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The Effects of Progesterone Receptor on Development of Serotonergic Circuits that Mediate Cognition

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Abstract

As the rate of premature births has been substantially increasing, progestin administration is becoming a common treatment for the prevention of preterm labor. However, not much is known regarding how these hormonal supplements may affect the development of the fetal brain. The developing brain is highly sensitive to progesterone as progesterone receptor (PR) is expressed in many regions during critical developmental periods. Steroid hormone receptors such as PR are powerful transcription factors and regulate gene expression to alter fundamental processes of neural development. During the developmental period of post-natal day one (P1) to P14 in rats, PR is transiently expressed in the medial prefrontal cortex (mPFC), suggesting an important developmental influence. The mPFC is an area critical area of the brain for higher-order cognition, so modulation of the effects of PR is assumed to have behavioral implications. In this study, male rat pups were treated daily from the day of birth (P1) to P14 with the progesterone receptor antagonist RU486 (20 mg/kg) or an equal volume of the sesame oil vehicle as a control (during the precise critical developmental period when PR is transiently expressed), and cognitive flexibility was tested using the attentional set-shift task plus maze. In the maze itself the arms are painted either light or dark and their texture is either smooth or rough. Animals are trained to receive a food reward using one cue (e.g., reward in light arms). After the task is learned the "rule" is changed (e.g. food reward only in rough arms) and the rats must "shift" to the new rule to receive a reward. They must inhibit the previously learned response and demonstrate cognitive flexibility. RU486 treated rats demonstrated a significant impairment in cognitive flexibility and an increase in perseveration. Specifically, they were slower to shift to the new rule and were

more likely to continue to use the old rule compared to control rats. This difference suggests a disruption in higher-order cognition.

Serotonin activity in the mPFC has been shown to affect cognitive flexibility, the ability to alter behavioral strategies after changes in reward contingencies. Therefore, we treated an additional cohort of rats with RU486 or oil during development, as described above and collected adult tissue to analyze serotonergic fiber density in the mPFC. Because PR is expressed in serotonergic midbrain nuclei and serotonergic target regions of the medial prefrontal cortex, this study tested the hypothesis that PR activity plays a role in development of mesocortical serotonergic pathways and in the display of complex cognitive behavior in adulthood. At P36, brain tissue was collected and sectioned on a sliding microtome at 50µm. Immunocytochemistry was used to detect serotonin fibers in the mPFC, which receives extensive serotonergic projections, and as mentioned previously, expresses PR during development. Analysis is still in progress, however it is predicted that RU486 treatment will significantly alter serotonin fiber density in this area, which is critical for complex cognitive behaviors. In conclusion, we hypothesize that PR activity during development is critical for cognitive functioning and that this may be due to its effects on the serotonergic system. This suggests the need for further research to determine the potential negative effects of hormone-based treatments for the prevention of premature births.

The Effects of Progesterone Receptor on Development of Serotonergic Circuits that

Mediate Cognition

Introduction

The administration of synthetic progestins to pregnant women to prevent premature delivery has increased dramatically despite little understanding of the effects of these progestins on fetal neural development. This increase in administration of progesterone to pregnant women in the United States has been concurrent with the increasing rates of premature birth (Ness, Dias, Damus, Burd & Berghella, 2006). Over the past two decades, there has been a 30% increase in the rate of preterm birth (<37 weeks), as well as a 76% increase in the number of maternal-fetal medicine specialists that reported using progesterone to prevent preterm birth (Ness, Dias, Damus, Burd & Berghella, 2006). Nevertheless, despite these alarming increases there is not much information known regarding the long-term effects of this exogenous progestin exposure. Several follow-up papers have been done regarding this treatment, but none focused on the after effects later in the child's life (Dodd, Flenady, Cincotta & Crowther, 2006; Meis & Connors, 2004; Spong et al., 2005). Unpublished data from our lab has shown that inhibition of progesterone receptor activity from postnatal day 1 (P1) through P14 impairs cognitive flexibility in adulthood, a measure of higher-order cognition. This connects the developmental administration of progestins to complex cognitive behavior controlled by the medial prefrontal cortex, and suggests a need for thorough investigation of long-term effects of progestin administration.

Progesterone Receptor Expression in the Developing Brain

Previous research has well established that steroid hormones, such as androgens, estrogens, and glucocorticoids have influential effects on the developing brain, and this effect is specifically well documented in sexually dimorphic areas. Due to this insight, research now is focusing on other less prevalent hormones to examine if they influence development as well. One important maternal hormone is progesterone, which is at high levels in maternal circulation during both gestation and lactation (Pepe and Rothchild, 1974; Sanyal et al., 1978). Furthermore, progesterone can readily cross the placenta into fetal circulation (Martin et al., 1977; Quadros and Wagner, 1999). Maternal progesterone can enter the fetal brain through shared circulation and bind to its nuclear receptor (Quadros and Wagner, 1999); thus, this suggests an influential role for progesterone during fetal brain development.

It is well known that fetal testosterone masculinizes sexually dimorphic areas of the male brain. However research now suggests that progesterone receptor (PR) expression also plays a crucial role in sexual differentiation. In one of the most sexually dimorphic areas of the brain, the medial preoptic nucleus (MPN), there is a stark contrast in PR-immunoreactivity (PRir) between males and females; males demonstrate high levels of PR expression in the MPN during perinatal life, while in females PR expression is virtually absent (Wagner, Nakayama & De Vries, 1998). This increased level of expression of PRir in males is observed from embryonic day (E) 19 through postnatal day (P) 28, whereas PRir is not observed in females until P10 (Quadros, Goldstein, Vries It & Wagner, 2002). This result was further confirmed in an additional study conducted in 2002 by Quadros, Goldstein, Vries, and Wagner (2002), which showed that castration of

male rats on the day of birth significantly reduced PRir levels in the MPN. Similarly, the

same study showed that ovariectomy in females before ovarian steroidogenesis

completely prevented PRir in the female MPN (Quadros, Goldstein, Vries & Wagner,

2002). These results suggest that PR expression may play an important role in the

development of sexually dimorphic areas of the brain.

In contrast, PR is also expressed in areas of the brain that do not regulate

reproductive or neuroendocrine function, including those regions important for higher-

order cognition. A study performed by Quadros, Pfau, and Wagner (2007) characterized

PRir in the cortex from E17 to P28. Furthermore, the expression in this important brain

area was shown to be transient, with PR expression virtually absent after P28. The

transient expression of PR in the cortex temporally coincides with the crucial

developmental period of the cortical connections, potentially implicating PR in the

mechanism of neural development affecting higher order cognition (Quadros, Pfau &

Wagner, 2007). An additional study performed by Jahagirdar and Wagner (2009) further

examined this influence on the cortex of rats. The results indicated that PR is transiently

expressed in the rat cortex during development, and this expression is initiated in the

developmentally critical layer of the subplate. PRir cells in the subplate of cortex are first

detectable on E18, the number of PRir cells peak at P2, and then the number steadily

declines until PRir is again not detectable in the subplate by P14. As mentioned

previously, this developmental window of PR expression within the subplate coincides

with establishment of early cortical circuitry and the gradual demise of subplate cells,

suggesting PR plays a critical role in mediating these fundamental developmental

processes (Jahagirdar & Wagner, 2009).

Exposure to Progestins and Higher-Order Cognition

Higher-order executive tasks such as learning, working memory, and behavioral flexibility depend on the prefrontal cortex (PFC), the most complex brain region in primates and humans (Puig & Gulledge, 2011). This study specifically focused on behavioral flexibility, which is a task controlled by the medial prefrontal cortex (mPFC). This behavior is often measured using the set-shift task; a manipulation of the mPFC in rodents has been shown to severely impair performance on this task (Floresco, Magyar, Ghods-Sharifi, Vexelman & Tse, 2006; Ragozzino, Detrick & Kesner, 1999; Stefani, Groth & Moghaddam, 2003). Cognitive flexibility is operationally defined as the ability to modify ongoing behavior in response to changing relevant environmental stimuli. The subject must behaviorally adapt and shift attention from the first set of stimuli to another set, making the initial stimuli irrelevant. This behavior has been variously called attentional set-shifting, strategy-shifting, and cognitive flexibility (Birrell & Brown, 2000; Dias, Robbins, & Roberts, 1997; Milner, 1963; Owen, Roberts, Polkey, Sahakian, & Robbins, 1991; Ragozzino, Detrick, & Kesner, 1999; Ragozzino, Wilcox, Raso, & Kesner, 1999; Shepp & Eimas, 1994).

As mentioned previously, this behavior is dependent on the mPFC, and manipulation of this crucial area impairs behavior. For example, one study performed by Birrell and Brown (2000) demonstrated lesions of the mPFC of rodents impaired extradimensional attentional shifts in the set-shift task. Therefore, since cognitive flexibility is dependent on the mPFC, behavioral discrepancies are indicative of physical deficits in this crucial area. PR is expressed during crucial developmental periods of the

cortex (Jahagirdar & Wagner, 2009), and the mPFC receives innervation from the VTA which also expresses PR. Therefore, it is hypothesized that PR modulates the development of neural circuits within the mPFC. Therefore, inhibition of PR activity during development could disrupt the function of mPFC connections, which can be measured through manifestation of these deficits in higher-order behavior. Interestingly, a number of neuropsychiatric disorders are associated with impairments in set-shifting, including schizophrenia (Pantelis, Barber, Barnes, Nelson, Owen & Robbins, 1999), depression (Austin, Mitchell & Goodwin, 2001), and attention deficit disorder (Yang, Chung, Chen & Chen, 2004).

Serotonergic Influence on Higher-Order Cognition

Higher-order executive tasks such as behavioral flexibility depend on the PFC. Due to the vast amount of serotonergic projections throughout the brain; serotonin has both direct and indirect influences on areas that control higher-order behavior. The serotonin neural system originates from the ten nuclei in the mid- and hindbrain regions, collectively known as the raphe nuclei (Bethea, Pecins-Thompson, Schutzer, Gundlah & Lu, 1999). The cells of the rostral nuclei project to almost every area of the forebrain, including the hypothalamus, limbic regions, basal ganglia, thalamic nuclei, and cortex (Bethea, Pecins-Thompson, Schutzer, Gundlah & Lu, 1999). Expression of the serotonergic phenotype begins on E12 in the raphe nuclei cells, detectable by immunoreactivity (Lauder, 1990). Ascending projections are detectable using by E12, and by E15 serotonergic immunoreactive (5HTir) fibers course between the mammillary complex and the ventral thalamic area in association with the medial forebrain bundle. By

E16 5-HTir fibers can be seen at the border of the diencephalon and telencephalon, and by E17 the fibers reach the frontal pole of the telencephalon (Lauder, 1990), and therefore innervate the mPFC. In contrast, the caudal nuclei project to the myelencephalon and spinal cord, and these cells express immunoreactivity around E14 (Bethea, Pecins-Thompson, Schutzer, Gundlah & Lu, 1999; Lauder, 1990).

This study examined two potential ways in which serotonin may be modulating cognitive flexibility: direct innervation of the cortex; and innervation of the ventral tegmental area (VTA), which has dopaminergic neurons that project to the prefrontal cortex. The prominent innervation of the PFC by serotonin fibers and the dense expression of serotonergic receptors in the PFC suggest that serotonin is a major modulator of its function (Puig & Gulledge, 2011). Additionally, serotonergic neurons projecting from the midbrain raphe nuclei also innervate the VTA, where serotonergic terminals make synaptic contact with dopaminergic neurons (Herve et al., 1987). Research has also shown that serotonin exerts phasic and tonic inhibitory control over the functional status of the mesocortical dopaminergic system through different serotonergic receptor types (Di Giovanni, Esposito & Di Matteo, 2010; Di Matteo, Cacchio, Di Giulio & Esposito, 2002). Furthermore, dopaminergic neurons that project from the VTA and innervate the mPFC have been implicated in the control of behavioral flexibility (Stefani & Moghaddam, 2006); creating an implicit connection between serotonin and the mPFC. In conclusion, serotonin may be affecting higher-order cognition through direct innervation of the cortex, or indirectly through innervation of the VTA where a connection is made with dopaminergic neurons that project to the PFC.

Effects of PR on Serotonergic Projections

Ovarian steroids are known to affect the dorsal raphe nuclei, thus directly affecting serotonin (5-HT) neurons (McEwen et al., 1998). Numerous studies, performed in adult females, have indicated ovarian steroids modulation the expression of several genes of the 5-HT system, including tryptophan hydroxylase, vesicular monoamine transporter, serotonin reuptake transporter and different 5-HT receptors (See review by Bethea, Pecins-Thompson, Schutzer, Gundlah & Lu, 1999). It is known that PR is expressed in the dorsal raphe nuclei from P1 to P7, abundantly expressed in the VTA from P1 to P14, and in the cortex from E17 to P28 (Quadros, Pfau & Wagner, 2007; Quadros, Schlueter & Wagner, 2008). Therefore, PR is expressed at both the source of serotonin neurons, and two of the areas the serotonergic neurons innervate and that are important for complex cognitive behavior. Therefore, it was hypothesized in this study that PR modulation during development would disrupt cognitive flexibility in the rats, and this effect could be due to modulation of serotonergic projections between the DRN, VTA, and PFC.

<center>**Materials and Methods**</center>

Animals

The Institutional Animal Care and Use Committee at the University at Albany, SUNY, approved all animal procedures used in these experiments. All animals were housed in a temperature- and light-controlled room (12-h light, 12-h dark) with food and water available *ad libitum*. All mated female Sprague-Dawley rats were ordered from Taconic Laboratories, Germantown, New York. All rats of prenatal age were obtained

from the pregnant dams. Pregnant females were housed individually in plastic tubs with bedding and were also given food and water *ad libitum*. The day of birth was designated at P1. After the pups were weaned from their mothers, they were housed in mixed sex cages until behavioral testing began.

Postnatal RU486 Treatment

Methods used regarding the RU486 treatment were similar to those used in the study by Lonstein, Quadros & Wagner (2001). Pregnant dams were checked multiple times per day near the expectance of birth. Once the pups were born, the mother was allowed to clean them and feed them before their first dosage of RU486 was administered subcutaneously at 20 mg/kg of body weight. The RU486 was dissolved in sesame oil, and control animals received an equivalent volume of sesame oil alone. Injections were given at the same time each day from P1 to P14 (Lonstein, Quadros & Wagner, 2001).

Behavioral Testing

The methods used for the attentional set-shift assay were taken from Stefani, Groth, and Moghaddam (2003). When the rats began the habituation for behavioral testing they were placed on a restricted diet of 15 g of rat chow per day per rat. This feeding schedule ensured the rats were at a lean, but healty body weight, but maintained a strong motivation for food reward. . All rats had *ad libium* access to water during the duration of the experiment.

A plus-maze with four arms was constructed out of a thin wood and painted with gray primer. A food well was located at the end of each arm, where the food reward was placed during the trials. The food well was sufficiently deep, so that the rat could not see the food pellet at the arm's entrance. Each arm of the maze varied along two properties:

brightness and texture (i.e.; dark and rough, dark and smooth, light and rough, light and smooth, see figure below). A standard plexiglass holding cage was used in between trials. All training and trials were completed with the luminescent light on in the room.

The rats completed a 3-week-long schedule of handling and habituation. During the first 5 days, each rat was individually handled for approximately five minutes per day. Approximately 5-6 food reward pellets (dextrose pellets, 45 mg) were given to the rats after handling to familiarize them with the given reward. On the sixth day the rats were individually placed into the open arm maze where 4 sugar pellets were placed in the food wells for each arm. The rats were given 5 minutes to habituate to the maze and exploration was rewarded with the food pellets, or until all the food pellets were eaten. On days 7 and 8 one sugar pellet was placed in the food well of each arm, rather than four, and again the rats were encouraged to explore. Finally, on days 9 through 11 the rats were habituated to the T-maze orientation where one arm was blocked off. Additionally, the rats were rewarded on a variable schedule (50% of the time) to show they wouldn't be rewarded for every arm they chose.

The set-shift assay consisted of two sessions held on two consecutive days (days 12 and 13). During the first day of testing (Set 1), the rats were trained to associate the food reward with a specific stimulus (i.e.; food pellets were placed in only light, dark, smooth, or rough arms). The rats had to discriminate between brightness (light or dark) and texture (rough and smooth). On the second day (Set 2) the rats were trained to the second discrimination stimulus. Therefore, if they were trained to light or dark on the first day, then they were trained to discriminate between smooth and rough during Set 2.

During Set 1, each rat was randomly assigned to its reward group (light, dark, rough, or smooth). In all cases, only one stimulus was associated with the food reward. The maze was in the T-configuration with one arm blocked off, so that after being placed in the starting arm the rat had a choice between one rough or smooth, or one dark or light arm made by a 90° turn. Rats were trained to this criterion until they successfully received the food reward for eight consecutive trials. If the rats exceeded 180 trials without learning the criterion and reaching eight consecutive trials they were removed from the study. Within each block of eight trials, there were an equal number of starts from each arm. The maze was rotated 90° after every trial to discourage the usage of extra-maze stimuli. Between trials the rat was placed in a standard plexiglass holding cage with bedding.

The subsequent day after Set 1, the rats were pseudo-randomly assigned to another stimuli, such that within each treatment group all extradimensional shifts were counterbalanced (i.e.; from light during Set 1 to rough during Set 2). As during Set 1, the maze was rotated 90° after each trial and only one stimulus is associated with the food reward. Each rat was given 80 trials to learn the second criterion of Set 2 regardless of their performance, however is was noted if the rat reached 8 consecutive trials before this upper limit. Only rats that were successful in reaching the criterion for Set 1 were included on Set 2. The data was analyzed for perseverance errors during the 8-trial blocks of Set 2, or the percent of trials the rat responded to the maze using the first criterion they learned, which produced an incorrect, non-rewarded response. The behavior of the rats was analyzed based on the percent of correct choices across trials, and perseverance responding, or the inability to shift attention from the rule learned in Set 1 to Set 2.

Perseveration represents impaired adaption, and can assess neuronal activity in the mPFC. A t-test was used to analyze the differences in trails to criterion between the two treatment groups. In Set 2 trials to criterion was again measured, but capped at 80 trials, so the Mann-Whitney Rank Sum Test was utilized for analysis. Also measured for Set 2 was the number of perseveration and omission errors, which was analyzed with t-tests. Percent accuracy was measured with a 2-way ANOVA using treatment group and trial block as variables.

Serotonergic Fiber Density Analysis

A second cohort of pregnant dams was ordered from Taconic Laboratories (Germantown, New York) in order to study the serotonin fiber density in the mPFC and VTA. The rat pups were treated with RU486 using the exact methods described above; again treated from P1 to P14 at a dosage of 20 mg/kg of body weight. The number of animals that were treated with RU486 was again counterbalanced with a group treated with the vehicle of just sesame oil. At an adult of P36, the brain tissue was taken and pericardially perfused with 4% paraformaldehyde. Each animal was perfused with approximately 70 mL of 4% paraformaldehyde; the brain was then removed manually, and immersed in postfix 4% paraformaldehyde for 24 hours. After 24 hours the brains were switched into 30% sucrose in 0.1 M PB (pH 7.6) for at least an additional 24 hours, until the brain tissue was sectioned at 50 μm on a rotatry microtome in the coronal plate. Only the brains of male rats were used for processing and analysis due to previous findings suggesting no sex differences in subplate PR expression (Lonstein, Quadros & Wagner, 2001).

The methods used for immunocytochemistry (ICC) came from the methods used by Jahagirdar and Wagner (2009), and were modified for serotonin. The tissue sections for the specific areas of the mPFC and VTA were selected prior to the ICC. Next, the sections were briefly washed in 0.05 M Tris-buffered saline (TBS; pH 7.6) 3 times for 5 minutes to remove any residual cryoprotectant solution, which the tissue was placed in after sectioning. The tissue was then incubated in TBS containing 20% normal goat serum (NGS), 1% H_2O_2, and 1% bovine serum albumin for 20 minutes to block any nonspecific binding. Serotonin antiserum was diluted to 1:2,000 in TBS containing 2% NGS and 0.3% Triton X-100, and the mPFC sections were submersed in this solution for 48 hours at 4° C. Similarly, serotonin antiserum was diluted to 1:5,000 in TBS containing 2% NGS and 0.3% Triton X-100, and the VTA sections were submersed in this solution for 48 hours at 4° C. Following 3 rinses at 5 minutes each in TBS containing 2% NGS and 0.3% Triton X-100, the sections were incubated for 90 minutes in biotinylated goat anti-rabbit IgG (5μl/ml of TBS; Vector Laboratories, Inc., Burlingame, CA). After 2 rinses (5 minutes each) in TBS containing 2% NGS and 0.3% Triton X-100 and two 5 minutes rinses in TBS, the sections were incubated in AB reagent for 60 minutes (Vectastain Elite Kit; Vector Laboratories). Following three 5 minutes rinses in TBS, the sections were incubated in 120 ml TBS containing 12 mL of diaminobenzidine (compensated for) and 2,040 μl of H_2O_2 for approximately 5 minutes. The sections were then rinsed in TBS one time for 1 minute, one time for 3 minutes, and five times at 5 minutes each. The sections were then mounted on gelatin-coated microscope slides and allowed to dry for 24 hours. Following dehydration in increasing concentrations of alcohol, the slides were coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

After the tissue sections were coverslipped digital photos were taken with a SPOT digital camera and analyzed using Scion Image imaging program. The sections were analyzed for degree of immunoreactivity in a density slice; the area covered by saturated pixels was measured. Serotonergic fibers were analyzed in dark field in the mPFC, and serotonin neuronal cell bodies were analyzed in bright field in the VTA.

Results

Analysis of the behavioral data revealed RU486 treatment during development impaired cognitive flexibility in adulthood. During Set 1 of the attentional set shift assay, rats treated with RU486 required significantly more trials to achieve the criteria of eight consecutive correct trials ($t(15) = -2.46$, $p = .026$) (see Figure 3). Similarly, in Set 2 animals treated with RU486 required the 8 consecutive mandatory correct choices to criterion, with an upper limit of 80 trials. There was a significant effect between treatment group and trials to criterion; $U = 162.5$, $p < .01$ (see Figure 4). Regarding the percent of accurate choices made in Set 2, there was no significant difference between the two treatment groups during blocks 1-5, however during the second half (blocks 6-10) there was a main effect for treatment, $F(1, 12) = 5.063$, $p = .042$; and a main effect for the trial block, $F(4, 12) = 9.808$, $p < .001$. Furthermore, there was no interaction between these two variables, $F (1, 4) = .286$, $p = .886$ (see Figure 5 below). During blocks 1-5 in Set 2 there was no significant difference between treatment group in preservative errors, $t(14) = .093$, $p = .093$, however in blocks 6-10 animals treated with RU486 demonstrated significantly more preservative errors, $t(14) = -2.15$, $p = .049$. To further validate this result, there was no difference in omission errors between treatment groups, $t(114) = -$

.058, p = .0567, which signified random errors made that did not represent perseverance of the rule learned in Set 1.

Analysis of serotinin immunoreactivity in VTA and mPFC suggesting that RU486 treatment during neonatal life significantly altered serotonergic connections. Student t-test revealed that RU486 treatment significantly decreased the density of 5HT-ir fibers in the mPFC compared to controls ($t(9)$ = 2.424, p = 0.038) (Figure 9). Although there was no significant difference between treatment groups in the total amount of 5HT-ir in the VTA ($t(22)$ = 1.967, p = 0.062) (Figure 10), there was a trend toward controls having slightly more 5HT-ir.

Discussion

Results from these studies demonstrate that inhibition of PR activity during neonatal life impairs complex cognitive behavior in adulthood and may do so through alterations in the development of serotonergic connections; thus, implicating the importance of studying the effects of progesterone administration during neural development. Treatment with the progesterone receptor antagonist, RU486, significantly decreased the density of serotonergic innervation of the mPFC, and there was a trend toward decreased serotonin immunoreactivity in the VTA. This alteration in serotonergic innervation may have directly or indirectly impaired mPFC control over cognitive flexibility, and although this was not directly measured, it suggests an atypical importance of serotonin in the cortical control of behavior.

Treating rats with the PR antagonist, RU486, during neurodevelopment impaired the normal function of progesterone receptor, thus altering its efficacy as a transcription factor and affecting its influence over developmental processes. The combination of

previous and new data from our lab has demonstrated PR's expression in both the cell

bodies and targets of mesocortical dopaminergic projections (Jahagirdar & Wagner,

2009; Willing- Unpublished, 2011), suggesting an important influence over the

establishment of this circuit and the respectively controlled behaviors. Treatment with

RU486 during postnatal development significantly impaired cognitive flexibility, a

behavior dependent on the mPFC; which is an important component in the mesocortical

circuit. Specifically, RU486 treatment decreased the ability to shift to a new rule in the

set shift task (i.e., reduced accuracy) and increased the likelihood that rats would continue

to use the old rule (i.e., increased perseverative errors); signified by the increase of

perseverance errors made by RU486-treated animals in the second day of testing, as well

as decreased accuracy in the second half of Set 2. The statistical analyses revealed that

RU486-treated rats were not making random omission errors, but rather preserved the

first criterion and lacked the cognitive flexibility to learn the second criterion. The

treatment with RU486 made a clear impairment in the behavioral capability controlled by

the mPFC.

The results of the behavioral part of this study agree with previously published

literature, although many of these studies focus on the dopaminergic control over

cognitive flexibility. Seamans and Yang (2004), and Stefani and Moghaddam (2006)

demonstrated the importance of dopamine release in the control of complex cognitive

behavior mediated by the mPFC. Dopaminergic activity and local metabolite activity in

the PFC increase during cognitively demanding tasks. This effect is observed in

behavioral assays that convey uncertainty in reward contingency, such as cognitive

flexibility (Endepols, Sommer, Backes, Wiedermann, Graf & Hauber, 2010; Stefani &

Moghaddam, 2006). Furthermore, targeted disruption of dopaminergic functioning in this region produces behavioral deficits similar to those caused by mPFC lesions (Naneix, Marchand, Di Scala, Pape & Coutureau, 2009; Stefani & Moghaddam, 2006). New data in our lab has shown that treatment with RU486 not only impairs cognitive flexibility, but also alters crucial mesocortical dopaminergic circuits (Doctoral research of Jari Willing). The alteration of such a critical neurotransmitter system may account for the disruption in behavior.

Serotonin also has an important influential role on this dopaminergic circuit controlling higher-order cognition through dense innervation of the VTA, as well as direct influence on cortical areas through direct innervation; thus suggesting another important influence on behavior controlled by the mPFC. Prior studies have not extensively examined how modulation of serotonergic circuits affect behaviors controlled by the mPFC, Though, the data provided by this study suggest it may be another important influence to examine. Serotonin accounts for a large percentage of innervation of the forebrain; innervating the hypothalamus, limbic regions, basal ganglia, thalamic nuclei, and cortex (Bethea, Pecins-Thompson, Schutzer, Gundlah & Lu, 1999). Due to this prominent innervation of the forebrain and the dense expression of serotonergic receptors in areas such as the PFC, it is suggested that serotonin is a major modulator of cortical function (Puig & Gulledge, 2011). Furthermore, the midbrain dopaminergic cell groups that project to the forebrain, specifically in the VTA, receive a dense serotonergic innervation from the DRN (Herve et al., 1987); and research has shown that serotonin exerts phasic and tonic inhibitory control over the functional status of the mesocortical dopaminergic system through different serotonergic receptor types (Di Giovanni,

Esposito & Di Matteo, 2010; Di Matteo, Cacchio, Di Giulio & Esposito, 2002).

Therefore, serotonin may be affecting higher-order cognition through direct innervation

of the cortex, or indirectly through innervation of the VTA where a connection is made

with dopaminergic neurons that project to the PFC.

One previous study did find that perinatal administration of exogenous serotonin

altered cognitive flexibility in adulthood, suggesting a complex interaction between

dopamine and serotonin to modulate this behavior controlled by the mPFC (Blazevic,

Colic, Culig & Hranilovic, 2012). In order to further investigate the specific influence of

serotonin on behavioral flexibility we examined the density of serotonergic innervation of

the mPFC, and VTA; two critical brain regions that influence higher-order cognition.

Blocking the effects of PR significantly reduced serotonergic innervation of the mPFC,

and seemed to decrease serotonin cell body presence in the VTA. The statistically

significant difference in the mPFC may have had direct effects on the behavioral

outcomes due to serotonin's known influence in the cortex (Puig & Gulledge, 2011); and

the trend seen in the VTA may have indirect effects on cognitive flexibility due to the

important influence on the mesocortical dopaminergic circuits, which is well documented

in affected the cognitive flexibility task (Stefani & Moghaddam, 2006). Overall, these

results combined with the results presented by Blazevic et al. (2012) propose serotonin

plays a critical role in directly influencing this cortically controlled behavior through

innervation of the mPFC, as well as an indirect role through its influence on the

mesocortical dopaminergic circuit in the VTA. Blocking the effects of PR reduced the

density of serotonergic innervation of the specified brain areas, which caused a

dysregulation in the control of the studied behavior.

Limitations of this study included a less than ideal number of subjects in each treatment group. With more rats in both the group treated with RU486, as well as the control group a more significant effect regarding serotonergic density may have been seen. Additionally, the antagonistic properties of RU486 exert antagonistic effects on progesterone receptors as well as glucocorticoid receptors. Though the drug has preferential binding affinity for PR, there may have been some effects caused by antagonizing the glucocorticoid receptors that were unaccounted for. In order to account for this lack of specificity our lab is taking advantage of antisense DNA to directly knockdown the PR expression in specified areas of the brain. This can help differentiate the effects of downregulation of PR, and whether the effects on cognitive flexibility were caused by differential PR expression in specific areas of the brain during development. Also, another future study could examine the behavior of mice or rats with PR-A or PR-B knockout genes to determine if the behavioral discrepancies observed are caused by one receptor or a combination of both.

In conclusion, progestin administration to pregnant women may modulate the neural development of the fetus. This study has shown impairment of the progesterone receptor P1 through P14 causes disturbances in regards to cognitive flexibility measured with the attentional set-shift assay. This connects the administration of progestins to higher-order behavior controlled by the mPFC. This study further proposes influence from serotonergic innervation of the mPFC and VTA on the cortical control of behavior. Fully understanding the mechanism of disruption has important clinical implications as a number of neuropsychiatric disorders are associated with impairments in set-shifting, including schizophrenia (Pantelis, Barber, Barnes, Nelson, Owen & Robbins, 1999),

depression (Austin, Mitchell & Goodwin, 2001), and attention deficit disorder (Yang,

Chung, Chen & Chen, 2004). These disorders are often associated with dopaminergic

dysfunction in cortical areas of the brain, but additional studies may reveal an additional

regulatory role of serotonin. Additional insight will provide better understanding of the

neurodevelopemental processes underlying these disorders; and future studies will further

determine the clinical implications of the manipulation of PR and the beneficial or

detrimental effects it has on neural development.

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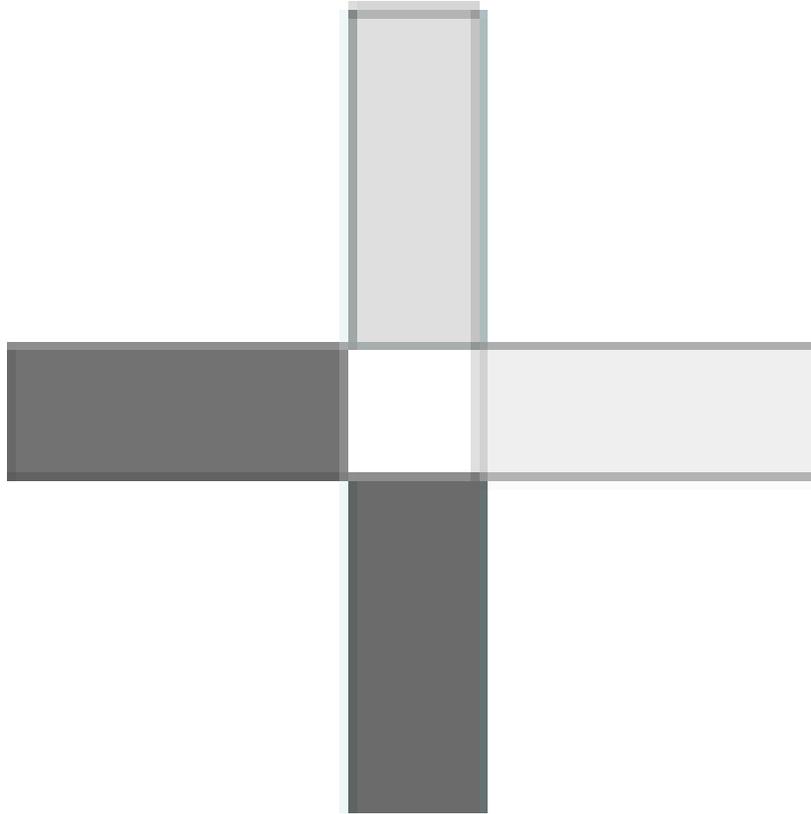
Figure 1. Visual schematic of the set-shift apparatus showing light, dark, smooth, and rough qualities.

Set 1
Initial Discrimination

Set 2
Extradimensional Shift

Relevant Stimulus Dimension:
Brightness: *Light*

Relevant Stimulus Dimension:
Texture: *Smooth*
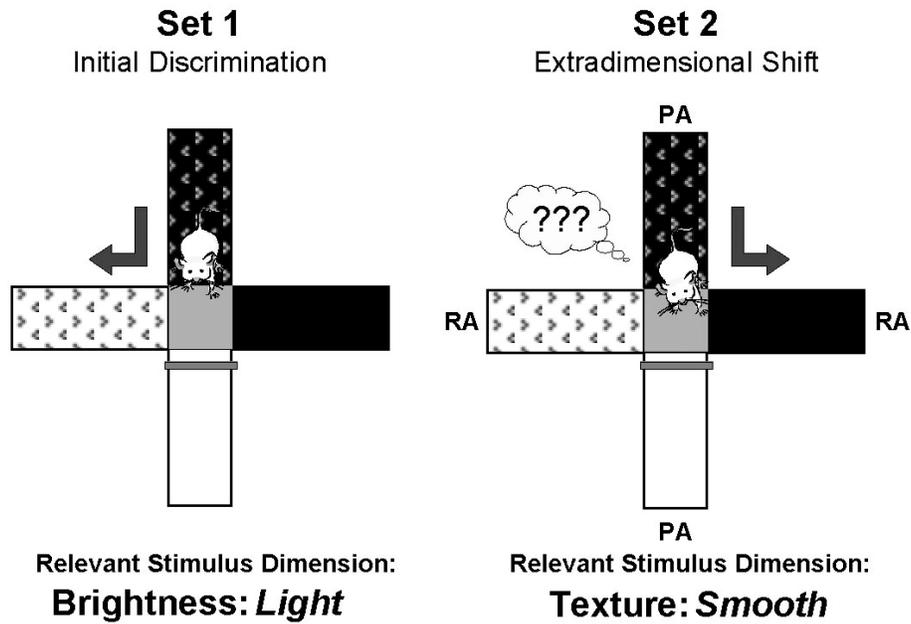
Figure 2. Visual schematic of the apparatus and method used to behaviorally test the rats. This figure was taken from the study performed by Stefani and Moghaddam (2005). PA = preservation arm and RA = reinforcement arm.

Trials to Criterion: Set 1
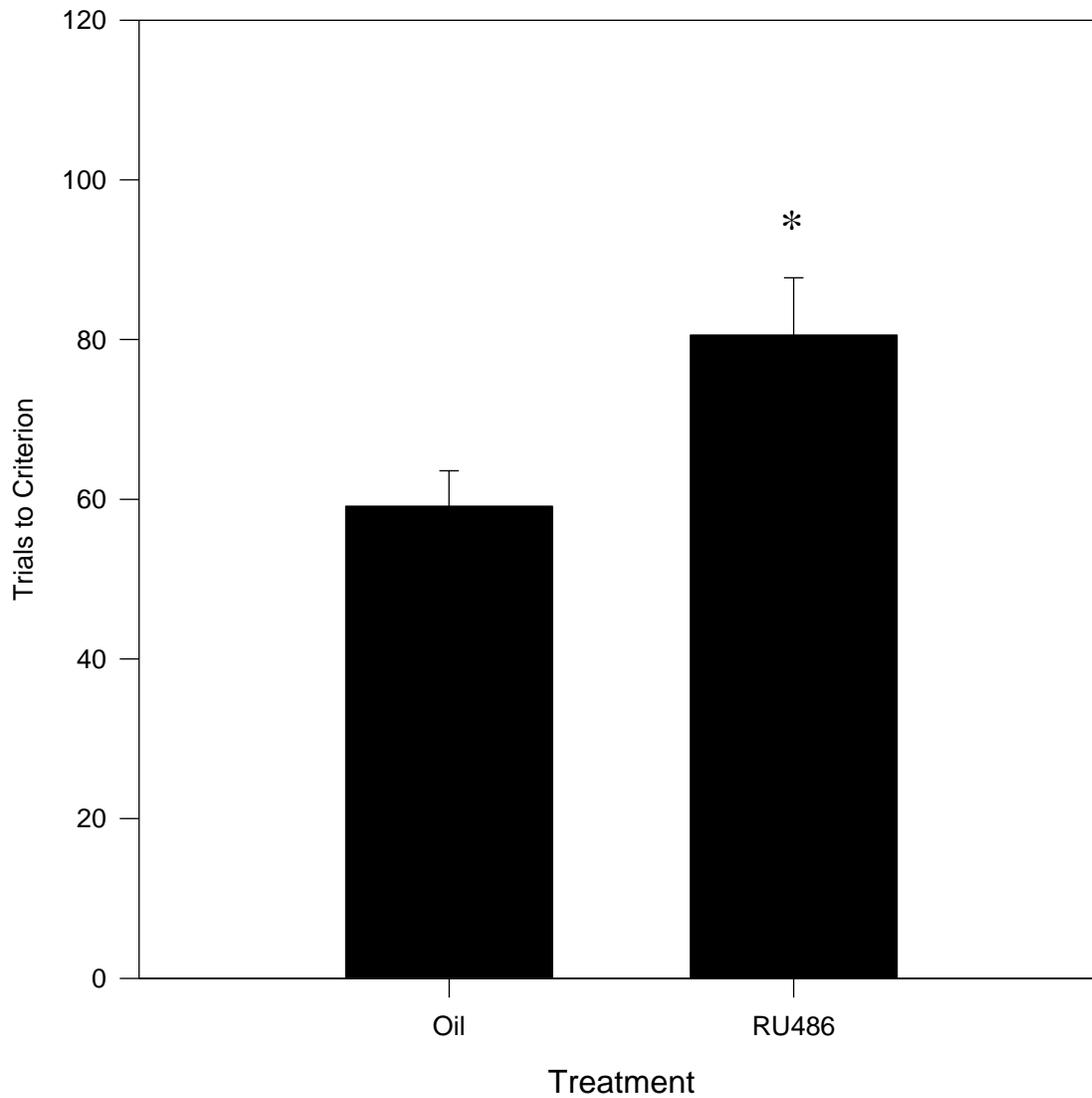
Figure 3. Pictorial representation of the number of trials to criterion in Set 1; $t(15) = -2.46$, $p = .026$.

Trials to Criterion:Set 2
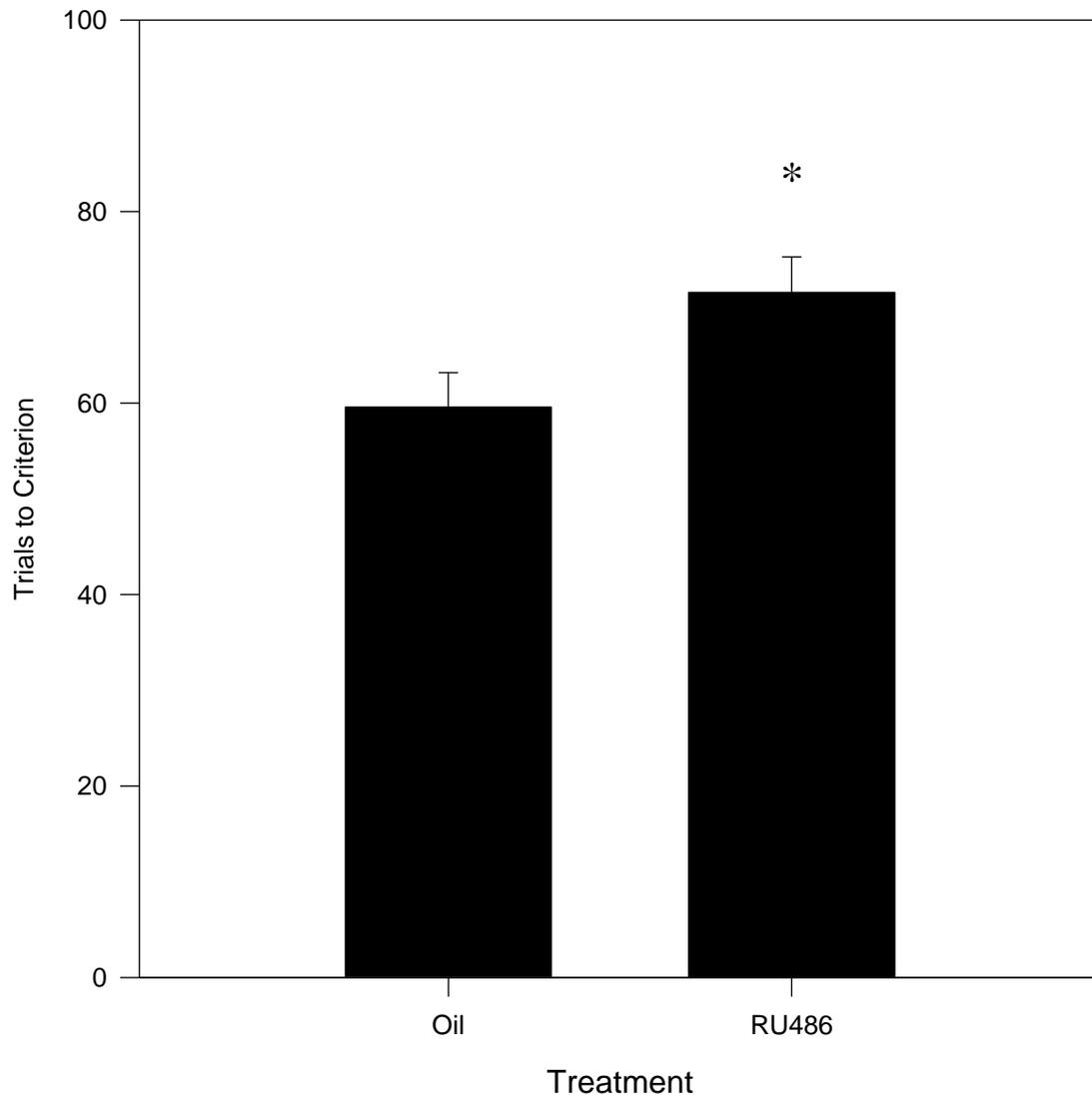
Figure 4. Trials to criterion in Set 2 with an upper limit of 80 trials. Significant effect observed using the Mann-Whitney U (Rank Sum) test carried out between treatment group and trials to criterion; U = 162.5, p < .01.

Set 2 Accuracy
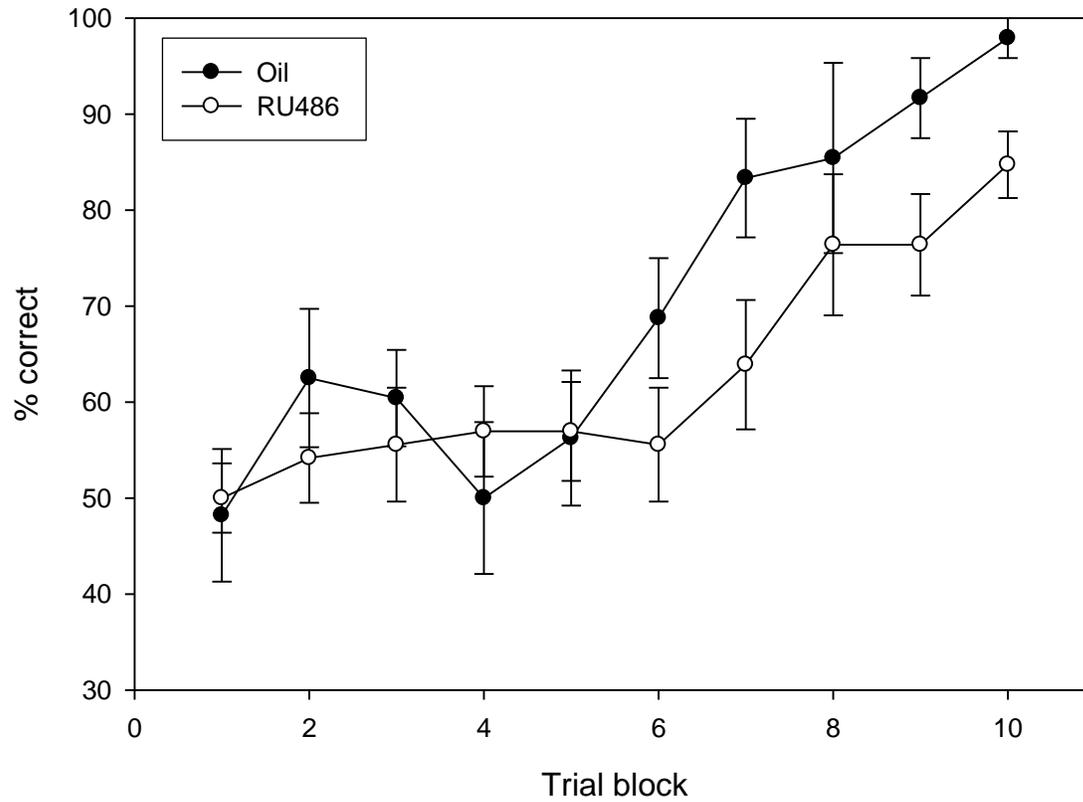
Figure 5. Percent accuracy of the choices made in Set 2. There was a main effect for treatment in the second half of Set 2 (blocks 6-10); $F(1, 12) = 5.063$, $p = .042$. Additionally, there was an effect for trial block in the second half of Set 2 with performance improvement in later trials; $F(4, 12) = 9.808$, $p < .001$. There was no interaction between treatment and trial block; $F(1, 4) = .286$, $p = .886$.

Perseverative errors: Blocks 1-5
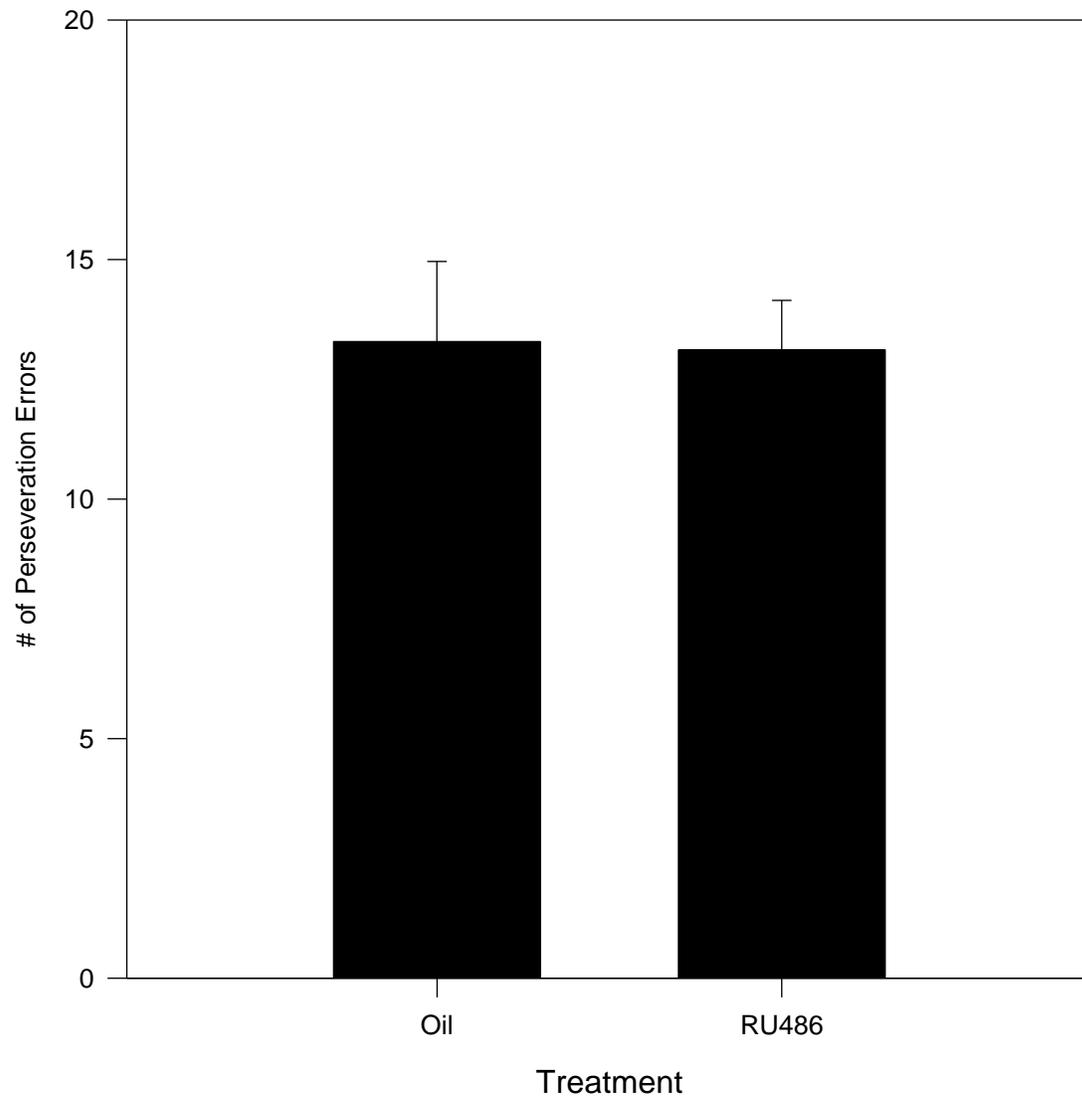
Figure 6. Perseveration errors in blocks 1-5 in Set 2; *t*(14) = .093, *p* = .093.

Perseverative Errors: Blocks 6-10
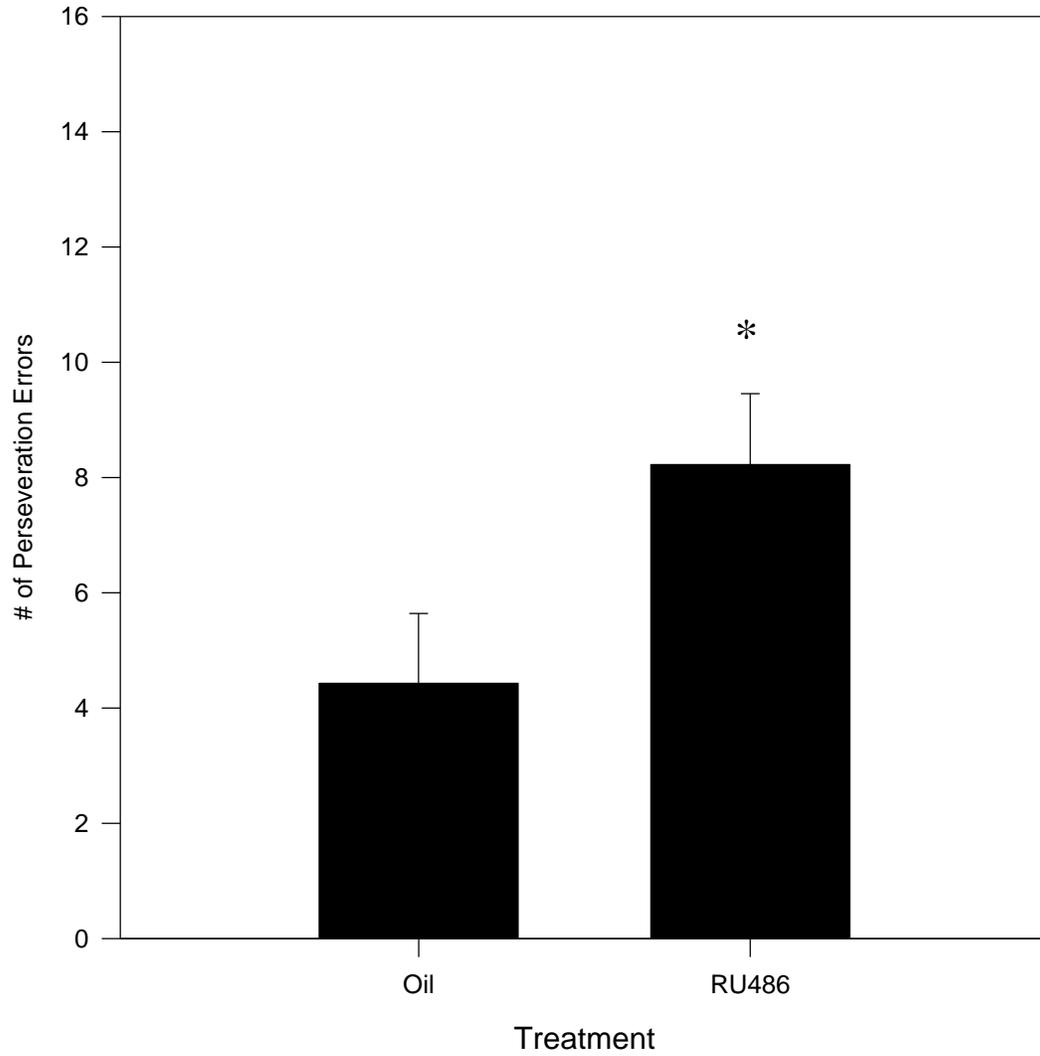
Figure 7. Perseveration errors in blocks 6-10 in Set 2; $t(14) = -2.15$, $p = .049$.
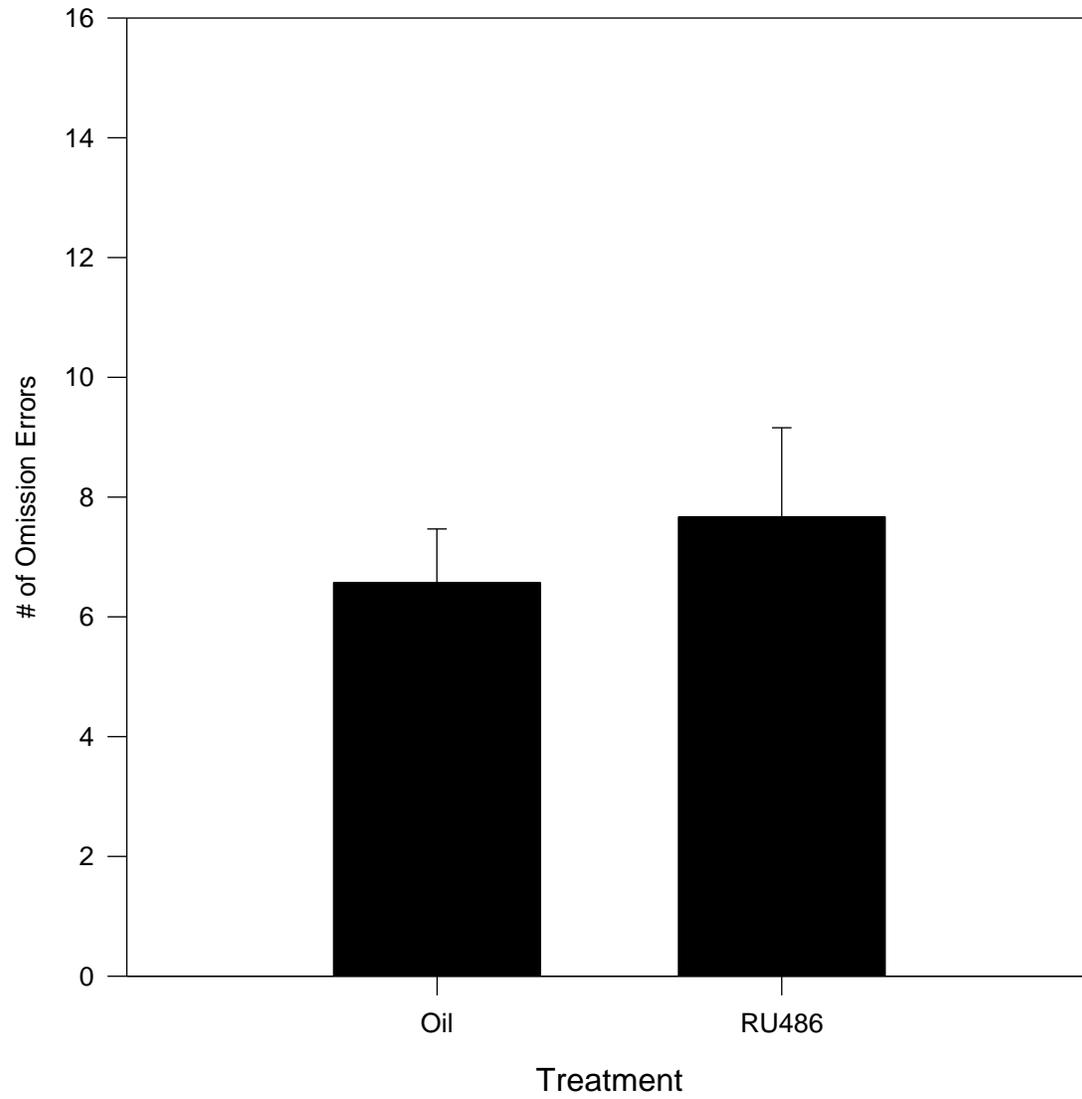
Omission Errors

Figure 8. Total number of omission errors during Set 2, no significant difference; $t(114)$
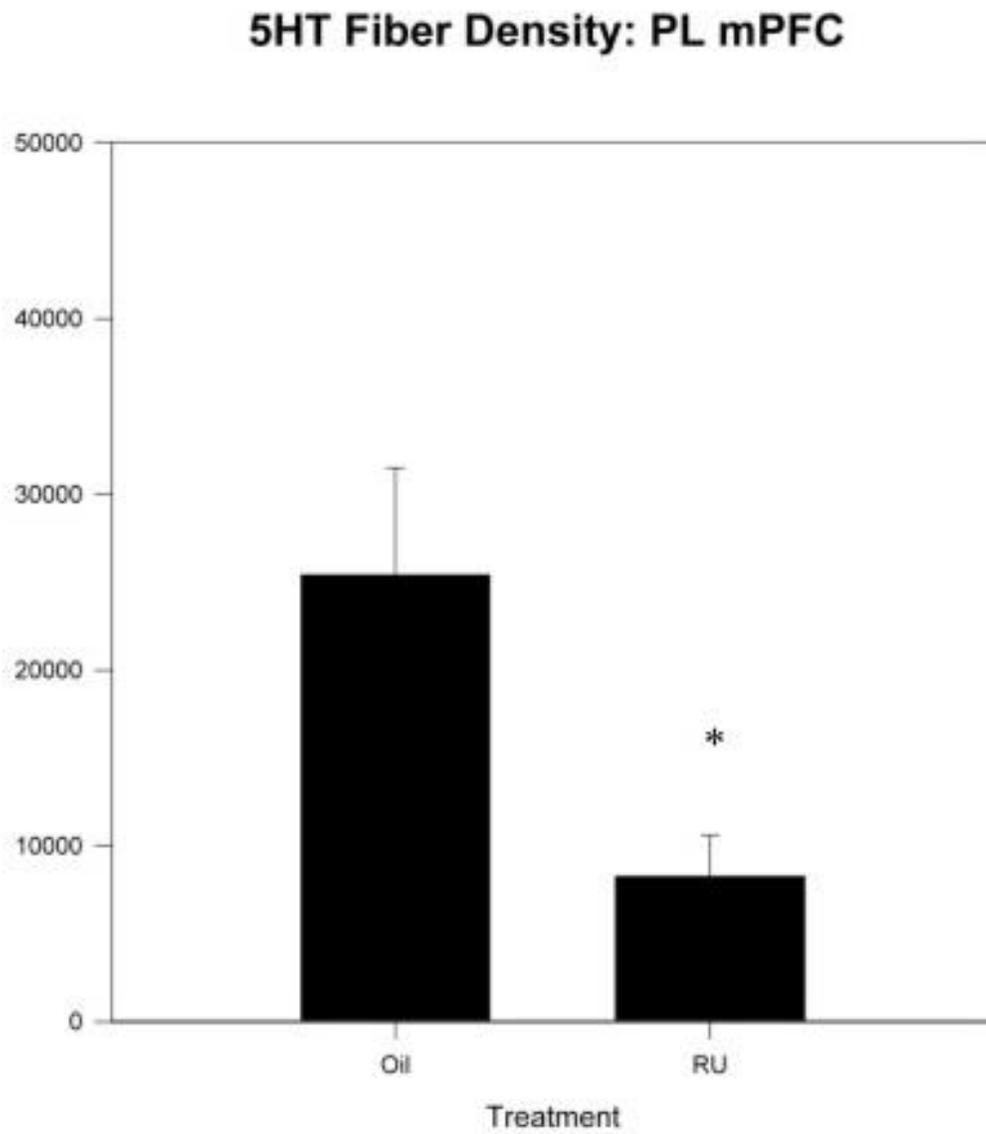
$= -.058$, $p = .0567$.

Figure 9. Measured serotonin immunoreactivity in the mPFC, $t(9) = 2.424$, $p = 0.038$.
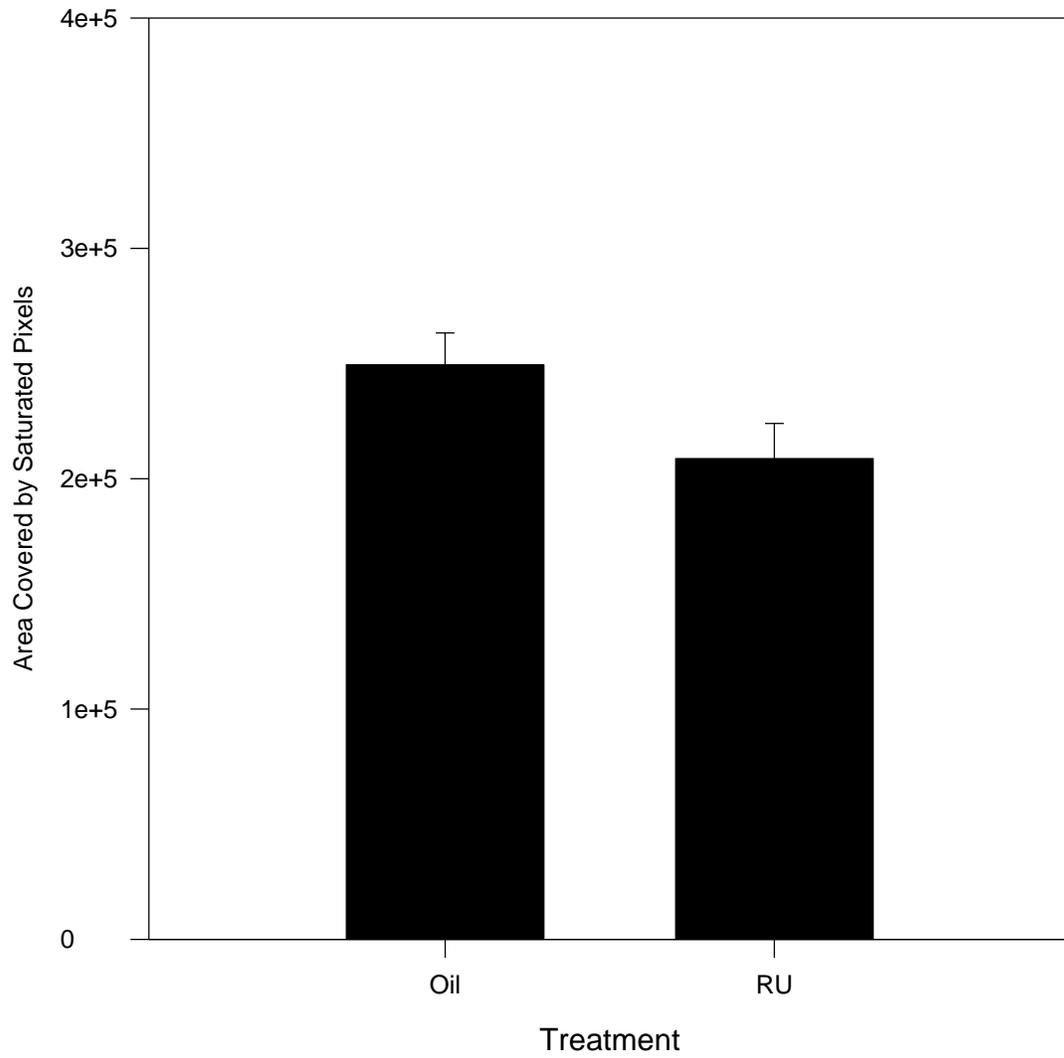
5HTir: VTA

Figure 10. Measured immunoreactivity in the VTA, $t(22) = 1.967$, $p = 0.062$.