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Estrogen Dependence of Cholinergic Systems That Regulate Lordosis in Cycling Female Rats

CHERYL S. MENARD[1] AND GARY P. DOHANICH

Department of Psychology and Neuroscience Program, Tulane University, New Orleans, LA 70118

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MENARD, C. S. AND G. P. DOHANICH. *Estrogen dependence of cholinergic systems that regulate lordosis in cycling female rats.* PHARMACOL BIOCHEM BEHAV 48(2) 417–421, 1994.—Previous evidence indicated that physostigmine, an acetylcholinesterase inhibitor, facilitated lordosis behavior when administered intraventricularly to cycling female rats on proestrus prior to the onset of natural sexual receptivity, but not when administered to rats on mid-diestrus or diestrus II. In the present experiments, intraventricular infusion of physostigmine (10 μg bilaterally) facilitated lordosis on mid-diestrus and diestrus II if females were primed with two injections of estradiol (0.2, 0.1, or 0.05 μg) administered 20 h and 32 h prior to infusion of physostigmine. Despite unequal levels of endogenous progesterone, physostigmine facilitated lordosis equally on mid-diestrus and diestrus II following estradiol priming. Finally, intraventricular infusion of the muscarinic receptor blocker scopolamine (20 μg bilaterally) reduced the incidence of lordosis in females that displayed lordosis on mid-diestrus following estrogen priming. Results confirm that cholinergic mechanisms influence sexual behavior displayed by cycling female rats. Data further indicate that sufficient estrogen stimulation is necessary for cholinergic neurons to facilitate lordosis. However, progesterone does not play a major role in the regulation of lordosis by cholinergic systems.

Estrogen Estrous cycle Sexual behavior Lordosis Acetylcholine Muscarinic receptors Physostigmine Scopolamine

STEROID hormones have been proposed to activate rodent sexual behaviors by regulating the activity of central neurotransmitter systems. In support of this hypothesis, intracerebral administration of various cholinergic agonists rapidly facilitates the display of the dorsoflexive mating posture lordosis in ovariectomized rats primed with low doses of estrogen (5,8–11). The facilitative effects of cholinergic agonists on lordosis are blocked completely by pretreatment with muscarinic receptor blockers (5,9,10). Furthermore, compounds that antagonize cholinergic activity inhibit lordosis in ovariectomized rats treated with estrogen and progesterone (5,7,11).

In order to determine if cholinergic systems play a role in occurrence of natural sexual receptivity, the effects of cholinergic manipulations on sexual behavior were studied in gonadally intact female rats. Lordosis normally can be elicited in intact female rats by males only during proestrus–estrus of the four-day estrous cycle. The muscarinic receptor antagonist scopolamine was found to reduce the incidence of lordosis displayed by intact females during proestrus–estrus within 15 min after systemic or intraventricular administration (13). This cholinergic treatment did not interrupt cyclicity as determined by vaginal cytology. In a second set of experiments (14) lordosis was facilitated in gonadally intact, unreceptive females after intraventricular infusion of physostigmine, an acetylcholinesterase inhibitor. However, this facilitation of lordosis in cycling females was dependent on the stage of the estrous cycle. Physostigmine activated lordosis during proestrus immediately prior to the onset of natural receptivity, and not on diestrous stages of the estrous cycle.

Several factors might account for the behavioral ineffectiveness of physostigmine when administered on diestrus. As a neurotransmitter, acetylcholine may facilitate lordosis only in the presence of additional endogenous compounds. The levels of these compounds, which could include neurotransmitters, peptides, and steroids, might be inadequate on certain days of the estrous cycle to act in concert with experimentally

[1] Requests for reprints should be addressed to Dr. Cheryl S. Menard, Department of Psychology, 2007 Percival Stern Hall, Tulane University, New Orleans, LA 70118.

elevated levels of acetylcholine. Alternatively, cholinergic agonists may fail to activate lordosis when administered during diestrus because of rhythmic changes in the availability of relevant endogenous proteins, such as enzymes or neurotransmitter receptors. Consequently, acetylcholine, as well as other neurotransmitters, may be able to facilitate sexual behavior only during a specific phase of the estrous cycle as determined by an endogenous pacemaker.

Previous evidence suggests that the ability of cholinergic agonists to facilitate lordosis in ovariectomized rats is enhanced by pretreatment with low levels of estrogen (6). Consequently, the release of endogenous estrogen prior to diestrus may provide insufficient hormonal stimulation to allow cholinergic facilitation of lordosis. To determine if the failure of physostigmine to activate lordosis at stages other than proestrus was due to inadequate estrogen stimulation, gonadally intact female rats were primed with minute amounts of estrogen prior to intraventricular administration of physostigmine in the present experiments. Specifically, several low doses of estradiol were administered to gonadally intact females via two pulse injections administered at 20 h and 32 h prior to behavioral testing on mid-diestrus or diestrus II. If the cholinergic mechanisms that contribute to the regulation of lordosis require sufficient estrogen stimulation, then administration of exogenous estradiol should permit the activation of lordosis on mid-diestrus and diestrus II by the acetylcholinesterase inhibitor physostigmine. In addition, since the ability of physostigmine to facilitate lordosis could depend on progesterone, circulating titers of endogenous progesterone were assessed in females. Lastly, the ability of the muscarinic receptor blocker scopolamine to inhibit lordosis and muscarinic receptor binding in specific brain regions was determined in those females that displayed sexual receptivity after estrogen priming on diestrus.

METHOD

Animals

Subjects were Long-Evans female rats, 175-200 g in weight, purchased from Harlan Sprague-Dawley Inc. (Indianapolis). The animals were individually housed and maintained on a 12-h light/dark cycle (lights off at 0800) in a temperature-controlled vivarium. Twenty Long-Evans males were used as stimulus animals in behavioral testing and were housed in pairs in the same vivarium with the females.

Procedure

Estrous cycling of female rats was determined by daily examination of vaginal cytology and sexual behavior. Vaginal smears were collected twice daily, at the beginning and end of each dark cycle. These Long-Evans female rats normally maintained diestrus I cytology for the first day, mid-diestrus cytology for the second day, diestrus II cytology for the third day, proestrus cytology during the morning of the fourth day, and proestrus-estrus cytology during the afternoon of the fourth day. Only a few of the females maintained five-day cycles with estrus cytology predominating on the fifth day (<5%). To determine sexual receptivity females were vaginally masked and received three mounts by a stimulus male daily during the afternoon of the dark cycle. Animals were considered to be successfully cycling when vaginal cytology and sexual behavior, corresponding to normal estrous cycles of four to five days in duration, occurred for at least two successive cycles.

On diestrus I, after successfully cycling twice, each rat was implanted stereotaxically with a bilateral indwelling cannula assembly that allowed delivery of drugs into the lateral ventricles [see (13,14)]. Prior to surgery, animals were anesthetized with ketamine (100 mg/kg; Bristol Laboratories, Syracuse, NY) in combination with xylazine (7 mg/kg; Miles Laboratories, Shawnee, KS).

Hormone and drug manipulations occurred during the third estrous cycle following surgery. Females received estrogen treatments on diestrus I and cholinergic treatments on mid-diestrus (n = 75) or estrogen treatments on mid-diestrus and cholinergic treatments on diestrus II (n = 50). Each female received one of four hormone treatments: 10% ethanol vehicle, 0.2 μg estradiol, 0.1 μg estradiol, or 0.05 μg estradiol (Sigma Chemical Co., St. Louis). Hormone treatments were administered by two SC injections delivered in 0.1-ml volumes at 0800 and 2000 (20 h and 32 h prior to drug testing). Administration of two pulses of free estradiol in 1-μg doses separated by 12 h has been reported to facilitate sexual receptivity in ovariectomized female rats when administered in conjunction with progesterone (3,4).

Behavioral testing was conducted in the vivarium room where the animals were housed. Testing arenas were 10-gal aquaria (50 cm length × 25 cm width × 30 cm height) lined with aspen wood chips. During behavioral testing to determine drug effects, vaginally masked females were placed at 1600 in an arena occupied by a male and received 10 mounts by the male. Females failing to receive 10 mounts within 10 min were transferred to another arena with a different male for the completion of the test. The lordosis quotient was computed for each female ([number of lordotic responses/number of mounts] × 100]).

On the day of drug testing, each female received 10 mounts by a male before cholinergic treatments were administered. If a female showed receptivity during this pretest (lordosis quotient > 50) she received bilateral intraventricular infusions of either saline vehicle or scopolamine hydrobromide (20 μg/cannula, Sigma) delivered in 0.5-μl volumes with Hamilton syringe driven by a microinfusion pump (13,14). The effects of scopolamine on lordosis were tested at 15 min after infusion. If a female failed to show receptivity (lordosis quotient < 50) she received bilateral intraventricular infusions of either saline vehicle or physostigmine hemisulfate (10 μg/cannula, Sigma). The effects of physostigmine on lordosis were tested at 15 min and 1 h after infusion. Immediately following behavioral testing, females were sacrificed and serum and brain tissue samples were taken.

Progesterone Radioimmunoassay

Trunk blood was collected immediately following sacrifice from females that were infused with saline (n = 61) or physostigmine (n = 55). The serum was centrifuged and the supernatant was extracted and frozen at −60°C until all samples were collected (2-26 weeks). A progesterone radioimmunoassay kit (Coat-a-Count, Diagnostic Products, Inc., Los Angeles) containing standard calibrators (0-40.0 ng/ml) and prescribed protocols was used to determine serum progesterone levels. A similar radioimmunoassay was conducted to determine serum estradiol levels. However, low titers of circulating estradiol produced highly variable results which are not reported.

Muscarinic Receptor Binding

In order to confirm the occupancy of muscarinic receptors following administration of scopolamine, muscarinic binding

was measured in tissue homogenates from females treated with saline (n = 22) or scopolamine (n = 9). Following sacrifice, the brains were extracted and the septum, medial preoptic area, medio-basal hypothalamus, and central gray were dissected. Each area was homogenized in 500 μl of 0.32-M sucrose and stored at −60°C (2–26 weeks). Muscarinic binding was determined in 75-μl aliquots of homogenate using the high affinity muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB; 42 Ci/mmol; Amersham Corp., Arlington Heights, IL). Incubations were conducted for 2 h at room temperature in 50 mM sodium-potassium phosphate buffer containing 1 nM [^{3}H]QNB in final volumes of 225 μl. Nonspecific binding was determined in parallel tubes containing 2 μM atropine sulfate (Sigma). Following vacuum filtration, radioactivity was measured using liquid scintillation technique. The amount of protein contained in each homogenate was determined by the method of Bradford (2).

RESULTS

Physostigmine Treatment and Lordosis

Figure 1 illustrates the mean lordosis quotients (± SEM) recorded before, 15 min after, and 1 h after administration of either saline or physostigmine (10 μg bilaterally) to female rats that were not receptive at pretest on mid-diestrus (n = 59) or diestrus II (n = 50). Significant interaction effects were obtained with a 2 × 4 × 2 (Day × Estradiol × Drug) analysis of variance (ANOVA) with repeated measures (behavioral test). When physostigmine was administered on mid-diestrus or diestrus II, lordosis quotients were increased significantly over pretests at 15 min after treatment in females primed with 0.2 μg ($p < .01$, $p < .01$), 0.1 μg ($p < .01$, $p < .03$), or 0.05 μg ($p < .02$, $p < .03$) estradiol, but not in females primed with ethanol vehicle (Newman–Keuls pairwise comparisons). The incidence of lordosis remained elevated at 1 h on mid-diestrus in those females that were primed with 0.2 μg estradiol ($p < .01$) and 0.1 μg estradiol ($p < .01$) and on diestrus II in those females primed with 0.1 μg estradiol ($p < .01$). On mid-diestrus, but not on diestrus II, pairwise comparisons of lordosis quotients at 15 min after physostigmine treatment indicated significant increases in lordosis quotients as the estradiol dose increased (0.2 μg > ethanol, 0.1 μg > ethanol, 0.05 μg > ethanol, 0.1 μg > 0.05 μg; $p < .01$).

Scopolamine Treatment and Lordosis

Figure 2 illustrates the mean lordosis quotients (± SEM) recorded before and 15 min after administration of either saline or scopolamine (20 μg bilaterally) to females that were receptive at pretest on mid-diestrus (n = 16). A 2 × 2 (Estradiol × Drug) ANOVA was performed on lordosis quotients recorded at 15 min after administration of saline or scopolamine in females that were primed with 0.2 or 0.1 μg estradiol. Independent comparisons of lordosis quotients at 15 min after saline or scopolamine at each estradiol level indicated that scopolamine treatment significantly inhibited lordosis in females primed with 0.2 μg estradiol or 0.1 μg estradiol ($p < .01$).

Progesterone Levels

Mean progesterone values (ng/ml; ±SEM) for females that received saline or physostigmine treatment on mid-diestrus or diestrus II are presented in Fig. 3. A 4 × 2 × 2 (Estradiol × Day × Drug) ANOVA revealed that progesterone levels did not vary significantly between females that received saline or physostigmine or between females that received different doses of estradiol. Overall, progesterone levels were higher on mid-diestrus than on diestrus II ($p <$

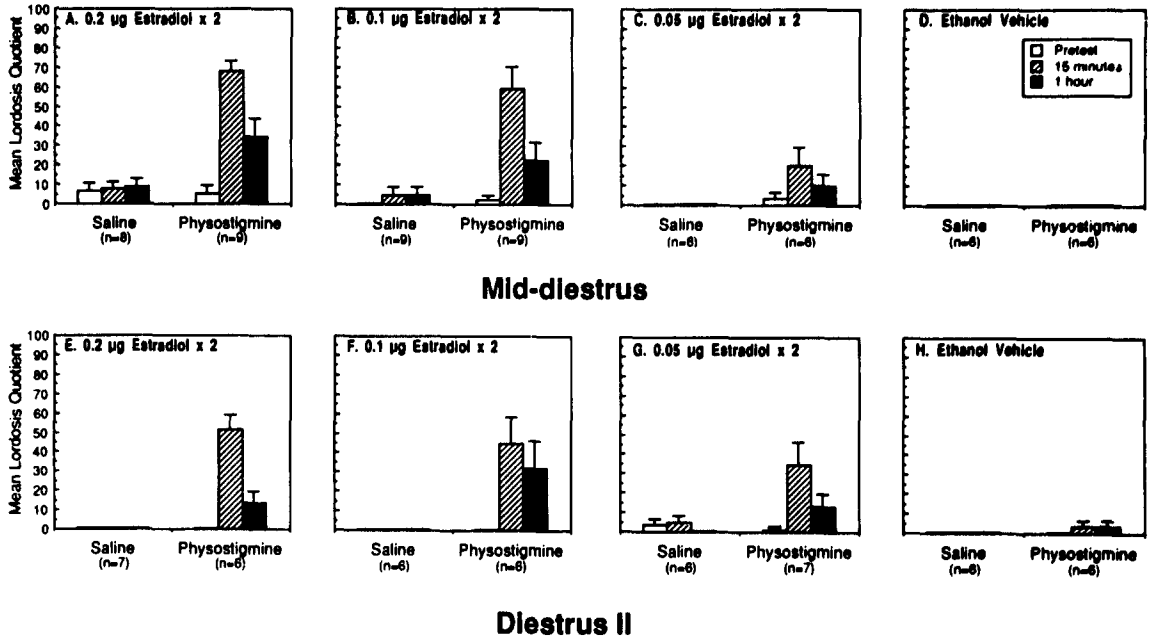

FIG. 1. Mean lordosis quotients (±SEM) of intact female rats recorded before, 15 min after, and 1 h after intraventricular infusion of either saline vehicle (0.5 μl bilaterally) or physostigmine (10 μg bilaterally) on mid-diestrus (A–D) or diestrus II (E–H). Each animal previously received two injections of either 0.2, 0.1, or 0.05 μg estradiol or 10% ethanol vehicle at 20 h and 32 h before behavioral testing on either day.

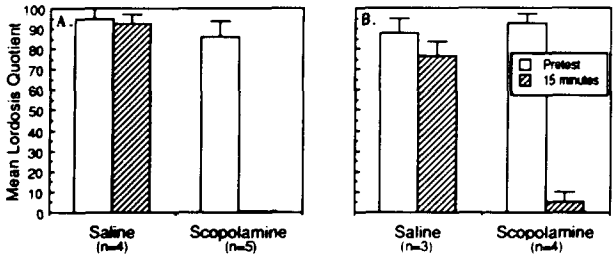

FIG. 2. Mean lordosis quotients (±SEM) of intact female rats recorded before and 15 min after intraventricular infusion of either saline vehicle (0.5 μl bilaterally) or scopolamine (20 μg bilaterally) on mid-diestrus. Each animal previously received two injections of either 0.2 (A) or 0.1 μg (B) estradiol at 20 h and 32 h before behavioral testing on either day.

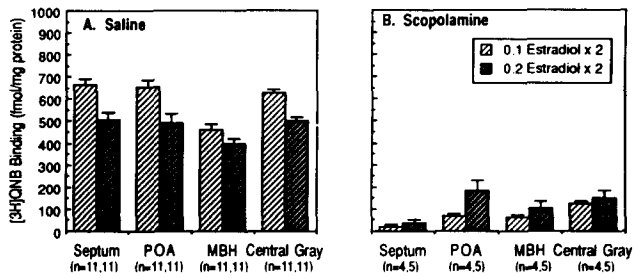

FIG. 4. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) binding (fmol/mg protein; ±SEM) in homogenates of the septum, preoptic area (POA), medio-basal hypothalamus (MBH), and central gray of intact female rats sacrificed on mid-diestrus. Each animal previously received two injections of either 0.2 or 0.1 μg estradiol at 20 h and 32 h before behavioral testing and intraventricular infusion of either saline vehicle (A, 0.5 μl bilaterally) or scopolamine (B, 20 μg bilaterally) 15 min before behavioral testing. Females were sacrificed approximately 20 min after intraventricular infusion.

.01), which may explain the pretest differences in lordosis responding across days of diestrus after estradiol priming. More females were receptive at mid-diestrus (22%) than at diestrus II (0%), and receptive females had received higher doses of estradiol than the nonreceptive females.

Scopolamine Treatment and Muscarinic Receptor Binding

Figure 4 illustrates mean [^{3}H]QNB binding (fmol/mg protein; ±SEM) in the septum, preoptic area, medio-basal hypothalamus, and central gray of females that were infused with either saline or scopolamine 15 min before behavioral testing and approximately 20 min before sacrifice. These females received 0.2 or 0.1 μg estradiol at 20 h and 32 h before behavioral testing. Independent 2 × 2 (Estradiol × Drug) ANOVAs conducted on [^{3}H]QNB binding in each brain region indicated significant suppression of muscarinic binding by scopolamine in all areas ($p < .01$).

DISCUSSION

The results of this study indicate that the cholinergic activation of lordosis in female rats depends on sufficient exposure to estrogen. Intraventricular infusion of the acetylcholinesterase inhibitor physostigmine (10 μg bilaterally) facilitated lordosis during diestrus only following priming with small amounts of free estradiol. In addition, the degree of activation was greater at higher doses of estradiol than at lower doses. These results suggest that circulating titers of estradiol above diestrous levels are necessary to prime the brain to allow cholinergic mechanisms to facilitate lordosis. However, only small increases in estradiol above endogenous levels appear to be necessary for a cholinergic agonist to facilitate lordosis. The results are consistent with previous evidence that physostigmine facilitated lordosis if administered during proestrus following exposure to sufficient levels of endogenous estradiol, but not when administered during diestrus, when prior exposure to endogenous estradiol is inadequate (14). Similarly, the facilitative effect of cholinergic agonists on lordosis in ovariectomized rats is enhanced by estrogen priming (6). However, the amount of estrogen necessary to prime ovariectomized females appears to be higher (0.125, 0.25, or 0.5 μg estradiol benzoate at 24, 48, and 72 h before testing) than that required to prime intact diestrous females (0.05, 0.1, or 0.2 μg estradiol at 20 h and 32 h before testing).

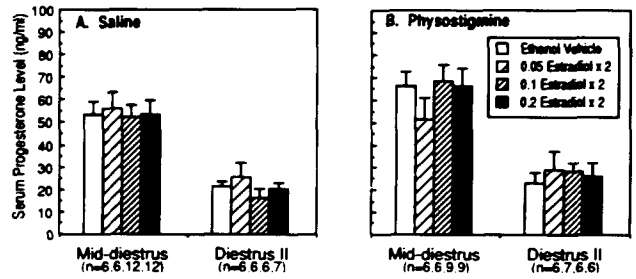

FIG. 3. Serum progesterone levels (ng/ml; ±SEM) of intact female rats sacrificed after intraventricular infusion of either saline vehicle (A, 0.5 μl bilaterally) or physostigmine (B, 10 μg bilaterally) on mid-diestrus or diestrus II. Each animal previously received two injections of either 0.2, 0.1, or 0.05 μg estradiol or 10% ethanol vehicle at 20 h and 32 h before behavioral testing on either day.

Although there were no differences in the ability of physostigmine to activate lordosis across days of diestrus, there were differences in the number of females that were receptive after estradiol treatment across days of diestrus. Significantly more females were receptive on mid-diestrus than on diestrus II before receiving any cholinergic treatment. Since most of these receptive females were primed with higher doses of estradiol, the ability of estradiol to induce lordosis may vary across days of diestrus. Progesterone radioimmunoassay indicated that serum progesterone levels were substantially higher during mid-diestrus than during diestrus II. Therefore, the differential activation of lordosis by estradiol across days of diestrus may occur as the consequence of higher endogenous progesterone levels during mid-diestrus. While females had significantly higher titers of progesterone at mid-diestrus than at diestrus II, estradiol priming 20 h and 32 h prior to mid-diestrus or diestrus II did not alter progesterone levels at either stage. These data indicate that exogenous estradiol did not affect normal endogenous secretion of progesterone through a feedback mechanism.

Although differences in progesterone levels across diestrus affected the percentage of females displaying lordosis prior to drug treatment, physostigmine facilitated lordosis independently of changes in progesterone levels. While levels of circulating progesterone differed across days of diestrus, physostig-

mine activated lordosis equally at mid-diestrus and at diestrus II following estradiol priming. These results suggest that the level of progesterone did not influence the ability of the cholinergic system to activate lordosis. A similar dissociation of progesterone and cholinergic activation of lordosis was demonstrated in an earlier report (8) in which the agonist carbachol activated lordosis equally in ovariectomized rats following priming with estrogen or estrogen and progesterone.

Intraventricular infusion of the muscarinic receptor blocker scopolamine (20 μg bilaterally) completely inhibited lordosis in females that were receptive on mid-diestrus following priming with the higher doses of estradiol (0.2 or 0.1 μg). This inhibition of lordosis by scopolamine at diestrus following exogenous estrogen treatment was similar to the inhibition found previously in ovariectomized rats following treatment with estrogen and progesterone (5,11) and in gonadally intact female rats at proestrus (13). The present results support the hypothesis that a functional cholinergic system is critical to the display of lordosis in intact female rats.

The brain sites at which cholinergic mechanisms act to influence the expression of sexual behavior in female rats have not been determined. Several brain regions that have been implicated in the regulation of lordosis are known to have cholinergic innervation. In addition, some of these areas, including the medial preoptic area, medio-basal hypothalamus, and midbrain central gray, manifest changes in the number of muscarinic binding sites over the estrous cycle and following estrogen treatments (1,12,15–17). However, previous experiments have not adequately characterized the role of these brain regions in the cholinergic regulation of lordosis. The relevant anatomical loci were not resolved by the results of the present study. In agreement with a previous report (11), intraventricular infusion of scopolamine substantially and significantly reduced muscarinic binding as measured in vitro with [^{3}H]QNB in the septum, medial preoptic area, medio-basal hypothalamus, and midbrain central gray. Consequently, scopolamine, administered by intraventricular infusion, accessed a wide area of the brain and inhibited muscarinic binding in several regions known to be implicated in the regulation of lordosis. However, the contributions of these or other brain areas to the regulation of lordosis by cholinergic systems remain to be determined.

While previous evidence indicated that manipulations of the cholinergic system in ovariectomized, hormone-primed rats affected the display of lordosis, the present experiments extend this model to gonadally intact, cycling female rats. Data support the hypothesis that cholinergic mechanisms contribute to the neural control of natural sexual behavior exhibited by female rats. The results of this study in addition offer evidence to suggest that sufficient levels of estrogen are necessary for the activation of central cholinergic mechanisms that regulate sexual receptivity in normally cycling female rats. However, progesterone does not appear to exert a significant influence on the regulation of lordosis by these cholinergic systems.

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