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Prenatal stress potentiates pilocarpine-induced epileptic behaviors in infant rats both time and sex dependently

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ABSTRACT

Stressful events during gestation have important effects on the later physical and mental health of the offspring. In the study described here, the pilocarpine-induced seizure model was used to test the hypothesis that prenatal stress affects seizure susceptibility in infant rats. Prenatal stress consisted of daily restraint of the dam under normal room conditions (for 120 minutes, twice daily) during the first, second, and third weeks of gestation. The pups were then compared with pups born to unstressed dams. Both second- and third-week-gestation stress significantly reduced pilocarpine-induced seizures in 19-day-old rat offspring, as compared with nonstressed control offspring. Mid- and late-gestation stress increased the rate and time of tonic–clonic seizures. Mortality rate 2 and 24 hours after pilocarpine administration increased significantly in all stressed rats. Stress induced a significant rise in circulating corticosterone levels (2- to 8-fold, $P<0.001$) in the offspring. Female offspring differed little from male offspring with respect to blood corticosterone levels and epileptic behaviors. These findings indicate that prenatal stress, particularly during the second and third weeks of pregnancy, may play an important role in increasing seizure vulnerability in the unborn offspring. Female rats are more resistant to stress than males probably because of the lower susceptibility of their hypothalamic–pituitary–adrenal axis.

1. Introduction

The major pathway implemented in coordinating the consequences of stress in most mammalian species is the hypothalamic–pituitary–adrenal (HPA) axis [1,2]. Corticotrophin-releasing factor (CRF)-expressing neurons within the paraventricular nucleus of the hypothalamus are a well known element in the stress response. The typical biochemical cascade in response to stresses involves the release of CRF from the paraventricular nucleus into the hypophysial portal system, which, in turn, stimulates the release of adrenocorticotrophic hormone (ACTH) into the systemic circulation [2,3]. Circulating ACTH can then interact with adrenal cortex receptors to stimulate the synthesis of steroids (steroidogenesis), as well as cause a marked elevation in plasma glucocorticoids [2,4]. Changes in neural plasticity to any of the key nuclei within the HPA circuitry can occur after the primary exposure to a variety of stressors [5,6]. The neuroplastic changes can be both adaptive and beneficial in nature; however, prolonged exposures to stressful stimuli could induce pathological changes within this circuit and lead to long-lasting or even elevated HPA activity. Alterations to the functions of HPA axis have been shown to occur in several clinical conditions, including epilepsy [2].

In rats, prenatal stress has been shown to lead to impaired sexual function [7,8], vulnerability to anxiety [9], increased propensity to self-administer drugs, and impaired feedback regulation of the HPA axis as a result of decreased numbers of hippocampal corticosteroid receptors [10]. With impaired feedback, animals show increased and prolonged corticosterone (COS) secretion in response to stress [11,12]. Thus, stressful events in early life appear to have important effects on HPA function and on the later physical and mental health of the unborn offspring. Altered HPA function might influence seizure susceptibility. In adult humans with epilepsy, subjective stress and/or unpleasant life events are positively associated with seizure frequency [10]. In adult animals, studies have similarly shown a direct proconvulsant effect of the corticosteroids. COS replacement after adrenalectomy, for example, lowers amygdala seizure thresholds and facilitates the development of kindled seizures in adult rats [10,12,13]. It has been reported that stress-induced events such as fear, worry, and frustration are commonly associated with elevated seizure occurrence [14]. Prolonged periods of stress markedly attenuate the efficacy of various anticonvulsant drugs [15]. Although stresses can influence the number of seizures reported by patients [2], the precise mechanism underlying the relationship between stress and epilepsy remains unclear.

There are some opposite reports of stress impact on seizures. Although the underlying mechanisms are poorly understood, it is well recognized that emotional stress can be a factor affecting seizure

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control in temporal lobe epilepsy and other seizure syndromes. Moreover, experimental stress, including swim stress, has been shown to have antiseizure effects in animals using hippocampal–enthorinal combined slices [16].

In infants, the effect of corticosteroids on seizure susceptibility is less clear. As prenatal stressors can alter regulation of the HPA axis, and as hormones of the HPA axis can affect seizure vulnerability, at least in adults [10], we hypothesized that prenatal stress might alter seizure susceptibility in infant rats. Previous investigators have demonstrated an effect of prenatal stress mostly on the mid- or late phase of gestation. No one to date, however, has tested the impact of stress during all 3 weeks of the rat pregnancy. Therefore, the present study was designed to test the effects of a prenatal restraint stressor on pilocarpine-induced seizures in infant rats.

2. Methods

2.1. Subjects

Male and female Wistar rats (200–250 g) were obtained from the Pastor Institute, Tehran, Iran. They were 10 weeks old on delivery. Rats were housed in groups of four per cage in our animal facility and kept under standard conditions as follows: 12-hour light/dark cycle, 22 ± 2 °C, food and water ad libitum. All experimental protocols and procedures complied with the guidelines of the 1975 Declaration of Helsinki as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, Iran. All females were mated at 12 weeks with a sexually experienced male of the same genotype. Each female was paired with one male at 9:00 AM, and we checked the plugged females at 3:00 PM, which were immediately housed individually in cages for the entire gestation. If a plug was not observed, the animal was returned to her home cage until the next morning for a new mating procedure. Pregnant rats were divided evenly into five groups ($n = 7$): control, first week stressed (FWS), second week stressed (SWS), third week stressed (TWS), and entire gestation stressed (EGS).

2.2. Restraint stress procedure

The FWS group was stressed daily on gestation days 4, 5, and 6 (i.e., early pregnancy stress). The SWS group was stressed daily on gestation days 11, 12, and 13 (i.e., midgestation stress).The TWS group was stressed daily on gestation days 18, 19, and 20 (i.e., late gestation stress). The EGS group was stressed on gestation days 4–6, 11–13, and 18–20 (i.e., complete gestation stress). In all stressed groups, stress involved transport of the home cage to the experimental room and placement of the pregnant female in a restraint chamber (transparent, plastic, cylindrical chamber, 6 cm in diameter, 16 cm long) under normal room conditions. Animals were restrained 120 minutes, two times a day (between 8:00 and 11:00 hours and between 15:00 and 18:00 hours). This procedure has previously been shown to cause alterations in the regulation of the HPA axis in the offspring [10,16]. The control group, consisting of seven pregnant females, was transported to the experimental room on gestation days 4–6, 11–13, and 18–20 and handled similarly to other groups, but were not stressed. After parturition, pups in each litter were counted and weighed at 09:00 hours on the first postnatal day (P1). Male and female pups from each litter were sorted into two uneven groups. The first group was subjected to pilocarpine injection on P18 and P19. To reduce possible litter effects, a maximum of four infant pups (two males and two females) from any litter were used as subjects in this experiment. On P16 and P17, the second group (a male and a female from each litter) were decapitated under halothane anesthesia at 08:30 hours to collect trunk blood into 1.5 mL of EDTA-coated microcentrifuge tubes. Samples were kept on ice and later centrifuged for 20 minutes at 9000 rpm at 4 °C. Plasma was transferred to clean 1.5-mL microcentrifuge tubes, and plasma samples were stored frozen at −20 °C until COS levels were determined [16,17]. Plasma COS was measured using the radioimmunoassay (RIA) commercial kit (Isotope, Budapest, Hungry) and the values are expressed as nanograms per milliliter. Weights of all pups were recorded at 08:30 hours on P15.

2.3. Behavior assessment

On P18 and 19, infant rats were injected subcutaneously with pilocarpine 100–200 mg/kg. Following injection, the behavior of each rat was observed and documented at least every 15 minutes for 120 minutes, and the seizure rating was assessed using a previously defined scale [18]: 1 = immobility; stage 2 = forelimb and/or tail extension; stage 3 = repetitive movements, head bobbing; stage 4 = rearing and falling; stage 5 = continuous rearing and falling; stage 6 = severe tonic–clonic seizures. Other parameters monitored included latency to first behavioral change and to first maximal seizure. Also, animals were monitored for fatal effect of pilocarpine until 24 hours after injection.

2.4. Statistical analyses

Results are expressed as means ± SEM. Data that were normally distributed were analyzed with parametric techniques. Two-group comparisons used *t* tests, whereas multiple-group comparisons used the one-way analysis of variance (ANOVA). When appropriate, post hoc analyses used the Tukey test. Data that were not normally distributed were analyzed using the Mann–Whitney *U* test and/or the Kruskal–Wallis one-way ANOVA. When appropriate, post hoc analyses used Dunn's test. Data related to mortality rate at both 2 and 24 hours were analyzed using K^2 and Fisher's exact test. All tests used a critical significance level of $P<0.05$.

3. Results

3.1. Effects of restraint stress on corticosterone levels in infant rats

The effects of gestational restraint stress on COS blood levels were determined in a male and a female from any litter of all groups. Stress significantly increased COS levels in pups at P16 and P17 (Table 1). Uneven elevation of COS levels in differently stressed groups indicates a time-dependent impact of restraint stress on brain structure and function.

There was no significant difference in litter size between experimental groups. The mean ± SEM number of pups per group

Table 1
Effect of gestational restraint stress in different phases of pregnancy on COS blood levels of infant rats.

Group	Sex	COS level	ANOVA
Control	Male	0.57 ± 0.041	ANOVA
	Female	0.54 ± 0.048	$F[9] = 36$, $P<0.001$ Tukey test $P < 0.01$
First week stressed	Male	1.07 ± 0.081	
	Female	0.83 ± 0.081	
Second week stressed	Male	2.26 ± 0.22	
	Female	1.16 ± 0.071	
Third week stressed	Male	3.36 ± 0.48	
	Female	1.84 ± 0.18	
Entire gestation stressed	Male	4.63 ± 0.29	
	Female	2.22 ± 0.25	

Note. There is significant difference between all male groups and some female animals. Also, there is a significant difference between males and females in all except the control and first week stressed groups.

was 7.8 ± 0.4 (control group), 7.2 ± 0.46 (FWS group), 7.8 ± 0.68 (SWS group), 7.6 ± 0.52 (TWS group), and 6.8 ± 0.54 (EGS group).

There was a significant reduction in weight of pups born to dams stressed during the second week, the third week, and the entire gestation, as compared with controls (ANOVA, $P<0.01$; Tukey, $P<0.05$). The mean ± SEM weight per group was 5.73 ± 0.14 g (controls), 5.65 ± 0.18 g (FWS), 4.58 ± 0.12 g (SWS), 3.86 ± 0.09 g (TWS), and 3.65 ± 0.08 g (EGS). This difference was gone by P15, and pups weighed, on average, 22.3 ± 2.4 g (mean ± SEM) at that time.

4. Effects of prenatal restraint stress on pilocarpine-induced seizures

After subcutaneous administration of 150 mg/kg pilocarpine, all five groups of rats displayed comparable seizures, but the time to onset of first epileptic behavior differed significantly between different groups. Within 5.43 ± 0.39 and 5.21 ± 0.26 minutes of injection, rats in the control and FWS groups showed immobility, respectively, whereas rats in the other stressed groups exhibited immobility faster (Table 2). There was no significant difference between male and female infant rats in each group with respect to immobility. This behavior was followed by myoclonic twitching and often frequent rearing and falling. All rats exhibited continuous tonic–clonic seizures but on different time schedules, as outlined in Table 2.

Mortality rates 2 and 24 hours after pilocarpine injection did not differ significantly between male and female infant rats in the control, FWS, and SWS groups, but did differ significantly in the TWS and EGS groups (Fisher's exact test, $P<0.01$) (Table 2). Comparison of all male and female rats revealed a significant difference in mortality rate at both 2 and 24 hours (K^2, $P<0.01$). Mortality rate 2 hours after subcutaneous administration of 150 mg/kg pilocarpine to control and stressed groups is illustrated in Fig. 1.

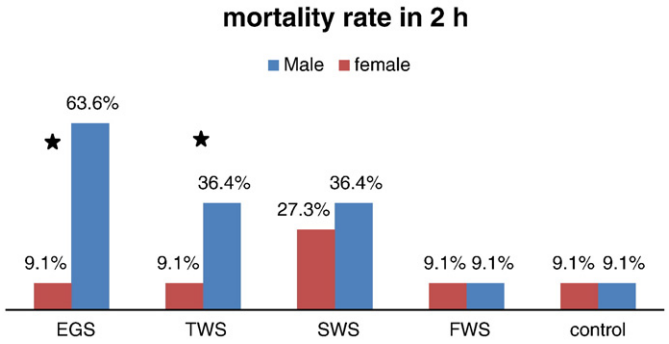

Fig. 1. Mortality rate 2 hours after subcutaneous administration of 150 mg/kg pilocarpine to control and stressed groups. There is a significant difference between male and female infant rats in the TWS and EGS groups. Also, there is significant difference between male animals in the EGS, TWS, and SWS groups compared with the FWS and control groups. FWS, first week stressed group; SWS, second week stressed group; TWS, third week stressed group; EGS, entire gestation stressed group.

5. Discussion

The present study was designed to determine whether prenatal manipulation affects seizure susceptibility, as measured by pilocarpine-induced seizure characteristics and development, in infant offspring. Late-gestation prenatal stress and entire-gestation stress, but not early-gestation manipulation, significantly potentiated the seizure parameters and facilitated the occurrence of maximum seizures. Male and female rats differed significantly with respect to most of the epileptic behaviors such as number and duration of immobility events, head bobs, tail extensions, and tonic–clonic seizures. Blood COS levels of SWS, TWS, and EGS groups differed significantly from those of the control and FWS groups. Blood COS levels of female rats were only slightly elevated compared with levels of male rats. The difference in COS level between male and female rats was significant in the SWS, TWS, and EGS groups.

Potentiation of seizures by prenatal stress has been reported by previous investigators [10,16]. Presumably, this facilitation is caused by an imbalance between the excitatory and inhibitory systems. What might this imbalance be? Three specific lines of compelling preclinical evidence suggest that there are neurobiological bases for stress-induced seizures: (1) exposure to stressors evokes hippocampal plasticity, (2) exposure to stressors induces noradrenergic neurotransmission, and (3) exposure to stressors facilitates adrenocortical hormone activation. A stress-exposed organism appears to be at higher risk of seizure onset in the event of hippocampal imbalance, adrenergic loss of function, or corticosteroid abundance [19].

The first candidate mechanism for stress-induced seizure susceptibility relates to hippocampal plasticity. Stress attenuates neurogenesis of dentate gyrus neurons, and long-lasting stress leads to a decrease in both size and number of dendrites in the CA3 region [20,21]. The hippocampal CA2 region is unique in being the only CA

Table 2
Classification of seizure parameters in infant rats of different groups after subcutaneous administration of 150 mg/kg pilocarpine.

Epileptic behavior	Sex	Control	FWS	SWS	TWS	EGS	ANOVA
Time to onset (min)	M	5.43 ± 0.39	5.21 ± 0.26	3.93 ± 0.4	4.09 ± 0.48	2.6 ± 0.34	$F[9]=7.7$
	F	5.46 ± 0.41	5.38 ± 0.34	3.77 ± 0.55	3.69 ± 0.44	2.65 ± 0.28	$P<0.001$
Number of tonic–clonic seizures	M	3.55 ± 0.49	5.36 ± 0.64	8.36 ± 0.95	8.82 ± 0.75	8.36 ± 0.95	$F[9]=7.2$
	F	3.82 ± 0.52	4.91 ± 0.64	5.27 ± 0.62	5.73 ± 0.66	5.73 ± 0.69	$P<0.001$
Duration of tonic–clonic seizure(s)	M	34.91 ± 4.26	43.18 ± 5.02	102.91 ± 8.06	186 ± 10.34	216.18 ± 15.8	$F[9]=68.7$
	F	32.18 ± 2.03	45.36 ± 4.12	92.27 ± 5.97	152.55 ± 9.57	106.93 ± 8.56	$P<0.001$
No. of immobility	M	3.18 ± 0.46	3.55 ± 0.56	5.09 ± 0.56	5.91 ± 0.67	6.64 ± 0.61	$F[9]=5.4$
	F	3.09 ± 0.44	3.64 ± 0.58	3.45 ± 0.47	3.73 ± 0.38	4.18 ± 0.44	$P<0.001$
Duration of immobility (min)	M	31.82 ± 4.7	34.27 ± 2.92	30.91 ± 3.9	43.45 ± 2.98	45.64 ± 2.5	$F[9]=1.9$
	F	30.91 ± 4.4	33.1 ± 4.1	37.82 ± 4.1	33.36 ± 4.33	33.73 ± 2.9	$P<0.001$
No. of head bobs	M	4.82 ± 0.61	5.45 ± 0.59	7.36 ± 0.74	10.09 ± 1.1	13.09 ± 0.92	$F[9]=13.8$
	F	5.27 ± 0.54	5.09 ± 0.67	5.647 ± 0.47	6.45 ± 0.8	8.64 ± 0.59	$P<0.001$
Duration of head bobbing	M	57.36 ± 6.2	53 ± 5.34	73.4 ± 5.98	96.8 ± 9.98	128.2 ± 9.38	$F[9]=11.6$
	F	52.36 ± 6.9	58.6 ± 6.83	60.3 ± 4.87	66.1 ± 8.27	88.3 ± 5.85	$P<0.001$
No. of tail extensions	M	3.9 ± 0.43	4.27 ± 0.56	5.27 ± 0.6	11.2 ± 1.03	12.6 ± 0.92	$F[9]=19.8$
	F	3.53 ± 0.44	4.91 ± 0.61	5.82 ± 0.55	8.1 ± 0.86	8.5 ± 0.78	$P<0.001$
Duration of tail extension	M	34.45 ± 3.2	39.36 ± 4.64	44 ± 4.44	113.8 ± 10.45	130.5 ± 10.58	$F[9]=25$
	F	31.73 ± 3.22	38.1 ± 4.17	50 ± 4.3	81.27 ± 10.11	80.6 ± 8.97	$P<0.001$
Mortality rate in 2 hours	M	9.1% (1/11)	9.1% (1 of 11)	36.4% (4/11)	36.4% (4/11)	63.6% (7/11)	Fisher's exact test
	F	9.1% (1/11)	9.1% (1/11)	27.3% (3/11)	9.1% (1/11)	9.1% (1/11)	$P<0.01$
24 hours	M	9.1% (1/11)	9.1% (1/11)	36.4% (4/11)	36.4% (4/11)	72.7% (8/11)	Fisher's exact test
	F	9.1% (1/11)	9.1% (1/11)	27.3% (3/11)	9.1% (1/11)	36.4% (4/11)	$P<0.001$

FWS, first week stressed group; SWS, second week stressed group; TWS, third week stressed group; EGS, entire gestation stressed group.

region receiving inputs from the hypothalamic supramammillary nucleus, of importance in modulating hippocampal theta rhythm, and is seizure resistant in temporal lobe epilepsy [22]. A prominent physiological state of the hippocampus, the theta rhythm, often accompanies voluntary motor behavior output and is associated with seizure resistance. Similarly, pharmacologically induced hippocampal theta activity is associated with behavioral arousal in freely moving rats [19]. Moreover, both physiologically and experimentally occurring hippocampal theta activity attenuates or terminates seizures, whereas lesions to the hippocampus that abolish theta rhythms are proconvulsant. These results suggest that the arousal-like effects of stressor exposure could modulate seizure-related behavioral output and onset of seizures themselves via a mechanism involving electrophysiological activation of the hippocampus [19].

Our findings in the present study are consistent with these data. As summarized in Table 2, time to onset of first epileptic behavior (more frequently immobility) decreased in TWS and EGS rats as compared with controls. As indicated earlier in this article, alterations in hippocampal structure and function might be the mechanism underlying rapid induction of seizures in stressed rats. Also, most of the changes in epileptic behavior parameters (Table 2) can be explained by alterations in hippocampal plasticity.

The second candidate mechanism for stress-induced seizure susceptibility relates to adrenergic neurotransmission. The major source of norepinephrine (NE) in the brain, the hindbrain locus coeruleus (LC), plays a pivotal role in stressor reactivity [23]. In particular, upregulation of NE neurotransmission derived from LC source neurons early in epileptogenesis may function as a compensatory or coping response. The importance of the inhibitory impact of the noradrenergic system in delaying the onset of seizure susceptibility is further supported by other studies as well. These studies reported that in rats with lesions of the forebrain catecholamine system, grafts of fetal LC cells, when placed into the amygdala and piriform cortex, were sufficient to provide dense noradrenergic reinnervation to the hippocampus, which delayed the development of seizures caused by hippocampal kindling [19]. Most convincingly, LC lesions convert sporadic seizures into limbic status epilepticus [24]. These findings suggest an anticonvulsive role for the adrenergic system, which is a stress-sensitive brain mechanism. Consistent with these data, several studies have reported an inhibitory effect of stress on epilepsy [16,25,26]. Our findings are not in agreement with these anticonvulsive effects of stress. Differences in stress procedures, times of stress application, and seizure-inducing methods, as well as whether the study was in vitro or in vivo, may be reasons for this inconsistency.

The third candidate mechanism for stress-induced seizure susceptibility relates to activation of adrenocortical hormones. There is strong evidence that stressors that trigger seizures increase glucocorticoid levels, which in turn lower the threshold for seizure induction [19]. Prenatal stress, most effectively during the third week of gestation, also alters the feedback regulation of the HPA axis [12,27], so that offspring have higher basal secretion of corticotropin-releasing factor (CRF) and adrenal corticosteroid production [10]. CRF is a potent convulsant in pups and has been shown to lower seizure threshold [28]. Prenatal stress is reported to produce 100–400% elevations in plasma COS [16,19]. Therefore, it seems that seizure severity may be a function, in part, of sensitivity to the excitatory effects of glucocorticoids. Our findings in the present study are consistent with these data. As outlined in Table 1, blood COS levels were increased in stressed groups, more prominently in the TWS and EGS groups, compared with control rats. In this context, the COS level of EGS rats was eightfold that of the control group. Therefore, the excitatory effect of prenatal stress on infant rats might, in part, implicate more glucocorticoid production. Taken together, prenatal stress increases COS levels and alters neurotransmitter systems in the hippocampus, including the serotonergic [10], cholinergic [27], and noradrenergic systems [10,12]. These alterations could potentially affect brain excitability and, hence, pilocarpine-induced seizures.

On the other hand, male and female rats differed significantly with respect to both COS levels and severity of epileptic behaviors. As summarized in Table 1, COS levels of female rats are lower than those of male rats. Also, female rats, particularly those in the TWS and EGS groups, are much more resistant to pilocarpine-induced seizures (Table 2).

It has been reported that men and women may respond differently to stressors. Women react to stressful situations with “tend-and-befriend” behaviors. In contrast, men display more of a “fight-or-flight response” in stressful situations [29]. Sex differences have also been observed in several animal models of stress [30,31]. It has been reported that overcrowded living conditions decrease COS levels in female rats, but increase COS levels in male rats, suggesting that crowding stresses males but not females [29,32]. These results suggest that even something as simple as housing conditions can model social stress and have a sex-selective impact on behavior.

Studies measuring seizure susceptibility have reported subtle sex differences in basal seizure risk between males and females, as well as differences within females across the estrus cycle [29]. It seems that differences in activity among sex hormones differentially influence seizures. Androgens exert early organizational and later activational effects that can amplify gender differences in the expression of some seizure disorders. Estrogen and progestins can exert acute activational effects to reduce convulsive seizures and these effects are mediated in part by the actions of steroids in the hippocampus [26]. Some of these anticonvulsive effects of sex steroids are related to their formation of ligands that have agonist-like actions at γ-aminobutyric acid ($GABA_A$) receptors or antagonist actions at glutamatergic receptors [29].

Differences in stress, developmental phase, reproductive status, endocrine status, and treatments such as antiepileptic drugs may alter levels of these ligands and/or the function of target sites (i.e., $GABA_A$ receptors), which may mitigate differences in sensitivity to, and/or tolerance of, steroids among some individuals.

In conclusion, this study of mixed physical–social (prenatal restraint) stress in an animal model of epilepsy revealed important sex differences in behavioral epilepsy. If these findings extrapolate to humans, they suggest that stressors, both social and nonsocial, may differentially influence the risk for seizures in men and women. Continuing studies will further elucidate basic mechanisms underlying stress to better delineate the importance of sex and stress interactions in increasing risk for a number of diseases, including epilepsy, mental disorders, and cardiovascular disease.

6. Ethical approval

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of interest statement

The authors have no conflicts of interest to declare regarding the study described in this article and the preparation of the article.

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