasing attention to tauopathy as a possibly central pathogenetic process in a number of neurodegenerative diseases[8]. Recently, stress has been linked to AD, because a higher susceptibility to distress led to increase the risk of AD[10–11]. Besides, it has been observed that the different acute stressful stimuli such as cold water swimming (CWS) and food deprivation, caused tau phosphorylation in brains of mouse, with characteristics possibly relevant to AD [9, 12–14]. These studies infer that stressors play roles

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in the AD pathogenesis.

The glucocorticoid cascade hypothesis is usually used to explain the hippocampus injury caused by stress [15-16]. However, in stress, tau phosphorylation caused by CWS seems not related to the over-activity of the hypothalamic-pituitary-adrenal axis, because interfering glucocorticoid system did not affect CWS induced tau phosphorylation [13]. Therefore, the mechanisms of CWS induced tau phosphorylation in brain remains elusive. A body of evidence suggested that excitotoxic effects evoked by excessive or prolonged activation of the excitatory amino acid receptors may be involved in pathogenesis of brain damage in acute insults or in chronic neurodegenerative diseases [17]. Meanwhile, the results of *in vitro* studies have demonstrated that glutamate influences the phosphorylation of tau in primary cultured neurons [18]. Considering the activation of glutamate system under stress conditions [19], in this study, we explored whether activation of glutamate receptors is involved in CWS caused tau phosphorylation.

1 Materials and methods

1.1 Reagents

Antagonists of glutamate receptors including 5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten-5, 10-imine maleate (MK-801), 6, 7-dinitroquino xaline-2,3-dione (DNQX) and (+)-2-amino-2-methyl-4-phosphonobutanoic acid (MAP-4) were purchased from Sigma (St. Louis, USA). MK-801, DNQX and MAP4 were dissolved in 0.1 mol/L phosphate buffer saline (PBS, pH7.4). Nimodipine was purchased from Shandong Xinhua Pharmaceutical Co, LTD. Anti-total tau antibody, anti-phosphorylated tau polyclonal antibody P202, anti-actin polyclonal antibody and c-fos polyclonal antibody were purchased from Sigma. Anti-phosphorylated tau monoclonal antibody C5 was kindly offered by Dr. Gu [20]. Second antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

1.2 Animal treatment and reagents administration

Male Chinese Kunming mice (25.0 ± 2.0 g; from the Experimental Animal Center of Fudan University, Shanghai, China) were housed in groups of four animals per cage and given food and water ad libitum. Mice were maintained at 23 ℃ and under the light period of 8∶00～20∶00. Cold water stress was given between 13∶00 and 18∶00. Mice were immersed up to the neck in ice-cold water of 5 cm depth in a 16 cm diameter container for 5 min, after then they were gently wiped dry and released in a new cage. At different time point after CWS, mice were sacrificed by cervical dislocation or intracardial perfusion under anesthesia condition for Western blotting or immunohistochemistry respectively. To interfere in CWS-induced tau phosphorylation, mice were injected intraperitoneally with the regents such as MK801, DNQX, MAP-4 or Nimodipine, 15 min before CWS performance. The mice injected with vehicle solution were used as controls.

1.3 Western blotting

The brains was removed immediately after sacrifice. After rinsing with ice-cold saline, hippocampus were dissected immediately and homogenized in 50 mmol/L Tris-HCl (pH 7.4) buffer containing 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L NaF, 0.5 mmol/L orthovanadate, 1 mg/L leupeptin, 1 mg/L aprotinin, 0.2 mmol/L PMSF, 1 mmol/L EDTA and 1 mmol/L EGTA. Homogenates were centrifuged at 12 000 *g* for 10 min at 4℃. The supernatants obtained were immediately placed in boiling water for 10 min. After recentrifugation at 12 000 *g* for 15 min at 4℃, the heat-stable supernatants were collected. Protein concentration was determined after 20 times dilution with saline by the Bio-Rad Protein Assay with bovine serum albumin (BSA) in lysis buffer as standard. Samples (20 μg protein/lane) were run on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked with tris-buffered saline containing 0.1% Tween-20 (TBST) and 10 % (*w/v*) skim milk for 1 h at room temperature, followed by incubation with primary antibodies for 1 h at room temperature and then incubated overnight at 4℃, washed in TBST, and then incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. Immunoreaction was visualized through ECL.

1.4 Immunohistochemistry

Animals were anesthetized with 10% chloral hydrate and perfused intracardially with 0.9% saline solution followed by ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brains were removed, immersed in 30% sucrose at 4℃ until sinking, and then the horizontal sections were cut at 30 μm on freezing microtome. Immunohistochemistry was performed in accordance with the free-floating method. Briefly, the sections were blocked in PBS containing 5% normal bovine serum and 0.2% Triton X-100 for 45 min, and then incubated with primary anti-C5 antibody (diluted 1 ∶ 200) overnight at 4℃. After rinsing in PBS, the sections were incubated with biotinylated anti-mouse IgG (1 ∶ 200) for 1 h at 37℃, followed by incubating in 1 ∶ 200 diluted avidin-biotin peroxidase complex for 1 h at 37℃. The peroxidase reaction was detected with 0.05% diaminobenzidine in 0.1 mol/L Tris-HCl buffer (pH 7.4) and 0.01% H_2O_2. As controls, sections received identical treatment except for incubation with the primary antibody and showed no specific staining. For double immunostaining, slices were sequentially incubated with anti-C5 antibody(1 ∶ 200) and anti-c-fos antibody (1 ∶ 500), and then with FITC and Texas Red-conjugate secondary antibodies (1 ∶ 200).

1.5 Measurement of amino acids by high performance liquid chromatography

The animals were killed by decapitation and the head snap frozen in liquid nitrogen. Hippocampus were dissected and weighed. Brain tissue was homogenized in 7% (*w*/*v*) perchloric acid and centrifuged at 4 000 *g* for 5 min. The procedure was repeated with dH_2O, the supernatants were pooled and neutralized with 1 mol/L KOH. High performance liquid chromatography (HPLC)-fluorometric system analysis to determine the amino acids including aspartate, glutamate, glycine, GABA and taurine were carried out using the Gold System from Beckman (Palo Alto, CA, USA) with fluorescence detection. Briefly, samples were derivatizated with o-phthaldialdehyde and separation on an Ultrasphere ODS column from Beckman (Fullerton, CA, USA) using 0.1 mol/L KH_2PO_4 and methanol as eluents. The fluorescence was measured at an excitation of 280 nm and an emission of 340 nm. The amino acid content was quantified from standard solution, and expressed as percentage of normal control samples.

1.6 Statistical analysis

All values were expressed as $\bar{x} \pm s$ and analyzed using Microsoft Excel 2000. Comparison between two experimental groups was made using Student's *t*-test, $P < 0.05$ was considered significant.

2 Results

2.1 Tau phosphorylation after cold water stress accompanied by c-fos expression

The mouse after CWS appeared static and stiff. If the tail was placed to direct upward, it will be kept to leave floor for 2～3 min (Figure 1a). As reported before[9, 12-13], the phosphorylated tau in hippocampus was significantly increased at 1 h after CWS stimulus, as detected by Western blot with phosphorylation dependent anti-tau antibodies, P202 and C5, which recognized the phosphorylated epitope at Ser202 and Ser396 of tau protein respectively (Figure 1c). Moreover, the expression of c-fos which is an indicator for neuronal activation also increased[21]. Immunostaining also showed that phosphorylated tau and c-fos were co-labeled in granular cell layer and CA3 region neurons in hippocampus after CWS (Figure 1b), implying tau phosphorylation after CWS related to neuronal activation.

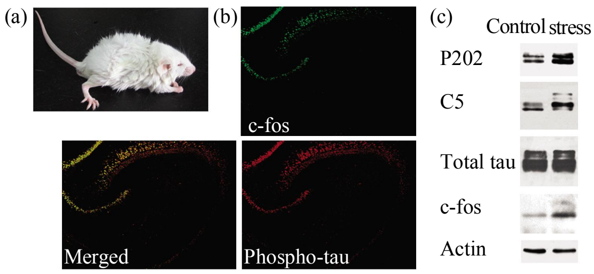

Fig. 1 Cold water stress induced tau phosphorylation and c-fos expression

The mouse has been stimulated with CWS (a). At 1 h after CWS, the distribution and expression of phosphorylated tau and c-fos in horizontal section of hippocampus was analyzed by immunofluorescence (b) and immunoblotting (c). P202 and C5 are anti-phosphorylation-dependent tau antibodies.

2.2 Amino acid transmitters in brain after cold water stress stimulation

For analyzing whether there were changes in the content of excitatory amino acid neurotransmitters in brain, we used HPLC to analyze them in hippocampus of CWS mice. HPLC detection showed that excitatory amino acid transmitters, glutamate and aspartate were decreased at 1 h after CWS, at this time point tau was phosphorylated significantly, although inhibitory amino acid transmitter such as GABA and taurine were also decreased. Then amino acid neurotransmitters in brain were checked at 15 min after CWS. At this time point, aspartate was significantly increased, whereas the increase of glutamate was not significant. The increase of GABA and taurine were also not significant at 15 min after CWS, but glycine is significantly increased (Figure 2).

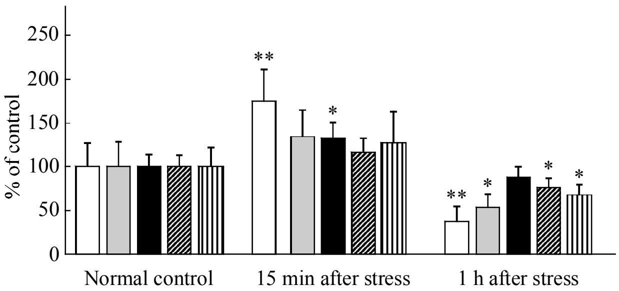

Fig. 2　The level of amino acid neurotransmitters in hippocampus after cold water stress were detected by HPLC

The statistically significant differences *vs* the control samples were marked with $^*P < 0.05$ and $^{**}P < 0.01$. Data were expressed as $\bar{x} \pm s$. The detailed numbers of individuals were: control mice, 5; stressed mice at 15 min after CWS, 5; stressed mice at 1 h after CWS, 4. □: Aspartate; ▨: Glutamate; ■: Glycine; ▨: Taurine; ▥: GABA.

2.3　The effect of the glutamate receptor antagonists on CWS caused tau phosphorylation

For convincing whether tau phosphorylation after CWS is related to excitatory neurotransmission system, the effect of glutamate receptor antigonists on CWS induced tau phosphorylation was tested. Immunoblotting results showed MK-801, an inhibitor of NMDA receptor, significantly reversed CWS caused tau phosphorylation with dosage of 5 mg/kg body weight (Figure 3a). Meanwhile, an AMPA/KA receptor antagonist DNQX also reversed CWS caused tau phosphorylation with dosage of 0.5 and 5 mg/kg (Figure 3b). When using MAP-4, a blocker of metabotrophic glutamate receptor, tau phosphorylation caused by CWS was not significantly affected (Figure 3c, 4e). Moreover, immunohistochemistry results confirmed the suppressing effect of MK-801 and DNQX on CWS caused tau phosphorylation (Figure 4c, d). These results indicated that the activation of ionic glutamate receptors were involved in CWS induced tau phosphorylation.

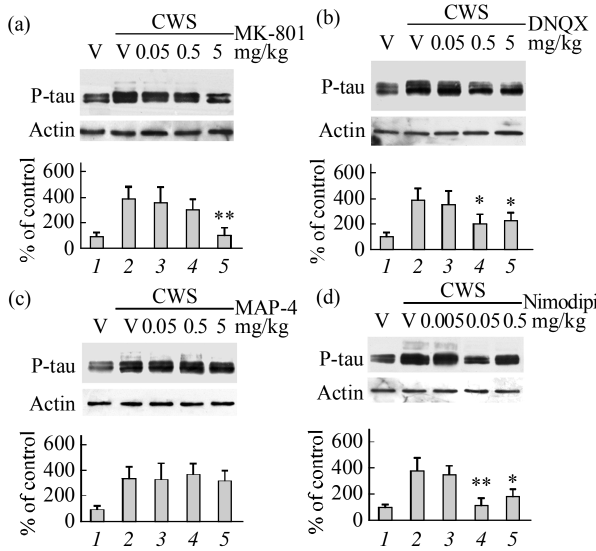

Fig. 3　The effect of antagonists of excitatory and inhibitory amino acid receptors and L-type calcium channel on cold water stress (CWS) induced tau phosphorylation in hippocampus

Data were expressed as $\bar{x} \pm s$ ($n = 3$). The statistically significant differences *vs* the samples from vehicle injected mice (V) marked with $^*P < 0.05$ and $^{**}P < 0.01$. *1*: Normal control mice were injected with vehicle; *2*～*5*: The CWS mice were pre-injected with vehicle or different dosage of drugs which are indicated in figure.

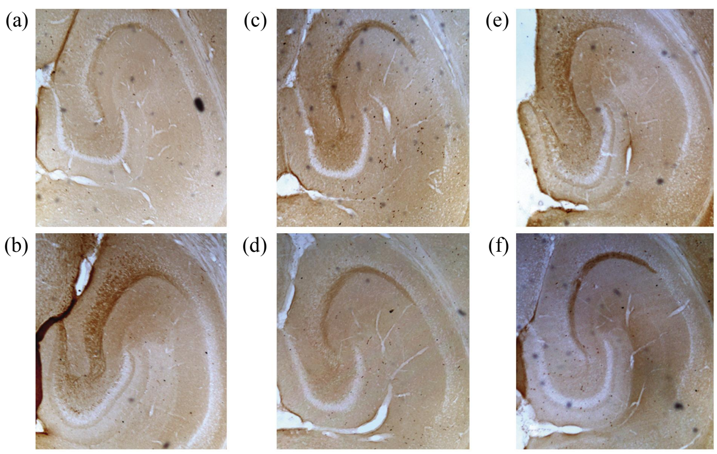

Fig. 4　Immunohistochemical staining showed the effect of excitatory and inhibitory amino acid receptors and L-type calcium channel on phosphorylated tau after cold water stress (CWS)

A phospho-tau specific antibody C5 was used to stain the horizontal sections of hippocampus, which from vehicle control mice (a), mice sacrificed at 1 h after CWS (b), preinjected with MK-801, a NMDA receptor antagonist(5 mg/kg, c), DNQX, an AMPA receptor antagonist (0.5 mg/kg, d), MAP-4, a metabolic glutamate receptor antagonist (5 mg/kg, e) and nimodipine, a L-type calcium channel blocker (0.5 mg/kg, f).

2.4 Abnormal excitation in brain related to tau phosphorylation caused by CWS

Tau phosphorylation could be mediated through calcium signaling, and the activation of L-type calcium channel has been linked to tau phosphorylation. In this study, for exploring the relationship between abnormal excitation in brain caused by CWS stimulation and tau phosphorylation, a L-type calcium channel blocker nimodipine was employed. Immunoblot analysis and immunohistochemistry showed that nimodipine (0.05, 0.5 mg/kg) was able to inhibit tau phosphorylation caused by CWS (Figure 3d, 4f).

3 Discussion

Phosphorylated tau protein accumulates in paired helical neurofilaments, the major constituent of neurofibrillary tangles observed in the brain of patients suffering AD [7–8]. The phosphorylation of tau in brain was directly linked to the functional change of memory and neurodegeneration [21]. Recently, psychological distress has been considered as a risk factor for AD, it is also been linked to the impairment of memory in AD patients [10–11]. The abnormal changes of tau in hippocampus after stress, which were observed in this study and reported data[9, 12–13], supported the relationship between stress and tauopathies. Because CWS induces a similar increase of tau phosphorylation in adrenalectomized and in cortisone subchronically treated animals comparing to intact animals [13], CWS caused tau phosphorylation seems independent of the activity of hypothalamic-pituitary-adrenal axis. The detailed mechanisms of tau phosphorylation in CWS model are poorly understood.

Accumulated data indicated that excitatory toxicity of glutamate neurotransmitter is related to neurodegenerative diseases including AD [17]. Experimental studies have linked excitotocity to tau phosphorylation[18]. The expression of "immediate early genes" such as c-fos is related to glutamate receptors mediated neurodegeneration under pathological conditions, and associates with neurofibrillary tangles, tau pathological change [22–23]. These observations committed our result of the relationship between c-fos expression and tau phosphorylation induced by CWS.

In this study, both excitatory and inhibitory amino acid neurotransmitters in hippocampus appeared an acute increase then decrease pattern after CWS stimulation. It should be noted that the detected neurotransmitters in this study were from extracts of brain tissue. So, the results reflected content of neurotransmitters in brain tissue, but not the release of neurotransmitters. However, our results were comparable to Engelmann's microdialysis study, which illustrated the changes of neurotransmitter content in extracellular of hypothalamic supraoptic nucleus following forced swimming[24]. At 15 min after CWS, aspartate and glycine appeared a significant increase. Glutamate also appeared an increase, but did not reach significance. Here, glycine might also take a role in modulating tau phosphorylation *via* NMDA receptors[25]. Although the detected amino acid neurotransmitters decreased at 1 h after CWS, at which time the level of phosphorylated tau was increased significantly. Notably, inhibitory amino acids including taurine and GABA decreased slower than excitatory amino acids at 1 h. Therefore, we argued that other mechanisms are also involved in the activation of excitatory neurotransmission. For instance, CWS might affect the affinity and density of glutamate receptors, because acute swim stress increased the binding of MK-801 to the NMDA subclass of glutamate receptors in brain[26].

For verifying glutamate receptors are involved in CWS related tau phosphorylation, different antagonists for glutamate receptors were used in this study. Our results showed that MK-801 and DNQX, antagonists for NMDAR and AMPA/KA respectively, suppressed CWS caused tau phosphorylation, while an antagonist of metabotropic glutamate receptor MAP4, showed no obvious effect on that. These results indicated that ionotropic glutamate receptors take roles in the process of tau phosphorylation induced by CWS. In addition, recent documents indicated that pharmacological activation of mGlu4 metabotropic glutamate receptors reduces neuronal degeneration[27], which is in agreement with no beneficial effect of mGluR4 antagonist MAP-4 on CWS caused tau phosphorylation. The imbalance of protein kinases and protein phosphatases activity is the direct reason for tau overphosphorylation in AD brain. Several tau phosphorylations related protein kinases were activated following CWS [9]. Moreover, it was found that glutamate inhibits protein phosphatases [28], implying that the inhibition of phosphatases might be also involved in CWS caused tau phosphorylation.

Stimulation of neurons *via* NMDA caused transient $[Ca^{2+}]_i$ responses and potential $[Ca^{2+}]_i$ responses [29]. Neuronal L-type calcium channel open quickly and admit Ca^{2+} influx into neuron during period of strong depolarization [30]. Because there was a relationship between intracellular calcium and tau phosphorylation[31], it was not surprised that the antagonist of L-type calcium channel inhibited CWS caused tau phosphorylation.

Taken together, our data indicated that the overactivation of excitatory neurotransmission is involved in CWS caused tau phosphorylation. Our results suggested that the antagonists of ionotropic glutamate receptors and L-type calcium channel could be used in inhibiting CWS caused tau phosphorylation with certain dosage. However, it was still unclear what kinds of these antagonists are most effective for suppressing CWS caused tau phosphorylation. Moreover, whether these antagonists have long-term beneficial effects for brain after stress warrants careful examination.

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兴奋性氨基酸受体的激活介导冷水应激诱导的tau 蛋白磷酸化 *

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摘要 为探讨兴奋性神经传递系统是否参与冷水应激引起的 tau 蛋白磷酸化，将小鼠于 4℃ 冷水应激 5 min．采用免疫印迹和免疫组织化学方法分析应激后脑内 c-fos 和磷酸化 tau 蛋白的表达情况；运用 HPLC 检测冷水应激后小鼠脑内兴奋性或抑制性神经递质的变化；同时分析兴奋性氨基酸受体和 L- 型钙通道拮抗剂预处理后冷水应激小鼠脑内磷酸化 tau 蛋白的水平．冷水应激后 1 h，海马内磷酸化 tau 蛋白的水平显著升高，同时伴 c-fos 的染色增加．HPLC 检测显示，兴奋性和抑制性神经递质呈现急剧上升而后又下降的趋势．冷水应激后 15 min，天冬氨酸和甘氨酸水平显著升高，1 h 后天冬氨酸、谷氨酸、牛磺酸和 γ- 氨基丁酸显著下降．NMDA 受体拮抗剂 MK-801(5 mg/kg)和 AMPA 受体拮抗剂 DNQX(0.5, 5 mg/kg)可显著抑制冷水应激引起的磷酸化 tau 蛋白水平的升高，代谢性谷氨酸受体拮抗剂 MAP-4 不影响 tau 蛋白的磷酸化，另外， L- 型钙通道阻断剂尼莫地平可抑制冷水应激引起的磷酸化 tau 蛋白水平的升高．这些结果表明，冷水应激可影响兴奋性神经传递系统，通过离子型兴奋性氨基酸受体和异常神经激活来调节 tau 蛋白的磷酸化．兴奋性神经传递系统的激活在冷水应激诱导的海马 tau 蛋白的磷酸化中发挥作用．

关键词 冷水应激，Tau 蛋白磷酸化，兴奋性氨基酸神经递质，HPLC

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