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Age-related decrease in aromatase and estrogen receptor (ERalpha, ERbeta, GPR30) expression on female rat hippocampus: protective effect of memory impairment during aging

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It is well known that local hippocampal estradiol (E2), mainly converted from testosterone (T) by aromatase, has been shown to play important roles in the regulation of learning and memory through action on synaptic plasticity. Although our previous study showed that age-related memory impairment is the effect of decreasing the hippocampal E2 level and its receptor (estrogen receptor, ER) expression, the age-related changes in the steroidogenesis remain largely unknown. This study is designed to examine the expression of aromatase, level of T, and expression of its receptor (androgen receptor, AR) in aged female rat brains of 2-, 5-, 10-, and 19-month-old. The results showed that T levels in the hippocampus were progressively and significantly increased with age (19-month-old), whereas the E2 levels were significantly decreased with age. Consistent with the pattern of E2 level, the expression of aromatase was significantly decreased with age. Moreover, we also showed a significantly increase of AR, but significantly decreased of ERα, ERβ, and G protein-coupled receptor1 (GPR30) expression in aged hippocampus. Since levels of aromatase activity indicating local synthesis of E2 in the brain, we conclude that decreased E2 in aged brain is aromatase dependent manner, leads to an impairment of spatial memory acquisition.

Keywords: Aromatase; Estradiol; Estrogen receptor: Testosterone; Aging brain

INTRODUCTION

Age-associated memory impairment has been considered a part of the normal aging. Female sex steroid hormones, estradiol (E2), play a key role in memory processing (Gale et al., 2016; Sasaki Russell et al., 2019). The elevating E2 levels enhances memory acquisition, and when decreasing E2 levels, memory is impaired (Daniel et al., 2006; Ooishi et al., 2012). In hippocampus, E2 increases synaptic plasticity, synaptic transmission, and the magnitude of long-term potentiation (Smith and McMahon, 2006; Oberlander and Woolley, 2016). The underlying mechanism of memory formation and maintenance is synaptic plasticity. Therefore, E2-mediated alteration in synaptic plasticity directly linked to hormone-induced memory acquisition and consolidation (Smith & McMahon, 2006; Tuscher et al., 2016). Although, the beneficial effect of E2 treatment is age-dependent manner (Gibbs, 2000), it mechanisms are poorly understood. Action of E2 occurs via estrogen receptor (ER) α and ERβ, which significantly decrease in aged hippocampus (Hojo et al., 2011; Chamniansawat and Sawatdiyaphanon, 2018). In

addition to the classical ER (ERα and ERβ) pathways, G protein-coupled receptor1 (GPR30) was recently shown to mediate the effects of E2 (Marcello & Didier, 2010). Within pyramidal hippocampal neurons, GPR30 expression has been generally associated with post-synaptic scaffolding proteins (Akama et al., 2013; Waters et al., 2015). It has been shown in ovariectomized rats to mediate spatial memory acquisition (Zhang et al., 2019). However, the expression GPR30 on aging brain still unknown.

Hippocampus is a center of learning and memory, hippocampal synthesis of sex steroid hormones is very attractive. Local hippocampal E2 production depends in large part on the enzymatic conversion of testosterone (T) to E2 by aromatase, a member of the cytochrome P450 family (Kimoto et al., 2010; Prange-Kiel et al., 2016). Moreover, the localization of other steroidogenic enzymes, including cytochrome P450scc and steroidogenic acute regulatory protein (StAR), was demonstrated in adult male and female hippocampus (Hojo et al., 2011; Chamniansawat & Sawatdiyaphanon, 2018). Expression of aromatase mRNA and protein decreased in Alzheimer's disease (AD) patient and a mouse model for AD (Prange-Kiel et al., 2016). Dendritic spines are up-regulated by T in gonadectomized rats. This could occur via aromatization of androgens to E2, by aromatase (Leranth et al., 2004). Although our previous study showed a decreased hippocampal E2 in aged female rat.

MATERIALS AND METHODS

Animals

Female Sprague–Dawley rats were purchased from National Laboratory Animal Center, Mahidol University, Thailand) and certified by the vendor to be free of rodent bacterial, viral, and parasitic pathogens. All animal use and accompanying procedures were in accordance with The Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee on Animal Experiment of Burapha University, Thailand. Rats were grouped into 2-, 5-, 10-, and 19-months old. The animals were kept under controlled conditions a 12 h/12 h dark/light cycle. Access to food and water was provided ad libitum. At the end, animals were anesthetized and decapitated. And then the brain was harvested by rapidly dissection and immediately frozen at -80 °C for Western blot and ELISA techniques or fixed in 4% paraformaldehyde for immunostaining, respectively.

Quantification T and E2 level in hippocampus

All collected tissue were homogenized in Pierce® RIPA lysis buffer mixed with 1% protease inhibitor cocktail. T and E2 in tissue lysates was assayed using the Sandwich ELISA kit (Biochemica GmbH, Germany) according to the manufacturer's instructions as described previously (Chamniansawat & Sawatdiyaphanon, 2018). Briefly, the sample was pipetted into pre-coated micro-assay wells, followed by incubation at 37°C for 30 min. To develop the reaction, a substrate was added and incubated for 15 min in the dark. The reaction was stopped and then absorbance at 540 nm was recorded. The concentration of T and E2 were quantified by interpolation in a standard curve**.**

Western blot analysis

Brains were dissected into 2 regions of interest: hippocampus and cerebral cortex. Small piece of cortex or hippocampus were washed with ice-cold PBS, and then lysed in Pierce® RIPA lysis buffer mixed with 1% protease inhibitor cocktail. The suspensions were lysed by sonication on ice for 4 minutes. The disrupted were centrifuged at 15,000 rpm for 15 min at 4°C. Thereafter, the supernatant was collected and then mixed with sample buffer and then heat for 5 min at 95 °C before being load onto a gel. Protein concentration measured by Bradford assay. Equal amounts of protein were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membrane were blocked with 5% non-fat milk for 2 h and then probed overnight at 4 °C with anti-ERα, -ERβ, -AR, -GPR30, -aromatase, and -β-actin antibodies. The membranes were probed with goat anti-rabbit or goat-anti mouse IgG-HRP secondary antibodies. Detect the blot by enhanced chemiluminescence (SuperSignal West Pico, Pierce) and visualize using autoradiography film.

Immunohistochemical staining

For Immunostaining analysis, paraffin-embed hippocampi from 2-month-old female rats were compared to 19-month-old female rats. Sections (4 um thick) of paraffin-embed hippocampi were mounted on glass slides and dry overnight at 37°C. Subsequently, the section were dehydrated in xylene, then rehydrated through a graded series of alcohol and rinsed with PBS; next, microwaved in 0.01 M citric acid buffer (pH 6.0) for 20 min to achieve antigen retrieval; and, following three washes with PBS, placed in blocking buffer (DAKO) for 30 min at room temperature. Subsequently, the sections were incubated with

anti-AR (diluted 1:100) at 4 °C overnight, and the next day, with secondary antibody, (i.e., biotinylated goat anti-mouse IgG) (diluted 1:200) for 60 min at room temperature. Subsequently, they were incubated with the avidin-biotinylated enzyme complex and then placed in peroxidase reaction solution containing diaminobenzidine (DAB). As a negative control, the sections were processed in the same manner except without the primary antibody. The positive area% of immunohistochemical staining for AR in randomly selected fields of vision were counted with the Image Pro Plus software (Image Pro Plus software, USA).

Statistical analysis

One-way ANOVA followed by a Turkey's post hoc test were used to determine statistical significance between experimental treatments. Data were analyzed using GraphPad Prism 5 software. In all analyses, a P value of <0.05 was considered statistically significant.compared to younger female (Chamniansawat & Sawatdiyaphanon, 2018), the age-related changes in the hippocampal steroidogenesis remain largely unknown. Therefore, this study aims to investigate the expression of aromatase, AR, ER and the level of T and E2 in hippocampus across broad range of age female rats, that may be an important factor in protect against age-related memory impairment.

RESULTS

Level of T and E2 in hippocampus of aged female rats

The concentrations of T and E2 in rat hippocampal lysates were determined using ELISA technique (Fig1). The result revealed that T level (A) was significantly increased in the hippocampus of 19-month-old rats compare to 5-month-old rats. In contrast, the level of E2 (B) in hippocampus was significantly decreased in 19-month-old rat compare to 5- and 10- month-old rats. This finding suggested that regulation of local neurosteroid biosynthesis is age-dependently. Notably, within the female brain, the amounts of T lower than E2 at each age.

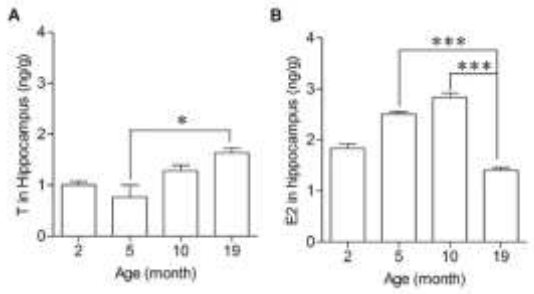

Figure1; Level of T and E2 in hippocampus of aged female rats.

Graphs of T level (A) and E2 level (B) in lysate of hippocampus from 2-, 5-, 10-, 19-month-old female rats. **P*<0.005; ****P*<0.0001vs the 5-month-old or 10-month-old group, n=5.

Expression of aromatase significantly decreased in hippocampus of aged female rats.

Next, to identify potential differences expression of hippocampal aromatase, the enzyme responsible for local E2 biosynthesis, between brains from various age 2-, 5-, 10-, and 19-month-old rats. The result showed that aromatase expression in hippocampus was changed at different phases of female rat life. As shown in Fig2, we showed a marked decrease in aromatase expression on hippocampus of 19-month old rats, whereas the middle age rat (5-10 months old) had little effect. This result indicating that the local synthesis of neurosteroid (including E2) in hippocampus may be aromatase-dependent manner.

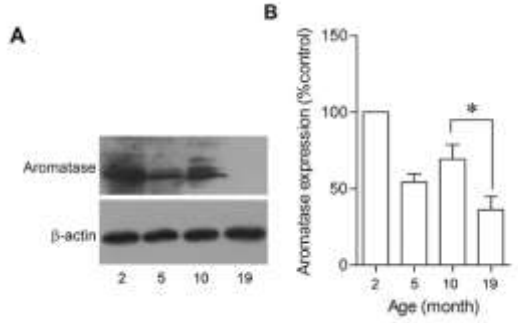

Fig2. Representative immunoblotting of aromatase in hippocampus of 2-, 5-, 10-, 19-month-old female rats (**A).** Densitometric analyses of aromatase expression in hippocampus (B) at each age of female rats normalized with β-actin. **P*<0.005 vs the 10-

month old group, n=5.

Expression of AR significantly increased in hippocampus and cerebral cortex of aged female rats

AR expression in female rat brains is progressive increased with age (Fig3). It significantly increased in both hippocampus and cerebral cortex at 10-and 19-month old female rats (A). To validate amount of AR expression, densitometry were analyzed. The total tissue level of AR increased 12-fold and 5-fold in the hippocampus (B) and cerebral cortex (C), respectively, of aged rats (19-month-old).

The analysis of positive immunostaining for AR was performed by immunohistochemistry technique (Fig4). The increased positive AR immunostaining was observed in aged hippocampal rats (19-month-old). In addition, this raise of AR proteins was augmented in the dentate gyrus and pyramidal cells in CA3 area. However, the expression of AR in the female rat did not differ between 5- and 10-month-old groups (data not shown). This result suggested increase in T activity and androgen receptors in aged female brains.

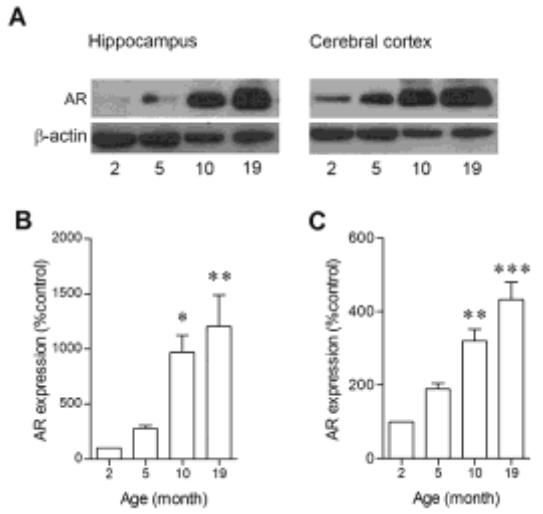

Figure3;Representative immunoblotting of AR in hippocampus and cerebral cortex of 2-, 5-, 10-and, 19-month-old female rats (A). Densitometric analyses of AR expression in hippocampus (B) and cerebral cortex (C) at each age of female rats normalized with β-actin. **P*<0.005, **P<0.0005 vs the 2-month old group, n=5.

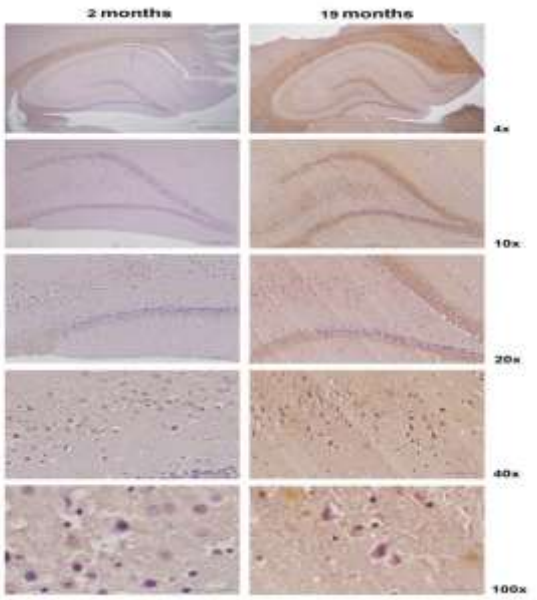

Figure4; The expression of AR in female rat hippocampus increased in aged rat. Representative immunostainings of AR of 2- and 19-month-old rats. Images are captured at 4X, 10X, 20X, 40, and 100X magnification as indicated. n=5.

Expression of ERα significantly decreased in hippocampus and cerebral cortex of aged female rats

To examine ERα expression in the brain across a broad range of ages, we measured ERα protein levels in intact females rat brain at five time points, including 2, 5, 10, and 19 months. The results showed a significant decline in ERα expression on hippocampus at 19 months old compared to 10 months old. (Fig5). Whereas, ERα expression on cerebral cortex remained intact in the 5-10-month-old rats and then decreased significantly in the 19-month-old rat. Notably, within the brain, the amounts of ERα in hippocampus lower than cerebral cortex.

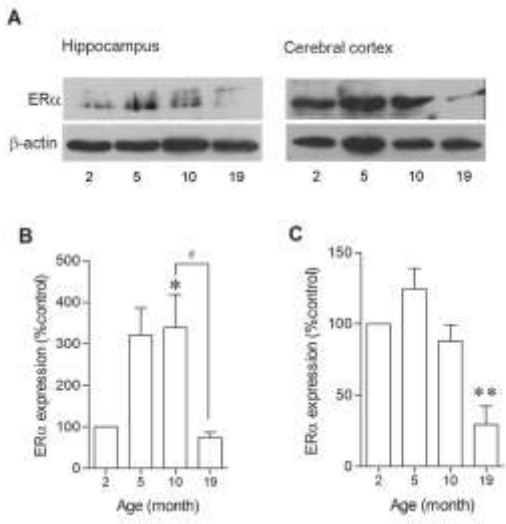

Figure 5; Representative immunoblotting of ERα expression in hippocampus and cerebral cortex of 2-, 5-, 10-, and 19-month-old female rats (A). Densitometric analyses of ERα expression in hippocampus (B) and cerebral cortex (C) at each age of female rats normalized with β-actin. **P*<0.005, ***P*<0.001 vs the 2-month-old group; #*P*<0.005 vs 10-month-old group, n=5.

Expression of ERβ significantly decreased in hippocampus and cerebral cortex of aged female rats

Similar to the expression of ERα, Western blot analysis of ERβ in hippocampus and cerebral cortex of female rat showed that the expression of ERβ significantly decreased on both hippocampus and cerebral cortex of 10-month-old rat in compared to 2-month-old rats (Fig6).

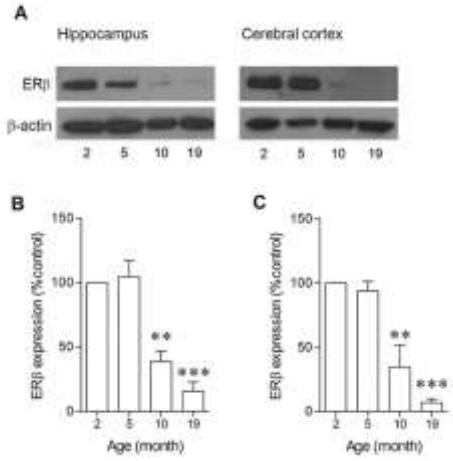

Figure6; Representative immunoblotting of ERβ expression in hippocampus and cerebral cortex of 2-, 5-, 10-, and 19-month-old female rats (A).

Densitometric analyses of ER**α** expression in hippocampus (B) and cerebral cortex (C) at each age of female rats normalized with β-actin. ***P*<0.001, ****P*<0.0001vs the 2-month-old group, n=5.

The significant decrease was sustained through 19 months in both cerebral cortex and hippocampus. Our results demonstrated different age-dependent spatial and temporal changes in the pattern of expression of ERα and ERβ, suggesting that each ER type may be involved in distinct roles across the neuronal function in different periods of time.

Expression GPR30 significantly decreased in hippocampus of aged female rats

In addition to transcriptional regulation, which occurs on time scale of hour, E2 also mediated cellular effect with response time from seconds to minutes. We next analyzed the effect of age on the expression of a transmembrane estrogen receptor, possibly of the 7-transmembrane G protein-coupled receptor family 1, GPR30, in tissues from hippocampus female rats of 5-, 10-, and 19-month-old by Western blot analysis (Fig7). The 19-month-old rats showed significantly decreased GPR30 protein level by about 30% with respect to 10-month-old rats. The result indicated that age female rats exhibited a decrease in GPR30 expression, which may contribute to rapidly decrease in dendritic spine plasticity underlies the formation and maintenance of memories in aged animal.

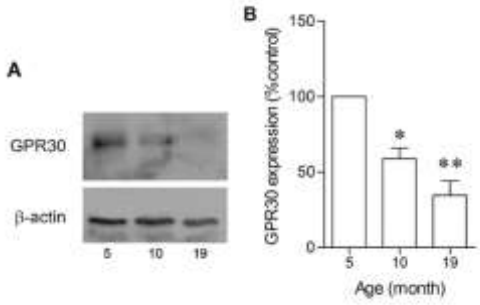

Figure7. Representative immunoblotting of GPR30 expression in hippocampus of 5-, 10-, and19-month-old female rats (A). Densitometric analyses of ERα expression in hippocampus (B) at each age of female rats normalized with β-actin. **P*<0.005, ***P*<0.001 vs the 5-month-old group, n=5.

DISCUSSION

Here, we demonstrated that age-related decrease in aromatase is associated with higher levels of T and lower levels of E2 in hippocampus of female rats. Furthermore, we also showed a significantly decreased of ER (ERα, ERβ, and GPR30) expressions and significantly increased of AR in aged female rats. The consistency of the patterns is suggesting of an underlying mechanism of a loss of local hippocampal E2 during aging in female rat hippocampus.

In human, more than 65 years are aged and memory impairment is found. Rats, approximately 2 years old, are typically considered as aged (Frick, 2009). Aged female rat, approximately 21-24 months old, showed impaired spatial task including Morris water maze (MWM) compare to 2-month old rats (Luine, 2011). Recently, we also showed that 19-month old rats significantly increased the latency time to the platform of MWM (Chamniansawat & Sawatdiyaphanon, 2018). This suggesting that 19-month old rat is suitable model to study the age-related memory decline.

It is well known that hippocampus synthesizes E2 in addition to the circulating form gonads (Prange-Kiel & Rune, 2006; Fester et al., 2011). Synaptic modulation by hippocampal-derived E2 is essential to maintain memory processes (Prange-Kiel & Rune, 2006). Our previous study demonstrated that aged rat elicited memory impairment whose serum E2 level was still high (Chamniansawat & Sawatdiyaphanon, 2018). Moreover, Exsogenous E2 treatment could not be restored the number of spine density in the brain of 22 month old female rats. These indicated that circulating E2 had no effect on improving memory impairment during aging (Adams et al., 2002; Rapp et al., 2003; Gibbs, 2000).

The concentration of hippocampal-derived E2 has been determined by mass-spectrometric analysis in combination with new steroid-derivatization methods (Ooishi et al., 2012). Normally, the E2 level in hippocampus is approximately 2-8 nM in adult rats, which much higher than in the circulation (Ooishi et al., 2012). Here, we showed that the concentration range of E2 in the hippocampus has a level of 2-4 ng/g. These concentrations are high enough to modulate synaptic plasticity and maintain normal memory processing (Kawato et al., 2004: Hojo et al., 2011).

Steroidogenesis in hippocampus is initiated by conversion of cholesterol to pregnenolone (PREG) by cytochrome P450scc. Then PREG was conversed to dehydroepiandrosterone (DHEA), and T, respectively. Localization of cytochrome P450scc was observed in pyramidal neurons in CA1-CA3, as well as granule cells in dentate gyrus in hippocampal slice from adult rat of 12 weeks old. The co-localization of cytochrome P450scc and NeuN (neuronal marker) confirm that expression of cytochrome P450scc is in this neuron. Moreover, StAR was co-localized with P450scc. These results implied that the steroidogenic system is completely occurred in neuron. In the female brain, T was converted to E2 by aromatase (Fester et al., 2009; Yague et al., 2010).

Reduced estrogen levels and decreased expression of related receptors is typical cerebral brain feature of aging. Previous study showed that ERα and ERβ significantly decrease in aged cerebral cortex and hippocampus (Hojo et al., 2011; Chamniansawat & Sawatdiyaphanon, 2018). In addition to the classical ER pathways, GPR30 was recently shown to mediate the effects of E2 (Marcello & Didier, 2010). It has been shown in ovariectomized rats to mediate spatial memory acquisition (Zhang et al., 2019). In 2018, Xu and colleague showed that the expression of GPR30 was reduced in middle-aged mice compared with young adult mice (Xu et al., 2018). Similar to our study, here, we showed that 19-month old showed a significantly decrease of GPR30 expression on hippocampus.

A loss of local hippocampal E2 and, consequently, a decrease in ERα and ERβ expression was recorded during aging in female rat hippocampus, which likely accounts for the decreased the spatial memory acquisition in Morris water maze (Chamniansawat & Sawatdiyaphanon, 2018). In parallel to the present study, we showed a sharp decrease in the aromatase expression during aging (19-month-old rats) in the female rat hippocampus. As increase age, aromatase expression levels tend to decrease. Similar to the other tissue, the expression of aromatase showed a markedly decreased in testis of 18-month old rat (Hamden et al., 2008). Moreover, aromatase expression was decreased in AD brain in average age of 77.5 years (Prange-Kiel et al., 2016). Aromatase is estrogen synthesizing enzyme by aromatizing of testosterone. We also determine level of testosterone and its receptors. In addition, an age-related increase in testosterone levels and AR has also been registered.

Over the past 10 years, a growing of information has emerged indicating that

androgens, especially testosterone, modulate the structure and functions of the hippocampus. Effects on hippocampal-dependent behavior, long-term potentiation, dendritic spine, and spine synapse density, patterns of dendritic arborization, as well as hippocampal neuronal survival have all been reported. AR expression and activity was previously reported in both male and female mice brain (Dart et al., 2013). However, there are only a few studies about level of region-specific changes in AR expression on female rat brain, especially during aging.

CONCLUSION

Here, we concluded that age-related decrease in aromatase is associated with higher levels of T and lower levels of E2. Moreover, the expression of AR is significantly increased, whereas the expressions of ERα, ERβ, and GPR30 are significantly decreased in aged female rats. This study suggested that the decreasing of E2 in aged brain is aromatase dependent manner, leads to memory impairment.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

CS performed experiments and analyzed the results; TN analyzed the results and edited the manuscript; SC designed and performed experiments, analyzed and interpreted the results, wrote and edited the manuscript.

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