

# Sex-specific effects of neonatal progesterin receptor antagonism on juvenile social behavior in rats.

Robin M Forbes-Lorman (✉ [forbes@ripon.edu](mailto:forbes@ripon.edu))

Ripon College <https://orcid.org/0000-0003-3935-6541>

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## Research Article

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# Abstract

Developing mammals are exposed to progesterone through several sources; however, the role of progesterone in early development is not well understood. Males express more progestin receptors (PRs) than females within several brain regions during early postnatal life, suggesting that PRs may be important for the organization of the sex differences in the brain and behavior. Indeed, previous studies showed cognitive impairments in male rats treated neonatally with a PR antagonist. In the present study, we examined the role of PRs in organizing juvenile behaviors. Social play behavior and social discrimination were examined in juvenile male and female rats that had been treated with CDB, a PR antagonist, during the first week of postnatal life. Interestingly, neonatal PR antagonism altered different juvenile behaviors in males and females. A transient disruption in PR signaling during development had no effect on social discrimination but increased play initiation and pins in females. These data suggest that PRs play an important role in the organization of sex differences in some social behaviors.

## Introduction

While it is clear that testosterone and its metabolites play an important role in the organization of the male brain and behavior through their actions on androgen receptors and estrogen receptors (1), less is known about the role of progesterone acting upon progestin receptors (PRs) in the developing brain.

Developing mammals are exposed to progesterone, both from fetal and maternal sources (reviewed in (2, 3)). Indeed, male and female rodents have approximately equivalent levels of circulating progesterone during development (4, 5). Furthermore, progesterone administration is also common during pregnancy for prevention of premature birth and contraceptive use in lactating women (reviewed in (6)).

Males express PRs as early as embryonic day 20 in many brain areas (7) and express more PRs than females within several hypothalamic regions on postnatal day (PN)1 but not PN10 (8, 9). This sex difference in PRs in the developing brain suggests that PRs are important for the organization of the sex differences in the brain. Indeed, blocking PRs using RU-486 during development increases male sex behavior and the expression of ARs in several regions of the adult male brain (10), although another study using a different PR antagonist, ZK 137616, found no effect on male mouse sex behavior (11). Neonatal PR antagonism using RU-486 also disrupts cognitive ability in adult male rats (12).

In the present study, we examined the organizational role of PRs on juvenile social discrimination and juvenile social play behavior by treating male and female rats with CDB, a specific PR antagonist, during the first week of postnatal life.

## Methods And Materials

### Subjects and treatment.

Sprague-Dawley rats supplied by Charles River Labs were bred in our animal facility. Animals were housed in standard lab cages with aspen shavings and no enrichment. Dams were checked daily to determine the day of birth and were allowed to deliver normally. Twenty-seven male and 25 female pups were pooled from five different litters and randomly assigned to each treatment group (13 CDB-treated females, 12 oil-treated females, 13 CDB-treated males, and 14 oil-treated males). Each litter contained animals of both sexes and treatment groups and a maximum of three animals from a single litter were assigned to each treatment group. Pups were foot-marked with India ink and treated subcutaneously with the 75µg/0.01mL/g body of the PR antagonist CDB-4124 or vehicle on PN0 (day of birth), PN2 and PN4. The vehicle was composed of 0.2% benzyl alcohol and 0.6% benzyl benzoate in sesame oil. This treatment regimen is similar to what has been previously used for RU-486 (10); however, we chose to reduce the number of administrations in order to minimize the injections. We have found that the sesame oil does not clear within a day, so treatment was likely continuous from ~ PN0-PN6. The weight of the pups ranged from 5–12 grams over the three days of treatment. CDB-4124 was used because it has a low binding affinity for glucocorticoid receptors (13). All pups remained with dams until weaning at PN21. On PN21, pups were separated into seven cages of six animals and two cages of five animals, each with at least one animal from each treatment group (*i.e.* CDB-treated females, CDB-treated males, vehicle-treated females, vehicle-treated males) and approximately half females and half males. An overview timeline of the experiment is shown in Fig. 1. The rats were housed under a 12:12 light/dark cycle with food and water available *ad libitum*. This research was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

## **Behavioral testing**

Behavioral tests were performed under dim red light approximately 1–2 hours after the beginning of the dark phase of the light cycle. Each behavior was recorded and then analyzed by a trained technician blind to all treatments using The Observer® (Noldus Information Technologies) or Stopwatch+ (Center for Behavioral Neuroscience, Atlanta, GA).

## **Social play behavior**

The play behavior paradigm and scoring criteria were adapted from (14, 15) which both use the focal observation method to capture “snapshot” of the play occurring in each home cage. On PN25-29, play behavior was digitally recorded in two 4-minute sessions per day in the home cage covered with a clear plastic lid. One play session was 2 hours after the beginning of the dark period and one play session was 4 hours after the beginning of the dark session. Therefore, we recorded 8-minutes of play occurring in each home cage every day for five days, for 40 minutes total. There were 5–6 animals in each cage, randomly numbered and coded by tail marks. An observer blind to the treatment groups scored the recordings for the following individual behaviors: pin, pounce, bite, and chase. We did not include boxing in the analysis, since we cannot determine which rat initiated this behavior. The frequency of play initiation was calculated by summing each animal’s play behaviors over the entire observation time. The animals from one cage were not used in the final analysis because this cage contained animals of two

different ages and social play changes with age (16, 17). Animal numbers for play behavior were therefore 12 CDB-treated females, 11 oil-treated females, 12 CDB-treated males, and 11 oil-treated males).

### **Social discrimination**

On PN32 or 33, rats were tested for social discrimination. Although it is typical to isolate adult animals for 1 to 10 days prior to testing (18–20), we isolated the juvenile animals for only 4 hours in this study, as social isolation is considered a severe stressor for juvenile animals (21). In trial 1, an age and sex-matched juvenile stimulus rat was placed in the home cage of the experimental animal and the experimental animal was allowed to freely investigate for five minutes. After five minutes, the stimulus juvenile was removed and the experimental animal was alone in its cage for 30 minutes. After this 30-minute intertrial interval, the stimulus juvenile from trial 1 and a stimulus novel juvenile were placed in the experimental animal's cage, and the experimental animal was again free to investigate for five minutes. The juvenile stimulus rats were distinguishable by unique tail marks drawn with permanent marker. Investigation of the stimulus juveniles was scored two ways, 1) body investigations, which included direct contact between the nose of the experimental animal and the body of the stimulus juvenile and 2) anogenital investigations, which included direct contact between the nose of the experimental animal and the anogenital region of the stimulus juvenile. Percent novel investigation was calculated by dividing the time spent investigating the novel animal divided by the time spent investigating either animal, multiplied by 100. Percentages greater than 50% indicate discrimination and larger scores indicate better discrimination. Animal numbers for social discrimination were 13 CDB-treated females, 12 oil-treated females, 13 CDB-treated males, and 14 oil-treated males.

### **Statistical analyses.**

All statistical comparisons were carried out using SPSS v. 27 (IMB). Statistical comparisons were carried out using a two-way ANOVA and follow up LSD posthoc tests were conducted when a significant or trend of an effect was found.  $p$  values of  $< 0.05$  were considered statistically significant and  $p$  values of  $< 0.1$  were considered trends.

## **Results**

### **Social play behavior.**

There was a trend in main effect of sex ( $F(1,47) = 3.1; p = 0.09$ ), and a trend in an interaction between sex and treatment ( $F(1,47) = 3.9, p = 0.05$ ) on the initiation of play behavior (Fig. 1A). Posthoc tests indicate that control males initiated played more than control females ( $p = 0.01$ ) and there was a trend of increased play initiation in CDB-treated females compared to control females ( $p = 0.06$ ). There was also a main effect of sex ( $F(1,47) = 5.4; p = 0.03$ ), a trend in main effect of treatment ( $F(1,47) = 3.3; p = 0.08$ ), and a trend in an interaction between sex and treatment ( $F(1,47) = 2.8, p = 0.09$ ) on pins (Fig. 1B). Posthoc tests indicate that control males pinned more than control females ( $p = 0.008$ ) and CDB-treated females pinned more than control females ( $p = 0.02$ ). There were no sex or treatment effects on chases, pounces, or bites (data not shown).

## Social discrimination

There was no effect of sex ( $F(1,50) = 0.47$ ;  $p = 0.50$ ) or treatment ( $F(1,50) = 2.5$ ;  $p = 0.12$ ) on percent novel anogenital investigations (Fig. 2A) and no effect of sex ( $F(1,50) = 0.36$ ;  $p = 0.42$ ) or treatment ( $F(1,50) = 1.1$ ;  $p = 0.75$ ) on percent novel body investigations (Fig. 2B).

## Discussion

The present data suggest that PRs play a role in organizing juvenile social play behavior; specifically, neonatal antagonism of PRs increased play behavior in females. Although previous data have demonstrated that PRs regulate social behavior in adults (19, 20), the present study is the first to show effects on juvenile social play behavior following a transient neonatal manipulation of PRs.

Males initiated play more than females in the present study, which is consistent with much of the previous literature using focal observation (14, 22, 23). Additionally, neonatal PR antagonism resulted in a trend of increased play behavior in females, essentially masculinizing their behavior. Even though levels of PRs in the developing female brain is lower than levels in developing male brain, its action may be important for preventing masculinization in females. That is, during the first week of postnatal life, PRs appear to be important for establishing female-typical levels of play.

The mechanism for the increase in play in females following neonatal manipulation of PRs is unclear, as little is known about the role of PRs in the developing postnatal female brain. Manipulation of PRs may affect a variety of signaling molecules, such as arginine vasopressin, opioids, endocannabinoids, dopamine, norepinephrine, serotonin, and GABA, which are all involved in social play behavior (24–26). Prior studies have found increase in arginine vasopressin expression in the lateral habenula of PR knockout mice (27), but it is unclear if these effects are due to altered PRs in development or adulthood. Further studies are necessary to elucidate the relationship between PRs and juvenile social play. Interestingly, the increase in play initiation in CDB-treated females appears to be driven primarily by increases in pins. This could indicate an increase in the type of attacks launched by these females.

In the present study, there was no effect on social discrimination following neonatal PR antagonism. Although previous data have demonstrated that PRs regulate social discrimination in adult male rats (19, 20), it appears that PRs do not play an organizational role in this behavior. It should be noted that social discrimination is a complex behavior, and several other signaling molecules have been shown to play a role in social discrimination, including oxytocin (28–30) and arginine vasopressin (18, 31–33).

The present study is the first to examine the specific role of PRs during early development on juvenile behaviors. In females, there was an increase in play initiation, while there was no effect in males. Future studies should examine the role of neonatal PRs on the neurobiology of the brain and behavior in both juvenile and adult animals.

## Abbreviations

progesterin receptor (PR), postnatal (PN)

## Declarations

Ethics approval and consent to participate- Not applicable.

Consent for publication- Not applicable.

Availability of data and materials- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests- The author declares that they have no competing interests.

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Authors' contributions- RFL analyzed and interpreted all data; and wrote and revised the manuscript.

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# Figures

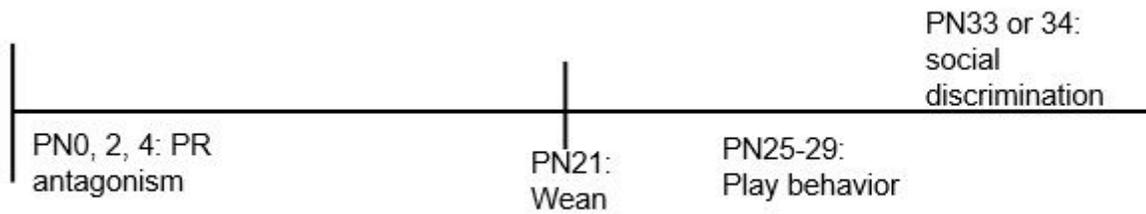


Figure 1

Experimental overview. Male and female rats were injected with the PR antagonist CDB or vehicle control on PN0, 2, and 4 then weaned into mixed-sex cages of 5-6 on PN21. Play behavior was observed in the home cage from PN25-29 and social discrimination was measured on PN33 or PN34.

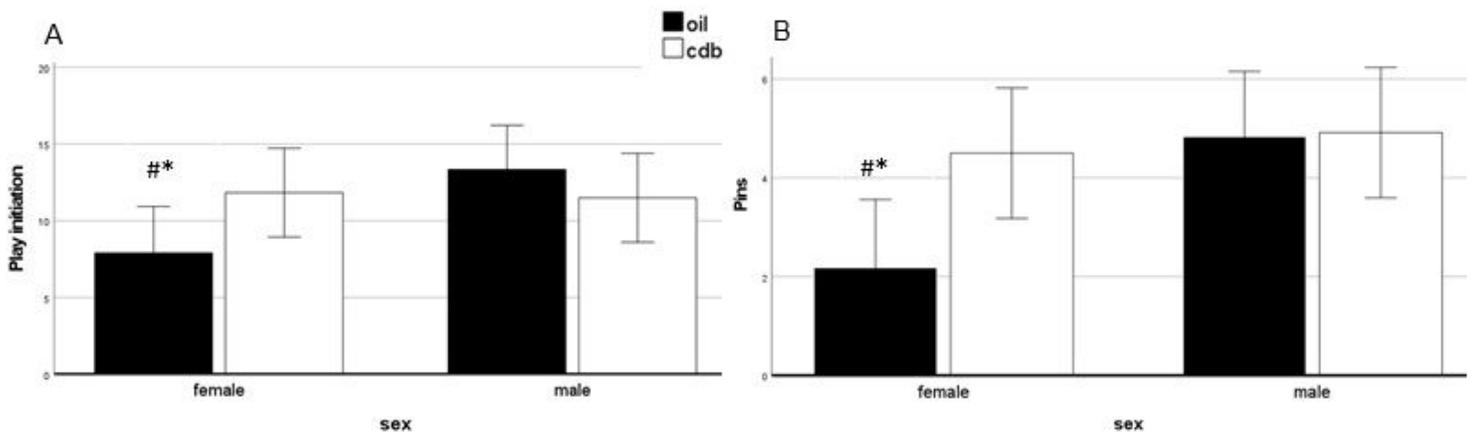
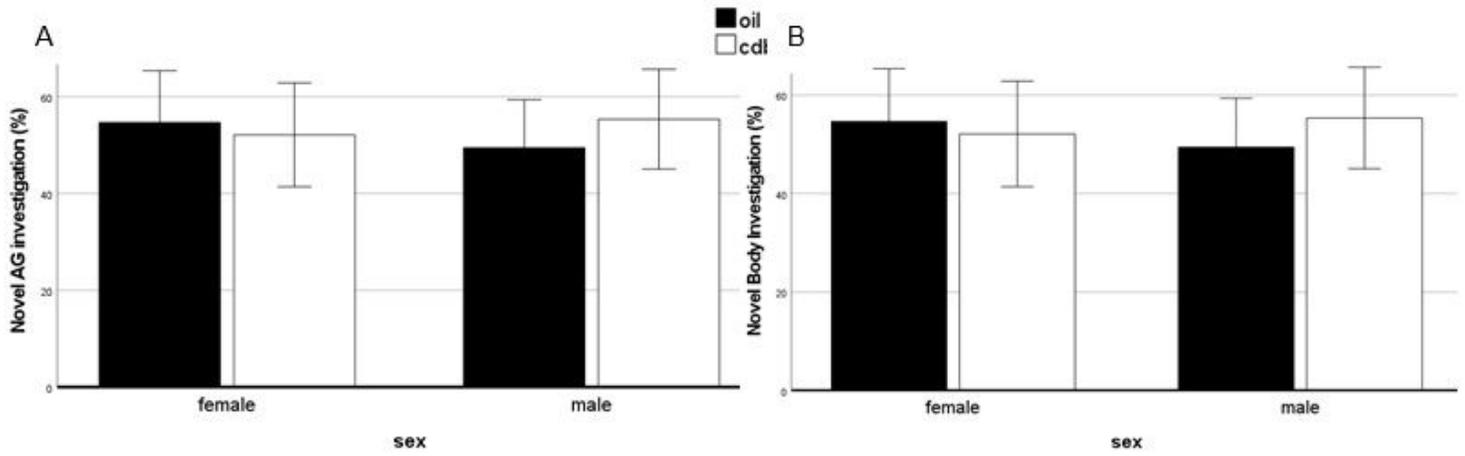


Figure 2

Social play in juvenile rats injected with CDB or control vehicle on PN0, 2, and 4. (A) Control females initiated play less than control males ( $*p=0.01$ ) and there was a trend of increased play initiation in females treated with CDB ( $\#p=0.06$ ). (B) Control females pinned less than control males ( $p=0.008$ ) and there were more pins in females treated with CDB ( $p=0.02$ ). Each bar represents the mean total number of instances of play. Error bars represent SEM.



**Figure 3**

There were no effects of sex or treatment on social discrimination in juvenile rats injected with CDB or control vehicle on PN0, 2, and 4. Each bar represents the mean percent of time that each group performed anogenital (AG) investigations (A) or body investigations (B) with the novel animal, with percentages greater than 50% indicating discrimination. Error bars represent SEM.