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Original Article

Doxepin improves stress-impaired long-term potentiation and gene expression of BDNF in the rat hippocampus

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Abstract

Introduction: Stress is associated with neurological and cognitive disorders. It has been suggested that doxepin, in addition to its influence on the content of neurotransmitters, has probable neuroprotective effects as well. Therefore, the aim of this study was to investigate the effects of doxepin on synaptic plasticity and brain-derived neurotrophic factor (BDNF) gene expression in the rat hippocampus following repeated restraint stress.

Methods: Male Wistar rats were divided into the control, the stress and the stress-doxepin 1 and 5 mg/kg groups. Stress was induced 6 hours/day for 21 days. Rats received daily ip injection of doxepin before induction of stress. Long-term potentiation (LTP) was induced in hippocampal dentate gyrus following stimulation of perforant pathway and then field excitatory postsynaptic potential was evaluated. Hippocampal gene expression of BDNF was measured by Real-Time PCR.

Results: Stress impaired LTP induction, but both doses of doxepin prevented those damages. Stress significantly decreased the expression of BDNF gene, but doxepin in both doses, increased it significantly.

Conclusion: The present results suggested that doxepin can prevented the harmful effects of stress on synaptic plasticity which may be related to changes in BDNF gene expression.

Keywords:
Doxepin;
Stress;
Hippocampus;
Long-term potentiation;
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Introduction

Stress is a stereotypic reaction of the organism to signals that tend to disturb dynamic homeostasis (Wales, 1995). When stress become severe and/or prolonged, it changes the structure and the function of the brain especially hippocampus (Ulrich-Lai et al., 2006; Sadeghi et al., 2015). These changes affect neurotransmission in the hippocampus (Vyas et al., 2002; Rosenbrock et al., 2005; Christian et al., 2011). Studies have shown that chronic stress produces negative effects in the hippocampus by affecting spine synapse turnover, dendritic shrinkage, suppression of adult neurogenesis in the dentate gyrus (DG) and finally damages long-term potentiation (LTP) (Chen et al., 2015; Shang et al., 2016).

Chronic stress affects the hypothalamic-pituitary-adrenal (HPA) axis and enhances weight of adrenal gland and production of glucocorticoids (Alario et al., 1987; Ulrich-Lai et al., 2006). Hippocampus that is an important areas for learning and memory (Reisi et al., 2009a; Moghaddasi et al., 2014), has direct and indirect anatomical connections with HPA axis (Christian et al., 2011) and has a large number of the glucocorticoid receptors as well (Xiong et al., 2004). In addition, hippocampus is innervated by serotonergic and noradrenergic neurons (Drzyzga et al., 2009) which can be affected by stress (Sabban and Kvetnansky, 2001).

Glucocorticoids promote neuronal death and disruption of neurogenesis in the hippocampus through affecting the expression of pro-apoptotic and anti-apoptotic genes (Drzyzga et al., 2009), the levels of neurotrophic factors (Haynes et al., 2004), the production of oxidative stress (Mohammadi et al., 2014; Soung et al., 2015) and other functions (Duman et al., 1999; Vyas et al., 2002; Christian et al., 2011) and eventually they impair learning and memory (Kanatsou et al., 2015; Lee et al., 2015).

Doxepin is a member of tricyclic antidepressants that inhibit serotonin and norepinephrine reuptake. Doxepin is used to treat depression and anxiety, sleep disorders in low dose (Krystal et al., 2010; Van Dongen and Kerkhof, 2011) and acute and chronic pain (as a local anesthetic) (Cheng et al., 2006). It has also anticonvulsant effects (Sun et al., 2009). In addition, anti-inflammatory (Drake et al., 1994; Hajak et al., 2001), anti-oxidative (Ji et al., 2004; Drzyzga et al., 2009) and neuroprotective effects of doxepin has been also suggested (Li et al., 2000).

According to previous studies, stress can damage the hippocampus and produces learning and memory impairments (Chen et al., 2015; Shang et al., 2016). A part of the effects of stress may be mediated through affecting neurotrophic factors including brain-derived neurotrophic factor (BDNF) (Taliaz et al., 2011). Since studies have shown a neuroprotective effect of some antidepressants and they can be effective in improving nervous system function by increasing neurotrophic factors; and also in our previous studies we have observed positive effects of doxepin in the rat model of stress (Azadbakht et al., 2015; Eidelkhani et al., 2015b), the aim of this study was to investigate the effect of doxepin on LTP and BDNF gene expression in the hippocampus of rats exposed to restraint stress.

Materials and methods

The experiments were carried out on male Wistar rats (200-250 g) that were housed under standard conditions of temperature (22±2 °C) and light (12 h light-dark cycle), with free access to food and water. The Ethic Committee for Animal Experiments at Isfahan University of Medical Sciences approved the study and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The animals were randomly divided into four groups (n=10 in each group): the control, the stress, the stress-doxepin 1 mg/kg (St-Dox 1 mg/kg) and the stress-doxepin 5 mg/kg (St-Dox 5 mg/kg).

In the stress groups, the rats were individually restrained for 6 hours/daily in plastic cylinders (20×7 cm) which had holes for air exchange at 7:30–13:30, for 21 days (Rosenbrock et al., 2005).

In the doxepin groups, doxepin (1 and 5 mg/kg; dissolved in saline; Ray Chemicals Pvt. Ltd.) was injected (intraperitoneal, ip) for 21 days (Azadbakht et al., 2015; Eidelkhani et al., 2015b). In St-Dox groups, doxepin was injected 5 min before the stress process. Animals in the control groups received the same volume of saline.

The rats were anaesthetized by an injection of urethane (1.8 g/kg, ip) (Dehghani and Reisi, 2015) and their heads fixed in a stereotaxic frame. Body temperature kept at 36.5±0.5 °C with a heating pad. The skull was exposed and two small holes were drilled at the positions of the stimulating and recording electrodes. The exposed cortex was kept moist by the application of paraffin oil. A concentric bipolar stimulating electrode (stainless steel, 0.125 mm diameter, Advent, UK) was placed in the right perforant pathway (AP=−8.1 mm; ML=4.3 mm; DV=3–3.2 mm) and a stainless steel recording electrode was lowered into the right dentate gyrus (AP=−3.8 mm; ML=2.3 mm; DV=2.7–3.2 mm) (Paxinos and Watson, 2006). The electrodes were adjusted at the level that field excitatory post-synaptic potential (fEPSP) obtained without any population spike. In order to minimize trauma to brain tissue, the electrodes were lowered very slowly (0.2 mm/min). Implantation of electrodes in the correct position was

determined by physiological and stereotaxic indicators.

Extracellular evoked responses were obtained from the dentate granule cell population following stimulation of the perforant pathway. Extracellular field potentials were amplified (1000 ×) and filtered (1 Hz to 3 kHz band pass). Signals were passed through an analogue to digital interface (Electromodule D3111 and eProbe provided by Science Beam Institute, Tehran, Iran). Stimulation intensity was adjusted to evoke about 40% of the maximal response of the fEPSP. As shown in Fig. 1, the fEPSP amplitude was measured as the difference in voltage between the positive peak of the fEPSP wave and the baseline (between A and B) in order to measure synaptic efficacy.

Stimulus–response or input/output (I/O) functions were acquired by systematic variation of the stimulus current (100–1000 μA) in order to evaluate synaptic potency before induction of LTP. The fEPSP were evoked in the DG region using 0.1 Hz stimulation. After ensuring that the responses are stable, baseline recordings were taken for 30 min (10 consecutive evoked responses were averaged at 10 s stimulus intervals every 5 min). Then, LTP was induced using high-frequency stimuli protocols of 400 Hz (10 bursts of 20 stimuli, 0.2 ms stimulus duration, 10 s interburst interval) at a stimulus intensity that evoked a fEPSP of approximately 80% of the maximum response. All potentials employed as baseline before and after high frequency stimuli were evoked at a stimulus intensity which produced 40% of this maximum (Reisi et al., 2010). The fEPSPs were recorded for the periods of 5, 15, 30, 60 and 120 min after the high frequency stimuli in order to determine any changes in the synaptic response of DG neurons. For each time-point, 10 consecutive evoked responses were averaged at 10 s stimulus intervals.

Then rats were decapitated, their brains were immediately removed from the skull and left hippocampus was instantly dissected. The extracted hippocampus tissues removed on a cold artificial cerebrospinal fluid and got deep freeze in liquid nitrogen and then stored at −80 °C until further studies. Also, the adrenal glands were removed and weighted.

Real-time polymerase chain reaction (PCR) was used to evaluate the expression of BDNF gene in hippocampus. Total RNA was isolated from

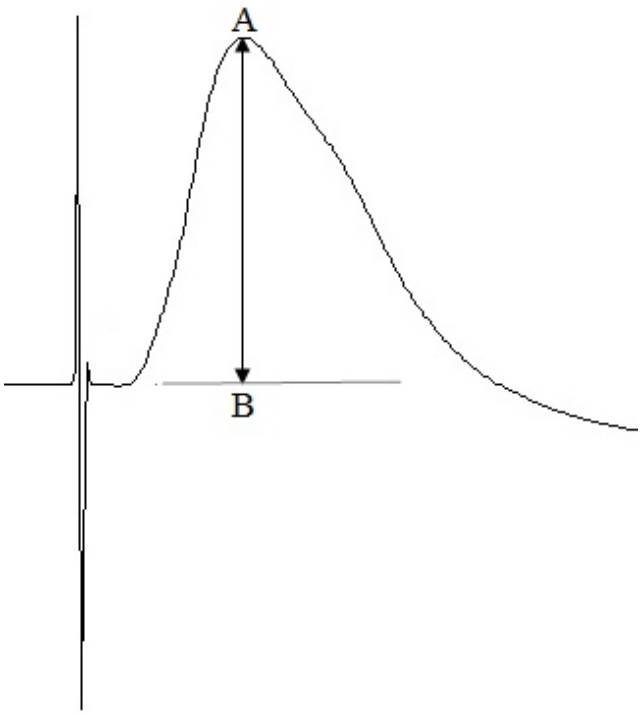

Fig.1. Schematic diagram of fEPSP. The fEPSP amplitude parameters analyzed as: voltage difference between A and B.

hippocampus tissues using YTA kit (Yekta Tajhiz Azma, IRAN). After isolation, the quality of messenger RNA (mRNA) was checked by gel electrophoresis and RNA quantity was measured using nanodrop (OD 260 nm). At the reverse transcription step, 5 ng of total RNA was used to synthesis the complementary DNA with random hexamers primer using the Reverta-L kit (Amplisens, Moscow, Russia), according to the manufacturer's manual. The real-time PCR was performed using the StepOnePlus real-time PCR System (Applied Biosystems). RealQ Plus 2x Master Mix Green with high ROX™ (Ampliqon) and specific primers were used (Table 1). Beta-actin (ACTB) was used as an internal control to normalize RNA input. Cycle parameters for real-time PCR included 95 °C for 1 min, 95 °C for 15 s and 60 °C for 60 s. The Ct value is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method presented as the fold expression change in treated experiment group relative to their corresponding control group after normalization to the ACTB endogenous control.

Data were analyzed using both the SPSS 21 (IBM Corporation) for Windows and the Rest 2009 (developed by M. Pfaffl [Technical University Munich]

Table 1: Primers used in real-time PCR experiments

Name	Sequences (5' to 3')
ACTB -F	AGGCCCCTCTGAACCCTAAG
ACTB -R	CCAGAGGCATACAGGGACAA
BDNF -F	AGAATGAGGGCGTTTGCGTA
BDNF -R	CCTGGTGGAACATTGTGGCT

ACTB was used as a housekeeping gene to compare the samples.

Table 2: Effects of doxepin and stress on adrenal gland weight.

Groups	Control	Stress	St-Dox 1 mg/kg	St-Dox 5 mg/kg
Weight of adrenals (g)	0.049±0.003	0.072±0.001***	0.062±0.003**##	0.071±0.002***

Data are expressed as mean±SEM. (n=9-10). St+Dox is stress-doxepin; **P<0.05 and ***P<0.01 with respect to the control group, $^{\#\#}P$<0.05 with respect to the stress group.

and QIAGEN). The data from electrophysiology study were statistically analyzed using two-way and multivariate of analysis of variance (ANOVA) followed by Tukey's test. The results analyzed for gene expression with one sample t-test between the treated and the control groups and with one-way ANOVA between the other groups followed by Tukey's test. The significant level was set at P<0.05. Results are expressed as mean±SEM.

Results

The adrenal gland weights of the stress and the St-Dox 1 and 5 mg/kg groups were significantly higher than the control group (P<0.001, P<0.01 and P<0.001; respectively). Also, there was a significant decrease in the adrenal glands weight in St-Dox 1 mg/kg group compared to the stress group (P<0.01) (Table 2).

A two-way ANOVA indicated that the I/O function curves in the DG measured as fEPSP amplitude in order to evaluate synaptic potency, had no significant difference between the groups.

As it is shown in Fig. 2, the effects of doxepin on LTP induction and maintenance in dentate gyrus of stressed rats were determined. A multivariate ANOVA test revealed that stress damaged LTP maintenance, as a significant reduction in fEPSP amplitude was observed at 60 (P=0.05) and 120 min (P=0.016) after high frequent stimulation with respect to the control group. Doxepin prevented these reductions in the St-Dox 1 mg/kg (P=0.053) and the St-Dox 5 mg/kg (P=0.036) groups with respect to the stress group after 120 min, so that there was no significant difference between St-Dox groups and the control (Fig. 2B).

As it is shown in Fig. 3, stress decreased the mRNA expression of BDNF significantly and the reduction was nearly 92 % (P<0.001) with respect to the control group. Doxepin increased BDNF in both St-Dox 1 mg/kg (P<0.01) and St-Dox 5 mg/kg (P<0.01) groups comparing to the stress group (Fig. 3).

Discussion

The results showed that the induced LTP following high frequent stimulation was significantly influenced by stress. Chronic stress had no effect on amplitude of fEPSP following LTP induction but impaired its maintenance, as the fEPSP amplitude showed a significant reduction after 60 to 120 minutes in the stress group (Fig. 2B).

The synaptic plasticity can be affected by several factors such as presynaptic release, number and affinity of postsynaptic receptors, signaling pathways, number of responsive neurons and etc. (Meyer et al., 2014). Chronic stress causes hyper activation of HPA axis and increases the adrenal weight (Ulrich-Lai et al., 2006). Hippocampus has high density of glucocorticoid receptors, therefore it is very susceptible to stress damages (McEwen, 1994). Some reports demonstrated that chronic stress

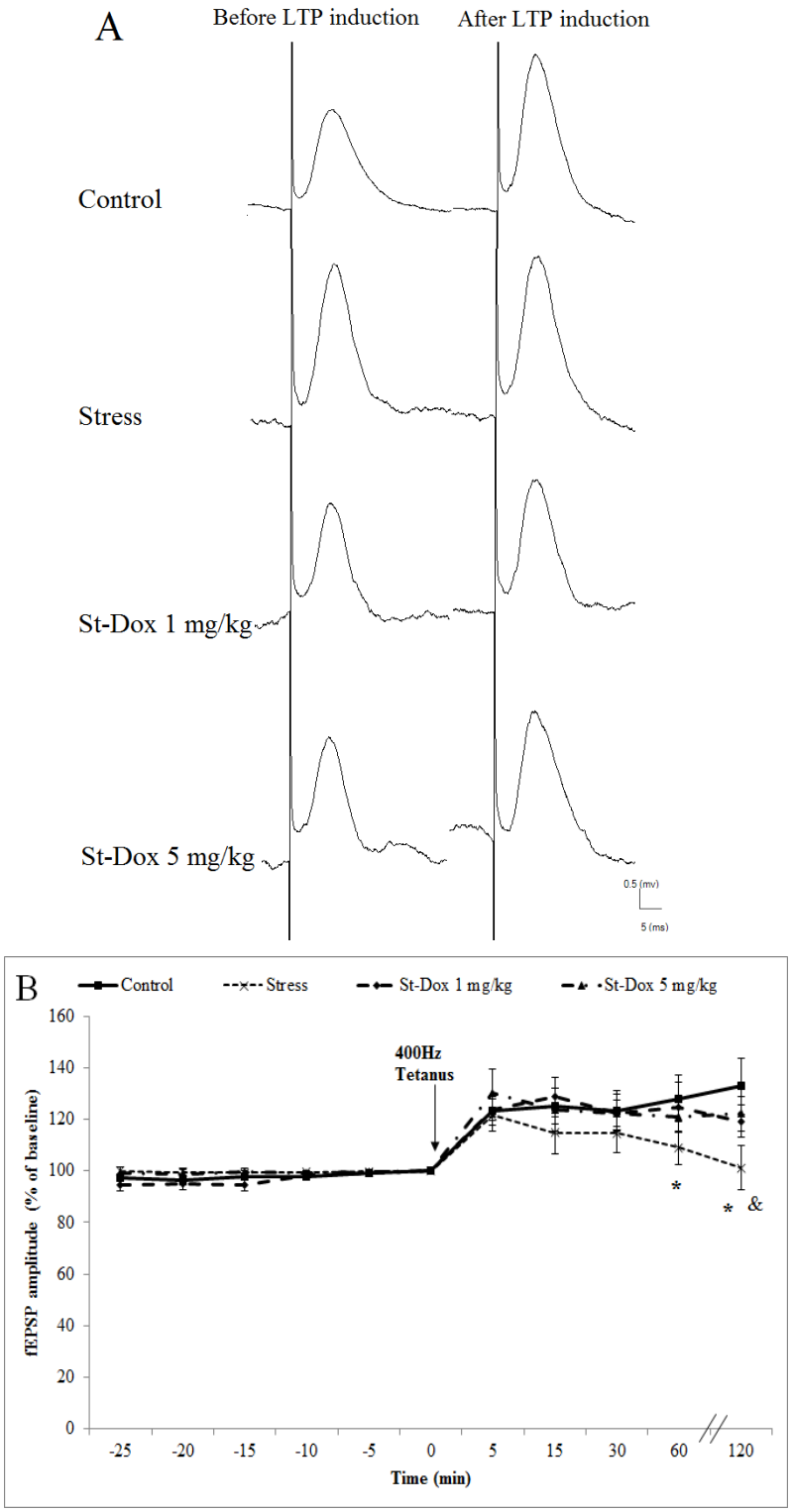

Fig.2. Effects of doxepin and stress on LTP induction and maintenance in dentate gyrus of the hippocampus using 400 Hz tetanic stimulation. Single traces recorded before and 120 min after induction of LTP in dentate gyrus of the hippocampus (A). The changes in amplitude of fEPSP (B). Data are plotted as the average percentage change from baseline responses. St+Dox is stress-doxepin; Values are % mean±SEM. *P<0.05, significant difference between the control and the stress groups; $^{\&}P$<0.05, significant difference between the stress and the St+Dox 5 mg/kg groups (n=9-10 in each group).

increased neuronal apoptosis (Drzyzga et al., 2009), and decreased neurogenesis (Rosenbrock et al., 2005) and the volume of hippocampus (Drzyzga et al., 2009). Glucocorticoids that are released in stress can activate apoptotic processes directly by increasing the expression of pro-apoptotic factors and reducing the expression of anti-apoptotic (Drzyzga et al., 2009) and neurotrophic factors such as BDNF

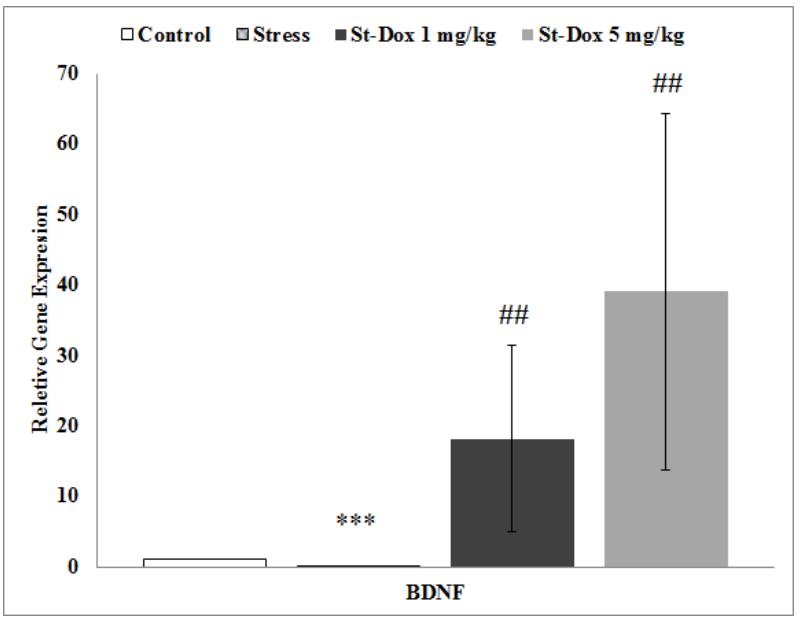

Fig.3. Effects of doxepin and stress on relative gene expression of brain-derived neurotrophic factor (BDNF) in rat hippocampus, the amount of expression was measured by real-time PCR. The mRNA expression data normalized to the Beta-actin (ACTB) signal. Fold changes relative to control are presented. St+Dox is stress-doxepin; Mean±SEM values of experiments are shown. ***P<0.001 with respect to the control group; $^{\#\#}P$<0.01 with respect to the stress group (n=7).

(Haynes et al., 2004). Also, it has been shown that stress increase glutamate toxicity (Skupio et al., 2015), produce neuronal apoptosis and dendritic retraction (Christian et al., 2011).

Studies have shown that chronic stress, particularly when it causes an increase in glucocorticoids, induces changes in glutamate and gamma-aminobutyric acid (GABA) neurotransmission in the hippocampus (Hu et al., 2010; Popoli et al., 2011). Since glutamatergic and GABAergic systems have especial roles in synaptic plasticity in the hippocampus (Reisi et al., 2009b; Zarei et al., 2014), it can be expected that these stress-induced dysfunctions can lead to cognitive deficits that are common in stress-related psychiatric disorders.

As a secondary observation, our study demonstrates that doxepin at both doses prevented stress-induced changes in the LTP induction and maintenance, so that there was no significant difference between the doxepin groups and the control group. As shown in Table 2, the adrenal gland weights significantly decreased by injection of the low-dose of doxepin (1 mg/kg) before each session of stress induction, however, failed to reach the level of intact animals. The high dose of doxepin (5 mg/kg) had no effect on adrenal glands weight in the stress group. Since, it has been demonstrated that low-dose of doxepin is effective in improvement of sleep (Krystal et al., 2010), there is a possibility that relaxing effect of low-dose of doxepin can reduce anxiety and induce sleep in the animals during the restraint stress. However, doxepin at both low and high doses could prevent the harmful effect of stress on synaptic plasticity. This represents that also other mechanisms may be involved.

In accordance to other studies (Murakami et al., 2005), the present study showed that stress significantly reduced the gene expression of BDNF in the hippocampus, but doxepin increased it in the stress rats. Since, stress increases neuronal apoptosis in the hippocampus (Abdanipour et al., 2015), therefore BDNF as a neuroprotective factor can possibly prevent neuronal death. It has been demonstrated that serotonin-noradrenaline reuptake inhibitors have positive effects on expression of neurotrophins and decrease proapoptotic factors in the mice hippocampus (Engel et al., 2013). However, in our previous study we observed that doxepin had no significant effects on mRNA expression of BDNF in hippocampus of intact rats (Eidelkhani et al., 2015a).

In addition, some previous studies demonstrated that stress leads to activation of inflammatory processes and the production of reactive oxygen species (Chen et al., 2016). It seems that doxepin through its anti-inflammatory and antioxidant effects can possibly

provide neuroprotection against stress (Ji et al., 2004; Azadbakht et al., 2015). Also, it is likely that doxepin by blocking the glutamate receptors (Kiefer et al., 1999) prevents stress-induced glutamate toxicity.

Conclusion

The results of this study suggest that doxepin can prevented the harmful effects of stress on synaptic plasticity which may be related to changes in BDNF gene expression. Therefore, doxepin may act through mechanisms other than inhibition of neurotransmitter reuptake.

Acknowledgments

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Conflict of interest

The authors declare that they don't have any conflict of interest.

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