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Actions of Selective Estrogenic Drugs Implanted Into the Medial Amygdala on Male Rat Mating Behavior

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ACTIONS OF SELECTIVE ESTROGENIC DRUGS IMPLANTED INTO THE MEDIAL AMYGDALA ON MALE RAT
MATING BEHAVIOR

by

ANNA DUNIGAN

Under the Direction of Andrew N. Clancy

ABSTRACT

Estrogen stimulation of the medial amygdala (MEA) of the brain promotes male rat mating behavior. However, selective stimulation of either of the estrogen receptor subtypes found in the MEA (ER α or ER β) does not support mating behavior. We tested the hypothesis that dual stimulation of ER α and ER β is required to activate estrogen-dependant neural circuits in the MEA responsible for mating by local treatment of MEA with a combination of selective estrogenic agonists: propyl pyrazole triol (PPT, an ER α agonist) and diarylpropionitrile (DPN, an ER β agonist) administered to castrated, DHT maintained male rats. Estradiol (E₂) or cholesterol (Chol) MEA implants served as positive and negative controls respectively. The animals receiving a mixture of PPT and DPN into the MEA displayed higher levels of mating behavior than the Chol treated animals but lower levels of mating behavior than the E₂ treated animals.

INDEX WORDS: Estradiol, Estrogen receptor, Medial amygdala, Sexual behavior, Propyl pyrazole triol (PPT), Diarylpropionitrile (DPN), Methyl-piperidino-pyrazole (MPP), Mating

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ANNA DUNIGAN

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1. INTRODUCTION

Testosterone (T) is required for male rat mating behavior to occur. Castrated males show a decline in mating behavior [Davidson, 1966a, 1966b] but mating can be restored with administration of exogenous T [Christensen and Clemens, 1974; Davidson, 1966a, 1966b]. At least two biologically active T derivatives are formed in the male rat brain, the estrogen estradiol (E_2) and the androgen dihydrotestosterone (DHT). E_2 is produced from T by aromatase [Naftolin et al., 1975], whereas DHT is produced by the conversion of T to DHT by 5α reductase [Martini, 1982; Massa et al., 1972]. Alone, neither E_2 [Baum and Vreeburg, 1973; Davidson, 1969] nor DHT [Davidson, 1966a; Feder, 1971; McGinnis and Dreifuss, 1989] are as effective as T in their ability to fully maintain mating. Nevertheless, combined treatment with physiological doses of E_2 and DHT restores mating behavior to levels comparable to T-treated animals [Baum and Vreeburg, 1973], suggesting that both androgenic and estrogenic derivatives of T jointly play a role in expression of copulatory behavior by male rats.

Fadrozole (Fad), a non-steroidal aromatase inhibitor, blocks the conversion of T into E_2 [Bonsall et al., 1992; Lipton et al., 1990]. Systemic administration of Fad and T to castrated male rats significantly reduces mating behavior compared to rats treated with water and T [Bonsall et al., 1992]; reinforcing the important role that E_2 plays in mating behavior. When gonadally intact male rats are treated with Fad infusions directly into the MEA, the animals displayed significantly fewer intromissions and ejaculations with significant increases in mount and intromission latencies and the post ejaculatory interval in comparison to controls that receive saline infusions into the MEA [Huddlestone et al., 2006], suggesting that estrogen is required in MEA to support mating. Mounts and intromission, but not ejaculations, were maintained at levels not significantly different from preoperative values in gonadally intact male rats treated with Fad systemically and bilateral E_2 implants in the MEA. In contrast, mounts, intromissions, and ejaculations declined significantly in gonadally intact rats receiving systemic Fad and blank MEA cannulae [Huddlestone et al., 2003]. The combined results of these experiments indicate that

aromatization of T into E₂ in the MEA is critical for expression of mounting and intromission and that E₂ contributes to activation of neural circuits in MEA presumed to be involved in the motivation of mating behavior.

For estrogen to have a physiological effect, it must bind to estrogen receptors (ER) in its target cells. Two types of ERs are well known and studied: estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) [Koike et al., 1987; Kuiper et al., 1996; Mosselman et al., 1996]. The two receptors are coded by different genes and exhibit a high degree of similarity in their ligand (E, 58%) and DNA binding domains (C, 96%) whereas other domains, A, B, D, and F, are poorly conserved [Mosselman et al., 1996]. Both receptors bind with high affinity to steroidal estrogens but vary in their ability to bind to nonsteroidal ligands [Charn et al., 2010; Lindberg et al., 2003; Paulmurugan et al., 2011]. A smaller ER β ligand binding cavity [Hillisch, 2004] may account for this difference and prove to be useful experimentally when trying to selectively activate one of the receptors while being assured that the other subtype remains inactive. It is known that estrogen receptors undergo dimerization and may form homodimers or heterodimers [Cowley et al., 1997]. The similarity in the C domain, a domain responsible for DNA binding and dimerization of receptors, may account for the ability of ER α and ER β to form heterodimers. Heterodimer formation is possible in cells where both ER subtypes colocalize. Findings reported by several laboratories indicate that ER α and ER β are co-expressed in MEA [Greco et al., 2001; Greco et al., 2003; Mitra et al., 2003; Shughrue et al., 1997; Shughrue et al., 1998]. Furthermore, in female rat's dorsal portion of posteriodorsal MEA (MEApd) both ER α and ER β containing cells are activated by mating and express mating-induced Fos-ir, a marker of neuron activity, whereas, in the ventral portion of MEApd, only ER β cells are Fos expressive [Greco et al., 2003]. This highlights the importance of both ER receptor subtypes in the MEA in the expression of mating behavior.

When an ER α antisense oligodeoxynucleotide (AS-ODN) complementary to the mRNA of ER α was infused locally into MEA, ER α expression was inhibited in the MEA but this did not significantly

reduce mating behavior in gonadally intact male rats [Paisley et al., 2012]. Conversely, AS-ODN infusion of the medial preoptic area (MPO) significantly reduced copulation, which suggests that the sexual response of the MPO to estrogen is mediated by ER α to a greater degree than the response to estrogen of the MEA [Paisley et al., 2012]. Moreover, neither MEA implants of propyl pyrazole triol (PPT [Stauffer et al., 2000]), an ER α agonist, nor diarylpropionitrile (DPN [Meyers et al., 2001]), an ER β agonist, maintained mating in castrated male rats administered DHT s.c. whereas E₂ MEA implants were effective [Russell et al., 2012]. Taken together, it appears that estrogen may not act through a single ER pathway in MEA because ER α alone is not necessary and ER β alone is not sufficient to promote the sexual response of MEA to estrogen. However, because estrogen is required in MEA, it is possible that ER α and ER β normally work together to mediate the sexual response of MEA to estrogen. Therefore, we hypothesized that, in MEA, both ER α and ER β must be simultaneously activated in order to promote expression of mating behavior.

To test this hypothesis we examined the effects of combined implantation of the selective non-steroidal estrogenic ER α and ER β agonistic drugs, PPT and DPN respectively, locally into the MEA on mating behavior of castrated DHT s.c. maintained male rats. As a positive control, E₂ was implanted into MEA, and as a negative control, cholesterol (Chol) was implanted into MEA. We expected that animals receiving a mixture of ER α and ER β agonists would show high levels of mating behavior comparable to animals receiving E₂, whereas Chol MEA implants would not maintain mating.

2. MATERIALS AND METHODS

2.1. Animals

Male and female Sprague Dawley rats obtained from Charles River Laboratories were housed in polycarbonate cages, 22 x 44 x 18 cm, with free access to food and water in the Georgia State University vivarium. Male rats were housed two per cage until surgery; post-operatively, male rats were housed in individual cages. Female rats were housed two per cage throughout the experiment except during post-operative recovery. The animals were kept in a climate controlled rat colony on a reversed 14:10 hour reverse light: dark cycle (lights off at 0930 hours EST). Animal care and surgical procedures were in accordance with the Georgia State University IACUC and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, revised 1985).

2.2. Female rat surgical procedures

Female rats were anesthetized with isoflurane (5% gas at 1.0 LPM oxygen exchange rate to induce and 2-3% gas at 0.3 LPM oxygen to maintain), ovariectomized through a midline abdominal incision and implanted s.c. in the scapular region with a 6 mm Silastic crystalline E₂-filled capsules. Postoperatively, the female rats received intramuscular injections of Penicillin (0.1 ml in benzathine penicillin G+ procaine penicillin G) and carprofen (5 mg/kg) to reduce the possibility of infection and to alleviate pain during recovery. The females were given at least 7 days to recover before the initiation of weekly sexual behavioral tests. On behavioral test days, female rats received s.c. an injection of progesterone (1 mg progesterone in 0.2 ml of sesame oil) at least 4-6 hours prior to being paired with males to render them sexually receptive.

2.3 Sexual behavior screening

Male rats were screened for the display of sexual behavior in weekly 30 minute mating tests over a 4 week period. The tests were performed under a red light illumination during the dark phase of the light: dark cycle. During the test each male was placed with a sexually receptive female in a 22 x 44 x

50 cm polycarbonate cage for a 30 minute period. The recorded data included: mount frequency (MF)- number of mounts without penetration; intromission frequency (IF) – number of mounts with penetration; ejaculation frequency (EF) – number of ejaculations; mount latency (MLAT)- time passed from the start of the test until the first mount or intromission; ejaculation latency (ELAT)- time passed from the first mount or intromission until the first ejaculation; post-ejaculatory interval (PEI) – time from the first ejaculation to the subsequent mount or intromission. The data were recorded by testers blind to the experimental status of the animals in order to minimize experimenter bias. Based on the obtained ejaculatory frequencies the animals were assigned into 3 matched groups, those receiving: 1) estradiol to the MEA (E₂ group), the positive control, 2) cholesterol to the MEA (Chol group), the negative control and 3) a 50%/50% wt/wt (50/50) mixture of PPT and DPN to the MEA (PD group).

2.4. Cannulae Implantation, Castration and Postoperative Screening

The male rats were anesthetized with isoflurane as described above and the testes were removed through an abdominal incision. The animals received a 10 mm Silastic capsule filled with DHT s.c. in the scapular region. DHT capsules of this size have been reported to produce physiological levels of circulating DHT [Ando et al., 1998; Lugg et al., 1995; Parte and Juneja, 1992]. While still deeply anesthetized, the male rats were then placed into a stereotaxic instrument and implanted intracranially with bilateral 22-gauge stainless steel, ethylene oxide sterilized guide cannulae aimed at the MEA (level skull coordinates: AP=-3.2 mm, ML=3.5 mm, DV=-8.2 mm [Swanson, 1998]). 28-gauge stainless steel, ethylene oxide sterilized inner cannulae filled with the appropriate drug, either a 50/50 mixture of PPT and DPN, or E₂, or Chol, were inserted extending 1 mm below the guide cannulae. Postoperatively, the male rats received intramuscular injections of Penicillin (0.1 ml in benzathine penicillin G+ procaine penicillin G) and carprofen (5 mg/kg) to reduce the possibility of infection and to alleviate pain during recovery. The males were given at least 7 days to recover prior to the onset of post-operative behavioral testing. The inner cannulae were replaced with fresh, drug filled and ethylene oxide sterilized

cannulae two days prior and one day following a behavior test to ensure that the MEA was chronically exposed to the appropriate drug. Microscopic inspection of the cannulae tips removed from the experimental animals showed that the drug was present at the cannulae tips at least 98% of the time. The animals were behaviorally tested for 30 minutes each week as described above for 7 consecutive weeks.

2.5. Histological Analysis

After the testing period was terminated, the male rats were euthanized with an overdose of sodium pentobarbital (100 mg/kg i.p.) and perfused transcardially for 5min with physiological saline followed by a minimum of 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution (pH 7.4). The skulls were partially opened and stored in the perfusion fixative overnight. The following day, the brains were dissected and stored in a solution of 30% sucrose in 0.1 M PB for at least 48 hrs. Frozen coronal brain sections (40 μ m) through the amygdala were collected in 0.1 M PB, mounted on gel-
albumin coated slides, and stained with toluidine blue to microscopically verify cannulae placement.

2.6. Statistical Analysis

Kruskal–Wallis one-way analysis of variance was used to determine if there was a significant in-between group difference during the terminal pre-operative, first post-operative, and terminal post-operative tests [Siegel, 1956]. If the results showed a significant group difference, pairwise Mann–Whitney U tests were used to determine the origin of the group difference by comparing E₂ to Chol, E₂ to PD, and PD to Chol groups. Wilcoxon signed-rank test was used to determine within group differences between the data values obtained during terminal pre-operative and terminal post-operative tests [Siegel, 1956]. Fisher’s test was used to compare the proportions of animals that mated in each group during the terminal post-operative test [Siegel, 1956].

3. RESULTS

The three groups showed robust mating during the pre-operative period and on the third pre-operative test (terminal pre-operative test) there were no significant group differences in any of the behavioral indices. Mating behavior diverged significantly between groups during the post-operative period. Specifically, castrated, DHT maintained male rats receiving Chol implants to the MEA showed a significant, progressive decline in all indices between the terminal pre-operative test and seventh post-operative test (terminal post-operative test). Conversely, except for an initial significant decline in all mating indices on the first post-operative test, E₂- MEA implanted rats continued mating such that, on most behavioral indices by the terminal post-operative test, these animals mated at levels not significantly different from those during the terminal pre-operative test. All behavioral indices of the PD group declined significantly between the last pre-operative test and 1st post-operative test but were stably maintained at this lower level throughout the post-operative period and were intermediate between the Chol and E₂ groups. The proportion of male rats in the PD group that mounted or intromitted during the terminal post-operative tests did not differ significantly from the proportion of male rats in the E₂ group that mounted or intromitted during the terminal post-operative test. Conversely, the proportion of animals receiving Chol-MEA implants mounted or intromitted at rates that were significantly lower than those in both the PD and E₂ groups.

Although the three groups did not differ significantly from each other in MF during the terminal pre-operative trial, all 3 groups showed a significant decline in MF on the first post-operative trial (Chol $p < 0.025$; E₂ $p < 0.029$; PD $p < 0.001$) but did not differ significantly from each other. By the terminal post-operative test, however, the MF displayed by the E₂ group returned to levels that were not significantly different from terminal pre-operative values. Conversely, the Chol group showed a steady decline in MF and by the terminal post-operative test, the frequency of mounts performed by the animals in this group was significantly lower compared to the values obtained during the terminal pre-operative and

first post-operative trials ($p < 0.003$ and $p < 0.005$ respectively). In the PD group, after an initial significant drop in MF between the terminal pre-operative and the first post-operative tests, MF was maintained at a steady rate during the post-operative trials such that the MF was not significantly different between the first and the terminal post-operative tests. However, MF was significantly lower ($p < 0.005$) on the terminal post-operative test than on the terminal pre-operative test. Between-group pairwise comparison conducted on the terminal post-operative tests showed that rats in E₂ group mounted at significantly higher frequencies of than rats in both PD and Chol groups ($p < 0.040$; $p < 0.003$ respectively), although MF measures for the PD group were numerically, but not significantly, higher than those of the Chol group. A similar pattern was seen for MIF (mount and intromission frequency) in all of the groups. Between-group pairwise comparison of MIF performed by male rats during the terminal post-operative trial showed that MIF rates were significantly higher in E₂ group than in Chol group ($p < 0.003$) but MIF was not significantly different in the PD group compared to the E₂ group, nor was the PD group significantly different compared to the Chol group (Figure 1A, 1C).

The three groups were not significantly different in the number of Intromissions during the terminal pre-operative trial. Immediately after the surgery, on the first post-operative test, IF in each of the groups decreased significantly (Chol $p < 0.003$; E₂ $p < 0.009$; PD $p < 0.001$) compared to the terminal pre-operative trial. Thereafter, however, the IF was maintained at a steady level in the E₂ and PD groups so that by terminal post-operative test the IF was not significantly different from the values obtained during the first post-operative test for each group. However, in both the E₂ and PD groups these values were significantly lower than those on the terminal pre-operative trial (E₂ $p < 0.009$; PD $p < 0.001$). The Chol group showed a progressive decline in IF throughout the post-operative trials such that the values obtained during the terminal post-operative trial were significantly lower compared to both terminal pre-operative and first post-operative trials ($p < 0.003$; $p < 0.005$ respectively). Between-group pairwise

comparison showed that three groups did not differ from each other in IF during the terminal post-operative trial (Figure 1B).

There were no significant between-group differences in ejaculation frequency (EF) on the terminal pre-operative trial. During the first post-operative trial the values for EF declined in all of the groups; although, only the Chol and PD groups show a significant reduction in EF compared to the terminal pre-operative trial ($p < 0.044$; $p < 0.007$ respectively). EF continued to decline in each of the three groups throughout the post-operative trials and were significantly lower by the terminal post-operative test compared to terminal pre-operative test (Chol $p < 0.005$; E_2 $p < 0.012$; PD $p < 0.002$). However, on the terminal post-operative trial EF displayed by male rats in the E_2 and PD groups did not differ significantly in comparison to EF seen during the first post-operative trial. On the other hand, Chol group ejaculated significantly fewer times on the terminal postoperative test compared to first post-operative test ($p < 0.018$). Pairwise between- group comparison of EF observed during the terminal post-operative trial showed no significant differences in ejaculatory rates between the three groups (Figure 1D).

During the terminal pre-operative trial, there were no significant differences between the three groups in any of the latency measures, MLAT, ELAT, and PEI. In-between and within-group differences developed after the male rats were castrated and received a DHT s.c. capsule and an implant of Chol, E_2 , or PPT and DPN into the MEA. Overall, Chol group displayed highest latency measures while E_2 group had the shortest latency measures and the PD group values were intermediate between the two other groups. Post-operatively, MLAT increased numerically in all three groups, but the elevation between the last pre-operative and the first post-operative tests was only significant in the PD group ($p < 0.008$). MLAT of the male rats in the Chol group continued to rise throughout the post-operative testing period and by the terminal post-operative trial was significantly higher than during the terminal pre-operative and the first post-operative trials ($p < 0.003$; $p < 0.005$ respectively). MLAT of the E_2 group remained slightly elevated numerically throughout the post-operative period compared to the terminal pre-operative

trial, but was not significant. The male rats that received PD-MEA implants displayed significantly longer MLAT during the terminal post-operative test when compared to the terminal pre-operative values ($p < 0.006$), but these values were not significantly different from those on the first post-operative test. Between-group pairwise comparison of MLAT observed during the terminal post-operative trial revealed that in the Chol group MLAT was significantly higher than that of E_2 group ($p < 0.003$) but no other significant differences in MLAT occurred between the Chol and PD or E_2 and PD groups (Figure 2A). Post-operatively, a similar trend was observed in ELAT as with MLAT (Figure 2B). During the first post-operative test, all of the groups showed a significant elevation of PEI compared to the terminal pre-operative trial (Chol $p < 0.026$; E_2 $p < 0.006$; PD $p < 0.001$). During the terminal post-operative trial, the PEI measure in the Chol group was significantly longer from the measures obtained during the terminal pre-operative and first post-operative tests ($p < 0.005$; $p < 0.017$ respectively). The lowest PEI measure occurred in the E_2 group and the PEI of PD group was intermediate compared to E_2 and Chol groups. Specifically, in both, the E_2 and the PD, groups the PEI was significantly longer during the terminal post-operative test when compared to the terminal pre-operative test ($p < 0.004$; $p < 0.001$ respectively) but were not significantly different from the values obtained during the first post-operative test. Between-group pairwise comparison of PEI observed during the terminal post-operative trial showed no significant differences between the three groups (Figure 1C).

The proportions of animals that mounted or intromitted during the terminal post-operative test in the E_2 (9 of 11) or PD (7 of 14) groups were significantly greater than the proportions of animals that that mounted or intromitted in the Chol group (1 of 11) ($p < 0.002$, $p < 0.042$ respectively). Moreover, the proportions of the animals in the E_2 group that mounted or intromitted on the terminal post-operative trial did not differ significantly from that in the PD group (Figure 3A). The proportion of animals that ejaculated during the terminal post-operative test was highest in the E_2 group (5 of 11) and lowest in the Chol group (1 of 11); the PD group values (4 of 14) were intermediate between the other two groups.

These values did not differ significantly between the three groups (Figure 3B). The histological analysis showed that the cannulae were located in or near the MEA in all three groups (Figure 4).

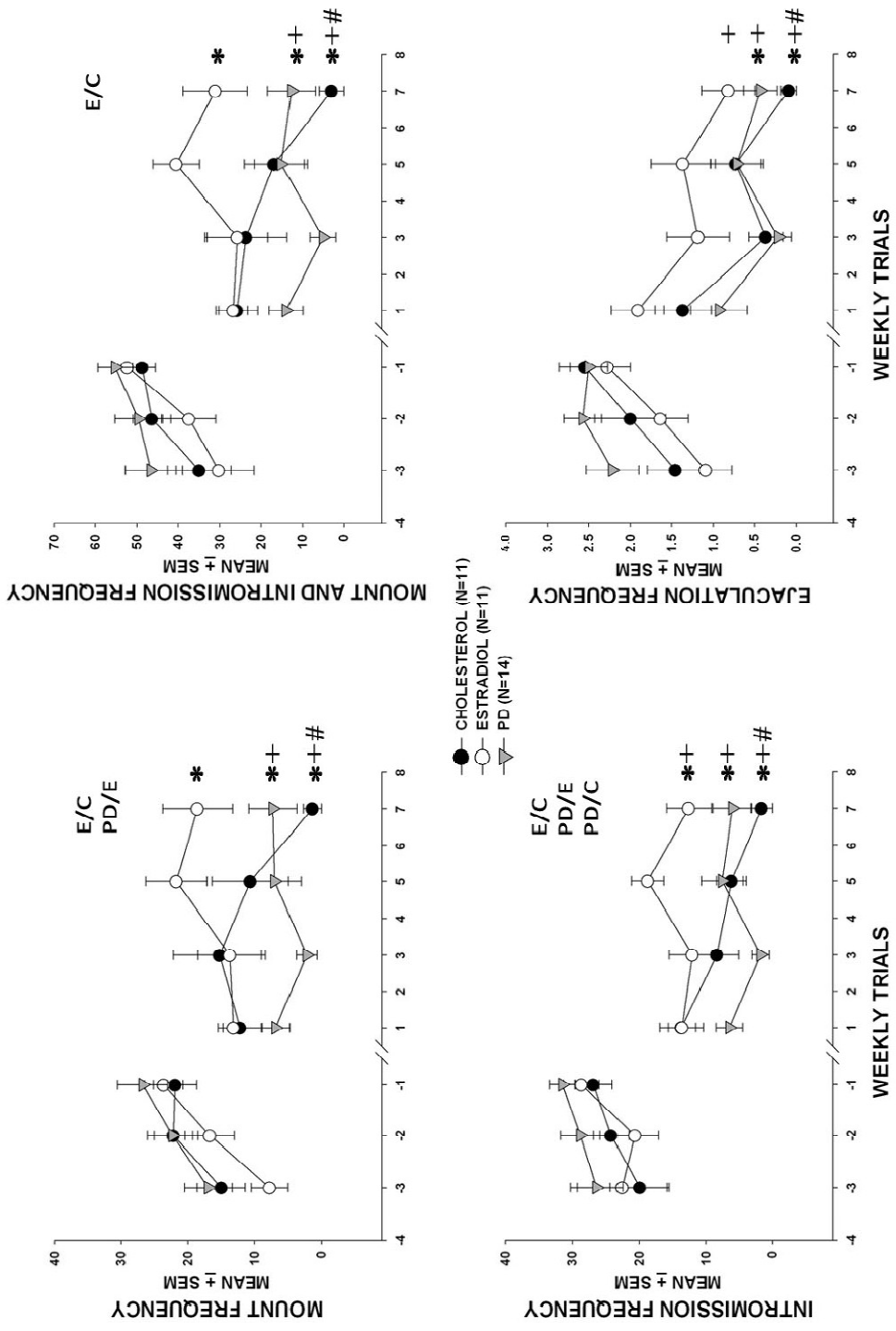


FIGURE 1: MALE SEXUAL BEHAVIORAL FREQUENCIES.

Time course of E2 (n=11), PPT and DPN (n=14) , and Chol (n=11) effects on mating frequencies of castrated, DHT maintained male rats. The three groups were not significantly different during the pre-operative trials. The differences in behavioral indices arose post-operatively. **A: Mount Frequency.** Post-operatively, all 3 groups showed a significant decline in MF between the last pre-operative and the first post-operative tests. MF returned to values not different from those obtained during the pre-operative trials in the E2-MEA group. Animals in the Chol-MEA group showed a steady decline in MF. Male rats that received PD-MEA implants were intermediate. **B: Intromission Frequency.** Post-operatively, all 3 groups showed a significant decline in IF between the last pre-operative and the first post-operative tests. IF did not return to pre-operative levels in any of the groups. **C: Mount and Intromission Frequency.** MIF follows the same pattern as MF. **D: Ejaculation Frequency.** Post-operatively, all 3 groups showed a significant decline in EF compared to pre-operative levels. EF continued to decrease throughout the post-operative trials so that by the terminal post-operative test all the groups showed significantly lower EF compared to terminal pre-operative test and the groups did not differ significantly from each other.

* Within-group difference during the terminal pre-operative test and first post-operative test, $p < 0.05$.

+ Within-group difference during the terminal pre-operative test and terminal post-operative test, $p < 0.05$.

Within-group difference during the first post-operative test and terminal post-operative test, $p < 0.05$.

E/C- Group difference between Estradiol and Cholesterol, $p < 0.05$.

PD/E- Group difference between PPT+DPN and Estradiol, $p < 0.05$.

PD/C- Group difference between PPT+DPN and Cholesterol, $p < 0.05$.

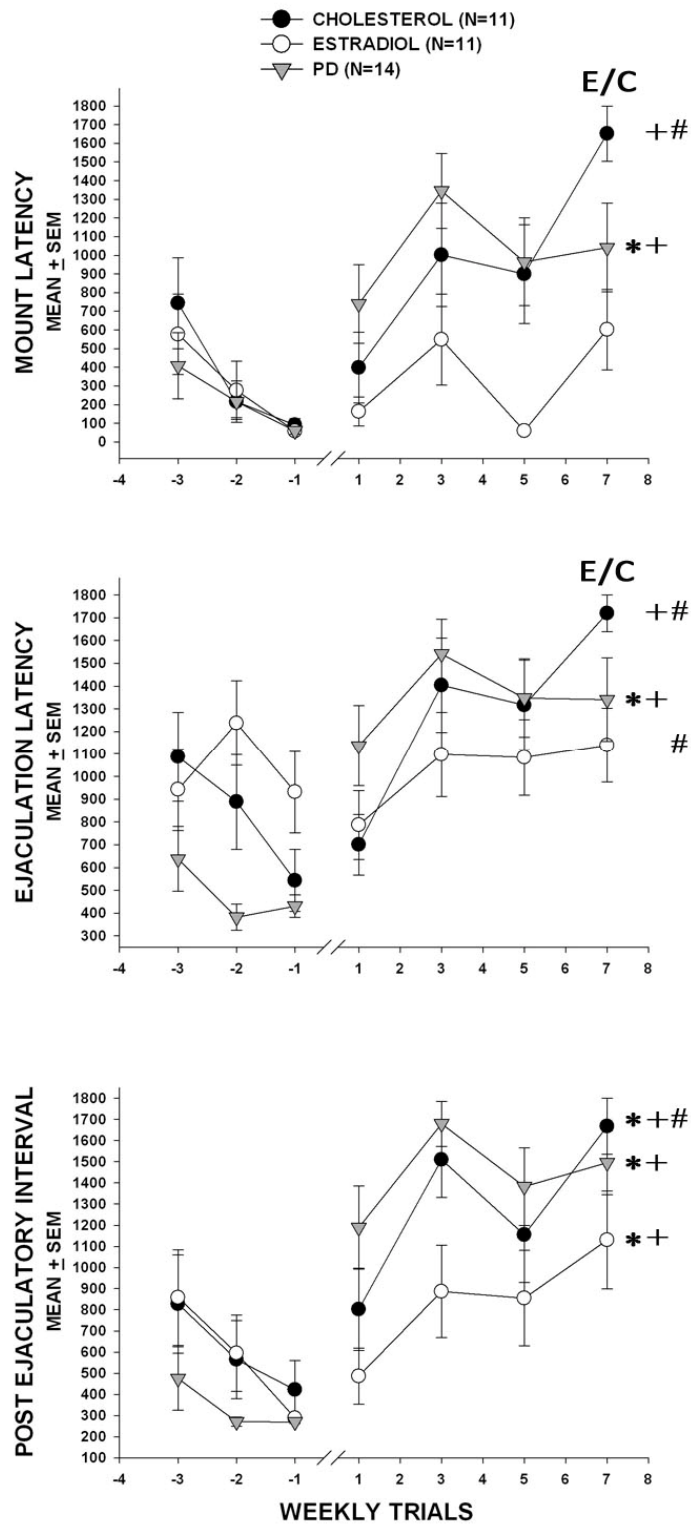


FIGURE 2: MALE SEXUAL BEHAVIORAL LATENCIES.

Time course of E2 (n=11), PPT and DPN (n=14) , and Chol (n=11) effects on mating latencies of castrated, DHT maintained male rats. The three groups were not significantly different during the pre-operative trials. The differences in behavioral indices arose post-operatively. **A: Mount Latency.** During the first post-operative test, MLAT increased for all of the groups, however, only in the PD group was this significant. Throughout the post-operative trials MLAT continued to rise for all of the groups numerically. During the terminal post-operative trial E2-MEA displayed the lowest MLAT values that were numerically but not significantly higher compared to the pre-operative values. PD-MEA and Chol-MEA groups showed significantly higher MLAT than on the terminal post-operative test compared to the terminal pre-operative values. These two groups were not significantly different from each other, yet numerically Chol-MEA group displayed longer MLAT than the PD-MEA group. **B: Ejaculation Latency.** ELAT MIF followed the same trend as MLAT. **C: Post Ejaculatory Interval.** Each group showed a significant elevation of PEI on the first post-operative trial. The values for PEI continued to rise throughout the post-operativetests and were significantly higer for all of the groups on the terminal post-operative test.

* Within-group difference during the terminal pre-operative test and first post-operative test, $p < 0.05$.

+ Within-group difference during the terminal pre-operative test and terminal post-operative test, $p < 0.05$.

Within-group difference during the first post-operative test and terminal post-operative test, $p < 0.05$.

E/C- Group difference between Estradiol and Cholesterol, $p < 0.05$.

PD/E- Group difference between PPT+DPN and Estradiol, $p < 0.05$.

PD/C- Group difference between PPT+DPN and Cholesterol, $p < 0.05$.

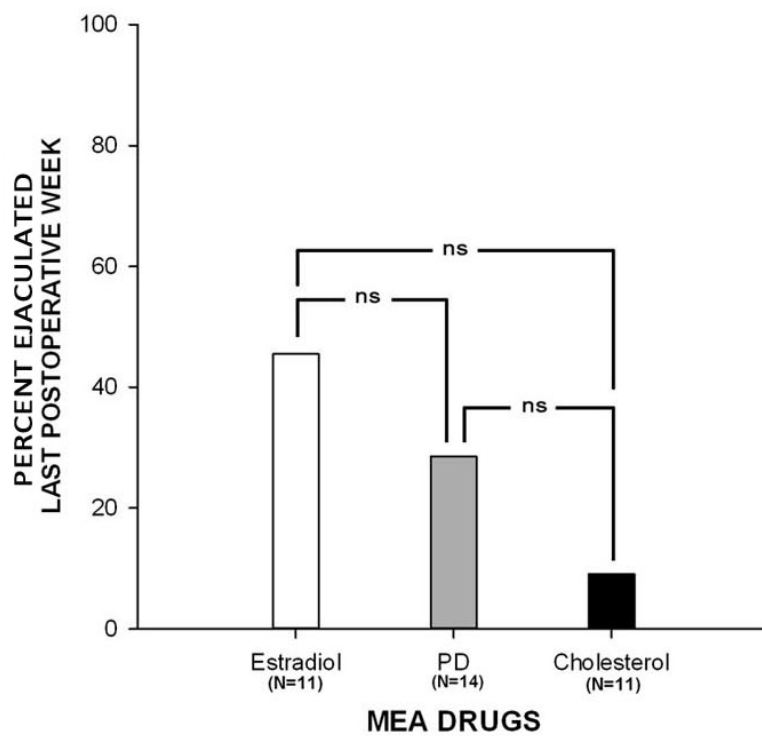
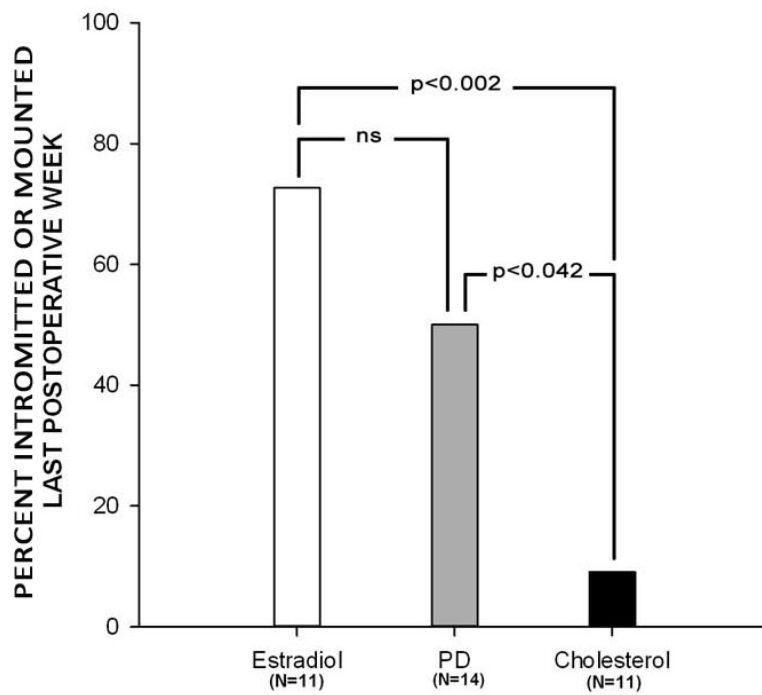


FIGURE 3: MALE SEXUAL BEHAVIOR.

Terminal post-operative mating behaviors (percentages) for the E₂, PD, and Chol groups. **A: Percent Intromitted or Mounted.** A large proportion (9 of 11, 81.8%) of castrated DHT maintained male rats given E₂-MEA implants mounted and intromitted and the proportion of rats receiving PD-MEA implants that mounted and intromitted (7 of 14, 50.0%) did not differ significantly from the E₂-MEA group. Only 1 out of 11 (9.09%) castrated male rats receiving Chol-MEA implants mounted and intromitted and the MIF proportion for this group was significantly lower than either the E₂ or PD group. **B: Percent Ejaculated.** None of the groups were significantly different from each other.

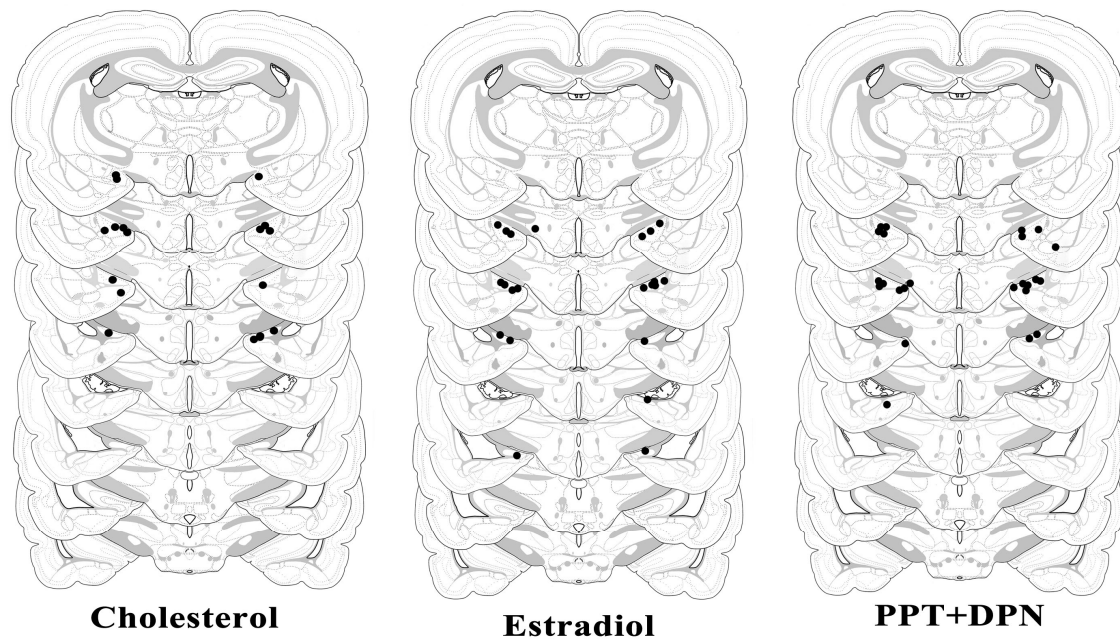


FIGURE 4: CANNULAE PLACEMENT TO THE MEA.

Location of the cannulae tips of male rats implanted with Chol, E2, or PPT and DPN into the MEA. Cannulae tips were positioned in or near the MEA in all groups. The black dots represent cannula tip locations.

4. DISCUSSION

Because, in the MPO, mating behavior is supported by activation of a single receptor (ER α), [Russell et al., 2012] it was expected that the estrogen-sensitive neural circuits of MEA might work similarly. However, this is not the case. Stimulation of MEA ER α alone by PPT (ER α agonist) did not mimic the actions of E₂ and did not support mating behavior [Russell et al., 2012]. Moreover, stimulation of MEA ER β by DPN (ER β agonist) alone also does not promote mating [Russell et al., 2012]. Therefore, because in the MEA the effect of either PPT or DPN is more limited and selective than that of E₂, the objective of this experiment was to determine if simultaneous stimulation of both ER subtypes is required for activation of the estrogen-sensitive neural circuits within MEA that mediate mating behavior.

The hypothesis was confirmed by examining the mating response of male rats implanted with a combination of selective estrogenic agonists of ER α and ER β (PPT and DPN respectively) into the MEA and by comparison of this group to controls receiving E₂ or Chol MEA implants. Because only MEA and not other brain areas important for mechanical execution of copulatory behavior, such as MPO, were stimulated, we expected post-operative behavioral indices to be lower for all of the groups; we also expected that only mounts and intromissions, but not ejaculations, would be maintained [Huggleston et.al., 2003]. Therefore, ejaculation frequency, ejaculation latency, and post ejaculatory interval did not figure prominently in the evaluation of the data although these data are included in the results.

During the pre-operative trials animals in all groups (PD, E₂, and Chol) mated vigorously and were not significantly different from each other on any behavioral indices. The number of mounts and intromissions declined significantly in all groups post-operatively as expected. Mounts and intromissions displayed by the E₂-MEA group returned to the pre-operative levels by the terminal post-operative trial, while the mounts and intromissions displayed by animals receiving Chol-MEA implants declined progressively throughout the postoperative period and were significantly lower on the terminal post-operative week compared to the terminal pre-operative and first post-operative trials. Although PD-

MEA implants failed to maintain mounts and intromissions at the pre-operative levels they did maintain these mating behavior indices at the levels not significantly different from those seen during the first pre-operative trial. Between-group statistical comparison of the mounts and intromissions exhibited by the rats in the three groups on the terminal post-operative week showed that PD group was not significantly different from the E₂ group, nor was it significantly different from the Chol group. Furthermore, on the terminal post-operative trial the proportions of animals that displayed mounts or intromissions in the PD-MEA and E₂-MEA groups were significantly higher than the proportion of animals displaying mounts or intromissions in the Chol-MEA group and the PD-MEA group did not differ significantly from the E₂-MEA group. These findings suggest that MEA responded to activation by combined administration of ER α and ER β selective estrogenic agonists, however, the response did not precisely mimic that of E₂. Nevertheless, we conclude that ER α and ER β play a role in activation of estrogen sensitive circuits in MEA responsible for mediation of mating behavior because combined application of PPT and DPN produced results that were numerically and in some instances significantly higher than those observed in Chol treated animals while singular application of either of the drugs did not produce these effects [Russell et al., 2012].

Although activation of MEA through simultaneous administration of PPT and DPN was successful, dual administration of these ER agonists did not mimic the action of E₂ in the MEA. The plausible explanations for this may lie in the dosage of drugs administered. The animals in the PD group were given a 50/50 mixture of PPT and DPN because an appropriate ratio of PPT/DPN required to activate E₂-dependent circuits in the MEA is not known. Perhaps, if the ratio of the drugs had been altered, we would have observed the rats displaying mating behavior at the levels not statistically different from rats receiving E₂-MEA treatment. When ER α expression was knocked out or knocked down in MEA, the mating was not affected [Paisley et al., 2012]. This previous finding, together with the results of this experiment, raise the possibility that ER α plays a secondary role in the MEA and is

required in lower concentrations than ER β . Thus, a 50/50 mixture of PPT and DPN might not have provided enough of ER β agonist to full activate the circuits in MEA.

Although our findings provide some information about the role of E₂ in MEA to promote mating behavior, the mechanisms of E₂ action in MEA are still not well understood. Several studies showed that ER α and ER β are co-expressed in cells of MEA and that in female rats the cells that co-express both receptor subtypes also exhibit FOS-ir in response to mating stimuli [Greco et al., 2003; Shughrue et al., 1997; Shughrue et al., 1998]. It is also known that ER α and ER β undergo dimerization to form homodimers and heterodimers during the reaction cascade that results in gene activation leading to expression of mating behavior [Cowley et al., 1997]. ERs are able to form heterodimers if both receptor subtypes are colocalized within a cell and it has been proposed that different functions are rendered based on the type of dimer that could be formed in an area [Shughrue et al., 1997; Shughrue et al., 1998]. However, a new finding showed that heterodimers may be formed when only one subtype is bound by a ligand [Paulmurugan et al., 2011]. Therefore, when PPT alone was given, it is possible that ER α /ER β heterodimer was formed along with an ER α homodimer and when DPN was given alone, it is possible that ER α /ER β heterodimer was formed along with an ER β homodimer. Neither of these pairs of dimers were sufficient to activate E₂-dependent neuronal circuits in MEA [Russell, et al., 2012]. However, when a combination of PPT and DPN was administered to MEA, it is possible that ER α /ER β heterodimer, an ER α /ER α and an ER β /ER β homodimers were also formed and that all three dimers are required to facilitate mating behavior.

The results of this experiment might appear to contradict previous findings because it was shown that, in the MEA, ER α is not necessary for male rats to display mating behavior [Paisley et al., 2012]. However, the current findings suggest that ER α nevertheless participates in the signaling pathway which leads to the display of mating behavior by male rats. There are multiple plausible explanations for these apparently conflicting findings. It is possible that ER β is required in MEA but cannot promote

mating behavior on its own and instead must be coupled to another receptor in order to generate the response. Although ER β might normally be coupled to ER α , there might be other possibilities. Because mating behavior persists when ER α mRNA expression is inhibited [Paisley et al., 2012], it is possible that ER β may couple to another non-ER α receptor (not yet identified). Another possibility could involve two different pathways, one of which engages dimers of ER α and ER β , while the other proceeds through a novel type of E₂ receptor not linked to ER α or ER β . Yet another interpretation may emerge from this experiment. It is reasonable to assume that when expression of ER α mRNA was inhibited in MEA by AS-ODN infusion [Paisley, et al., 2012], it did not completely knock out ER α , but rather only knocked down ER α expression. The reduced level of ER α in MPO may have been too low to support the mating behavior if ER α is primarily responsible for E₂ actions in MPO [Paisley et al., 2012; Russell et al., 2012]. However, low residual levels of ER α in MEA might still be enough to sustain the response of the MEA to E₂ because they may aid ER β even if ER α is not the primary E₂ receptor of MEA. To further investigate the actions of E₂ in MEA an anatomical study of male MEA to test if the cells co-expressing ER α and ER β also would exhibit Fos-ir upon mating stimulation might be valuable. Nevertheless, it now appears that E₂ dependent circuits in the MEA responsible for activation of mating behavior in male rats proceed through simultaneous activation of both ER α or ER β and that both receptor subtypes are important in the MEA.

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