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The Role of Progesterone Receptor in Development of Medial Prefrontal Cortex and Impulsive Behavior

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Abstract

Human behaviors are regulated by the complex functions of the brain and many behavioral disorders are assumed to be the result of alterations or deficits in neural function. Attention Deficit Hyperactivity Disorder (ADHD), characterized by differences in complex behaviors such as attention, memory and impulse control has been linked to alterations in dopaminergic activity in the medial prefrontal cortex (mPFC). Therefore, factors that regulate development of this region may be implicated in ADHD. For example, steroid hormones can alter fundamental processes of neural development through interaction with their nuclear receptors, which act as transcription factors. Progesterone receptor (PR) is transiently expressed in cerebral cortex during fetal and neonatal life in the laboratory rat, suggesting that progesterone and its receptor may play an important role in cortical development. The current study tested the hypothesis that PR activity during development plays an important role in the dopaminergic innervation of mPFC and in complex cognitive behaviors in adulthood. To test this hypothesis, animals were treated with a PR antagonist, RU486, or a control vehicle during postnatal development. On postnatal day 21 (P21) dopaminergic innervation of the mPFC was examined using immunocytochemistry for tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis. Animals treated with RU486 had a significantly lower density of TH immunoreactive fibers in the prelimbic region of the mPFC, suggesting incomplete development of a key dopaminergic pathway. In addition, RU486 treated animals showed deficits on an inhibitory avoidance task, indicating that impulse control, memory and decision making may be impaired in these animals, behaviors that are regulated by dopaminergic afferents to the mPFC from the midbrain ventral tegmental area. These findings suggest that progesterone and its receptor play a previously overlooked role in the normal development cortical connectivity and subsequent

cognitive behaviors. These findings may have implications for our understanding of developmental behavioral disorders involving the mPFC such as ADHD.

The Role of Progesterone Receptor in Development of

Medial Prefrontal Cortex and Impulsive Behavior

Steroid hormones such as testosterone, estrogen, and progesterone profoundly influence the development of the nervous system through many different biological processes. These hormones facilitate the regulation of key developmental processes involved in neuronal maturation through interactions with nuclear receptors. It has been shown that steroids can alter cell death (Forger, 2006), dendritic morphology, axon outgrowth, and neurotransmitter phenotype (Kinsley et al., 2006; Choi et al., 2003; Wooley et al., 1990; Gould et al., 1990). Steroid hormones substantially influence these neural processes, especially in the brain of the developing neonates (Sakamoto et al., 2001; Murphy and Segal, 2000; Woolley, 2000; Mong et al., 2001). Specifically, the actions of progesterone at its nuclear receptor play a role in regulating many developmental mechanisms in the brain. Studies have shown that many regions of the brain are sensitive to progesterone during development (Wagner, 2006; Wagner 2008). Recent data from my lab have suggested that dopaminergic nuclei of the midbrain such as the ventral tegmental area (VTA), and the cerebral cortex transiently express progesterone receptor (PR) during fetal and neonatal life in rodents, which indicates that PR may play an important role in the development of the mesocortical dopaminergic system (Lopez and Wagner, 2009; Quadros et al, 2007; Quadros et al, 2008). The mesocortical dopamine system, dopaminergic connections from VTA to frontal cortex, is known to be involved in complex cognitive processes. It can be hypothesized that exposure to exogenous progestins, or inhibition of PR during perinatal life could permanently alter critical mechanisms that underlie the normal development of important neural circuits, and could disrupt subsequent cognitive ability.

Previous studies have shown that PR is transiently expressed within the developing cortex. PR appears in subplate cell layers of the cortex as early as embryonic day 18 (E18). PR peaks in pyramidal cell layers of cortex at postnatal day 9 or 10 (P9-P10), and by P24-28 PR expression is virtually absent (Jahagirdar and Wagner, 2008; Quadros et al., 2007; Lopez and Wagner, 2009). This transient PR expression within all functional areas of cortex coincides with key processes in cortical development, such as dendritic maturation and synaptogenesis, which suggests that PR activity is critical for proper development of cortical connectivity. Recent evidence also indicates that PR is colocalized with MAP-2, a neuronal marker, but not with GABA, an inhibitory neurotransmitter, suggesting that PR is found in excitatory pyramidal cells (Lopez and Wagner, 2009). Since PR is transiently expressed during the development of the cortex, PR activity in pyramidal cell bodies may be necessary for the proper development of functional cortical circuits involved in complex cognitive behaviors.

In humans, the prefrontal cortex (PFC) guides executive functions, including regulation of attention, planning, impulse control, mental flexibility, and self-monitoring (Arnsten and Li, 2005). The PFC receives robust dopaminergic input from the VTA, referred to as the mesocortical dopamine system. In rats, the medial prefrontal cortex (mPFC) is one of the few neocortical areas that receive dopaminergic projections (Kalsbeek et al., 1988; Benes et al., 2000). Dopaminergic fibers are first detected in the PFC on E16 and dopaminergic innervation reaches maturity between P20 and P35. By P60, dopamine fiber density is fully mature (Kalsbeek et al., 1988). Tyrosine hydroxylase (TH) is an enzyme required for the synthesis of dopamine, and therefore is a reliable way to detect dopaminergic fibers; TH immunoreactivity (THir) can be used as a selective marker for cortical dopaminergic innervation (Verney et al., 1982). TH fibers can be observed in all cortical layers by P9, and a high density of TH fibers are

present in the mPFC by P14 (Verney et al., 1982). Disruption of these dopaminergic connections could lead to deficits in the cognitive behaviors mediated by the mPFC. In accordance with this idea, evidence indicates that the mesocortical dopaminergic system plays a role in reward-based learning. Previous studies have demonstrated that the PFC is involved in goal-directed behavior and planning (Naneix, 2009). Alterations in the PFC often cause disturbances in attentional processes, which may have implications for attention deficit/hyperactivity disorder (ADHD) in humans (Boulougouris and Tsaltas, 2008; Brennan and Arnsten, 2008).

The present study tested the hypothesis that PR activity is required for the normal development of the mesocortical dopamine pathway and for adult cognitive behaviors such as emotional memory and behavioral inhibition. To test this hypothesis, the experiments examine the effects of RU486, a progesterone receptor antagonist, on the development of dopaminergic innervation in the mPFC in neonatal rats and subsequent cognitive behaviors in adulthood. In experiment 1, TH immunocytochemistry was used to visualize dopaminergic fibers in the mPFC. It was hypothesized that animals treated with RU486 will show a decreased amount of TH fibers in the mPFC, suggesting that dopaminergic innervation is underdeveloped in these animals. In experiment 2, another cohort was treated with RU486 during development and tested in an inhibitory avoidance task as adults, a task that assesses emotional memory and behavioral inhibition. The behavior task was used to compliment the immunocytochemistry technique because dopaminergic innervation of the mPFC presumably plays a role in regulating these behaviors. It was hypothesized that RU486 treated animals will show deficits on the inhibitory avoidance task as a result of disrupted cortical development. Results suggest that PR activity during postnatal development is important for the proper development of dopaminergic innervation of the mPFC and for subsequent adult cognitive behavior.

Materials and Methods

Animals

The animals used in this study were male offspring of timed pregnant female Sprague Dawley rats. Animals were housed on a 12-h light, 12-h dark cycle at a constant temperature of $25 \pm 2°$ C, with food and water available *ad libitum*. All females were allowed to deliver their pups normally. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee at the State University of New York at Albany.

Experiment 1: Effects of RU486 on dopaminergic innervation of mPFC

Drug treatment. Animals were treated daily with RU486, a progesterone receptor antagonist, (20mg/kg in sesame oil, s.c.) ($n = 11$) or an equal volume of the oil vehicle alone ($n = 12$), from P1-P10. Treatment groups were distributed randomly between at least three different liters to control for litter effects.

Tissue collection and preparation. Animals were given an overdose of Nembutal and killed by rapid decapitation on P21, when the mPFC shows mature dopaminergic innervation patterns. Brains were fixed in 5% acrolein in 0.1M phosphate buffer (PB) for 6 hours. The brains were then cryoprotected in 30% sucrose in 0.1M PB. The brains were cut at 50µm in the coronal plane using a freezing microtome, and sections were stored in cryoprotectant (30% sucrose, 0.1% polyvinyl-pyrrolidone-40 in ethylene glycol and 0.1M PB) at -20°C until immunocytochemistry was conducted.

Immunocytochemistry. Immunocytochemistry was performed on free floating brain sections using a rabbit antiserum directed against the enzyme TH. All incubations were performed at room temperature unless otherwise stated. Sections were rinsed in Tris-buffered saline (TBS) (3 x 5 min), incubated in 1% sodium borohydride in TBS (10 min), rinsed in TBS

(4 x 5 min), and were incubated in a blocking solution (20% normal goat serum, 1% H_2O_2, and 1% bovine albumin serum made in TBS) for 30 minutes. Tissue was incubated in primary antisera against tyrosine hydroxylase (Millipore, AB152, rabbit polyclonal) diluted 1:1000 in TBS containing 0.3% Triton X-100, 2% normal goat serum (TTG) for 24 hours at 4°C. Sections were rinsed in TTG (3 x 5 min) and incubated in biotinylated goat antirabbit IgG, at a concentration of 3µg/ml in TTG for 90 minutes. Afterward, sections were rinsed in TTG (2 x 5 min) and TBS (2 x 5 min), and incubated in avidin-biotin complex (ABC) reagent for 60 minutes. Sections were then rinsed in TBS (3 x 5 min) and stained with 0.05% diaminobenzidine (DAB), and 1% H_2O_2 in TBS for approximately 18 min. Sections were then rinsed with TBS (5 x 5 min), mounted on gelatin-coated slides, and coverslipped with Permount.

Analysis. Sections through the mPFC were selected and anatomically matched across animals. Image analysis was performed by an experimenter blind to treatment groups. Images were collected using a Nikon Eclipse E600 microscope at 20X using a darkfield condenser fitted with a SPOT Insight camera connected to a Dell Inspiron 8600 laptop. Three regions in the mPFC were selected for analysis: the infralimbic (IL), prelimbic (PL), and anterior cinculate (ACd) cortex were used in this study (Kritzer, 2003). Scion Image (NIH image software) was used to quantify THir fibers. Counting boxes were placed within the regions of interest (400 pixels x 925 pixels) to quantify percentage of the area covered by THir fibers. Box sizes were selected to encompass the majority of the area for each brain region. Using the density slice function to highlight THir fibers, the area of covered pixels within the box was calculated. This value was divided by the box size to give the percent total area covered by THir fibers. Group comparisons were made within each area of mPFC using a Student's t test ($p < 0.05$).

Experiment 2: Effects of RU486 on the Inhibitory Avoidance Task

Drug treatment. Animals were treated daily with RU486, a progesterone receptor antagonist, (20mg/kg in sesame oil, s.c.) ($n = 8$) or an equal volume of the oil vehicle alone ($n = 9$), from P1-P14. Treatment groups were distributed randomly between at least three different liters to control for litter effects.

Inhibitory avoidance task. The inhibitory avoidance task was used in this study to measure impulsivity/behavioral inhibition and emotional memory by assessing the rats' ability to inhibit a motivated behavior based on their memory of a past consequence. The testing apparatus consisted of a rectangular alley divided into two compartments by a sliding door: a well lit chamber and a dark chamber. Both compartments were covered by a ceiling. Parallel stainless-steel bars spaced approximately 1 cm apart constituted the floor of the dark compartment; the floor of the dark compartment was connected to a power source. Rats prefer the dark chamber and will typically enter the dark chamber with very short latencies (McNay and Gold, 1998).

Animals were tested at approximately two months of age. On day 1 of testing (baseline), animals were placed in the lighted compartment, with the door to the dark compartment closed, and given 30 sec to acclimate. At 30 sec, the sliding door was retracted and the latency to enter the dark compartment was measured (360 sec maximum). Once the animal had completely entered the dark compartment, the door to the lighted compartment closed and a mild, but aversive, foot shock (0.5mA, 3 sec) was delivered. Upon being shocked, animals were immediately removed and returned to their home cage. The latency to enter the dark compartment was recorded. On day 2 of testing, 48 hours after the baseline measure, the testing procedure was repeated (900 sec maximum) but no shock was administered.

Statistical analysis. Group comparisons were made using a Student's t test ($p<0.05$).

Results

Experiment 1: Effects of RU486 on dopaminergic innervation of mPFC

In the PL mPFC, RU486 treatment significantly reduced THir fiber density compared to oil treated controls ($p < 0.05$) (Figure 1). There were no significant differences between groups in the other regions of mPFC.

Experiment 2: Effects of RU486 on the Inhibitory Avoidance Task

RU486 treatment significantly increased the latency to enter the dark compartment on day 2 of testing, but did not alter the baseline latencies. The t-test revealed a significant effect of treatment group ($p < 0.03$) (Figure 2).

Discussion

The results of this study suggest that PR activity during postnatal life is necessary for the normal development of dopaminergic innervation of the mPFC and for adult cognitive behavior. Postnatal exposure to RU486 significantly decreased the density of THir fibers in specific areas of the mPFC and impaired performance on the inhibitory avoidance task, a measure of emotional memory and behavioral inhibition, mediated by the mPFC.

Because THir fiber density was significantly decreased by RU486 treatment, it can be inferred that these animals have an underdeveloped cortical dopaminergic system. These findings suggest that progesterone facilitates the development of dopamine systems in the mPFC, particularly in regions involved in memory processes; the prelimbic area helps to orchestrate working and long-term memory functions (Kritzer, 2003). The other regions that were analyzed were the anterior cingulate cortex, which is also involved in memory functions, and the infralimbic cortex, which plays a role in sequential or temporal ordering tasks.

The second experiment, the inhibitory avoidance task, serves to compliment the findings of the TH immunocytochemistry study. In humans, the mPFC is responsible for behaviors such as impulse control, attention regulation, and self monitoring (Arnsten and Li, 2005). Although the rodent medial prefrontal cortex may not regulate the more complex behaviors, some of these behaviors can be tested in rats through the use of the inhibitory avoidance task. No differences were found between the groups in the first trial, suggesting that RU486 animals did not differ in their initial motivation to enter the dark compartment nor had decreased motor behavior. There were significant differences in the latency to enter the dark compartment between the treatment groups during the second trial. The RU486 group showed a significantly decreased latency to enter the dark compartment during the second trial, which indicates that these animals may have deficits in decision making, behavior inhibition, and/or long-term memory of the past consequence. Since the RU486 animals also showed decreased TH fiber density compared to control animals, it is possible that the disrupted development of the dopaminergic system may underlie changes in these behaviors. Both experiments suggest the role of active PR in the development of dopaminergic systems in the mPFC during early life.

Although this study uses an animal model to demonstrate changes in dopamine innervation and behavior as a result of PR antagonism, the findings could be applied to complex human disorders. Alterations in the PFC often cause disturbances in attentional processes, which may have implications for ADHD (Boulougouris and Tsaltas, 2008; Brennan and Arnsten, 2008). Children with ADHD exhibit impairments in executive function (Lawrence et al., 2004) and show abnormalities in the PFC, particularly in regard to dopamine levels (Prince, 2008). Previous studies have shown that individuals with ADHD perform significantly worse than control subjects on tasks sensitive to frontal lobe dysfunction (Reeve & Schandler, 2001),

suggesting that the frontal lobe may be altered in these individuals. The findings of this study indicate that PR antagonism produces symptoms in rodents that may be analogous to ADHD; changes in dopamine levels also mimic those found in ADHD. It is possible that in individuals with ADHD progesterone levels and/or receptors are altered during development, which disrupts the development of dopamine circuits in the mPFC, causing behaviors regulated by this brain area to go awry.

It is difficult to examine development in the absence of hormones because endogenous hormones are abundant in the brain. Although RU486 is a PR antagonist and receptors are not being activated, progesterone is still present in the brain. One limitation of this study is the absence of non-treated and progesterone treated groups. Although the non-treated group and the oil treated vehicle animals should be identical, it is possible that the groups will yield different results. In addition, it is unclear whether excessive exogenous progesterone will have a positive or negative effect on the development of neural circuits in the mPFC. Since RU486 treated animals show decreased TH fiber density in the mPFC and perform worse on the inhibitory avoidance task, progesterone treated animals may show increased TH fiber density and perform better on the task. However, it is possible that any alteration to progesterone levels or PR may disrupt development in a negative way. Therefore, it is unclear how progesterone would affect dopaminergic systems in the mPFC and performance on the inhibitory avoidance task. A second limitation is that dopamine projections in the VTA were not analyzed. Dopamine fibers that are sent to the mPFC originate in the VTA (Kalsbeek et al., 1988). RU486 may change TH fiber densities in the VTA, which then alters the morphology of the mPFC. PR in the mPFC may help direct incoming dopamine fibers from the VTA. As a result of RU486, target cells in the mPFC may not provide proper signals resulting in abnormal connectivity between VTA and PFC. In

order to make causal conclusions about which brain area is altered by RU486, THir cell bodies could be counted in the VTA. Future studies in this area should examine the effects of RU486 on TH fiber density in the hippocampus and the VTA, as well as the behavioral consequences of RU486 on other measures of behavior. To mimic development in the absolute absence of PR, progesterone receptor knock-out mice may be used in place of RU486 treated animals.

In conclusion, our data indicate that PR is necessary for the normal development of dopaminergic fibers in the mPFC. In the absence of functional PR during early development, neural circuits in the mPFC disrupted, leading to disrupted dopamine innervation. Behavioral deficits in impulse control and decision making are also observed as a result of PR antagonism. These findings have implications for understanding the behavioral deficits seen in complex human psychiatric disorders, such as ADHD. Recognizing potential causal factors that contribute to the development of complex human disorders may drive future research to develop treatments for these disorders.

Acknowledgments

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Figure 1. TH fiber density in the prelimbic area of the medial prefrontal cortex.
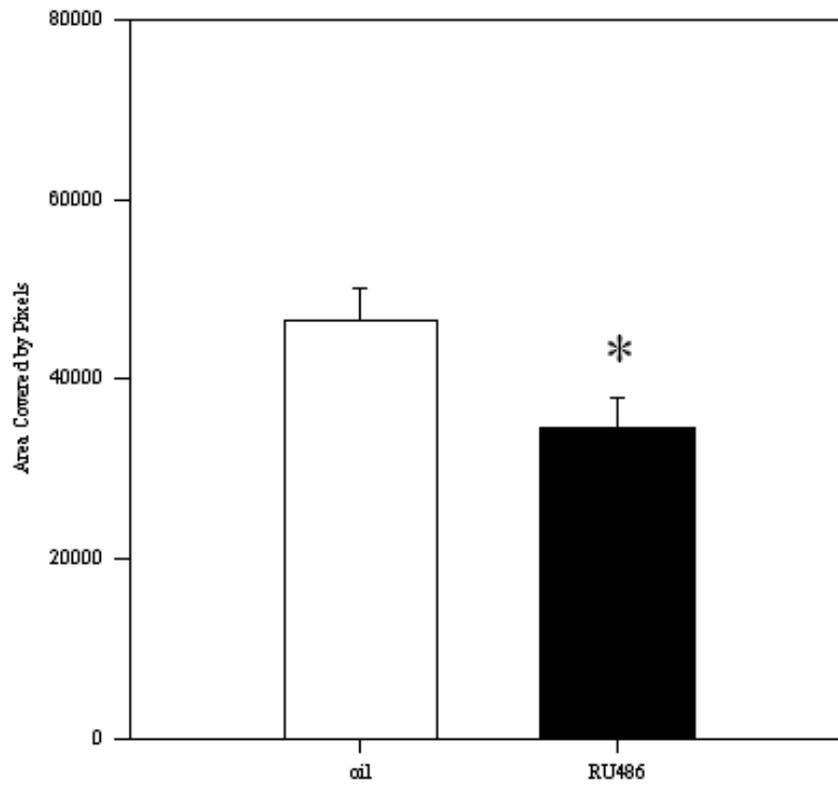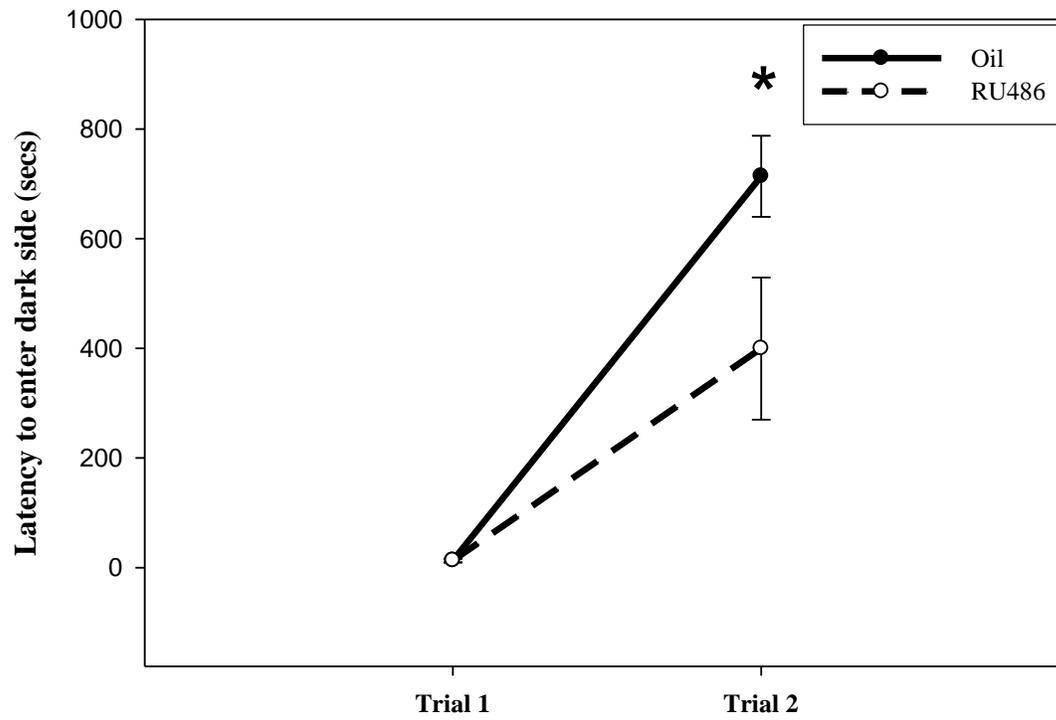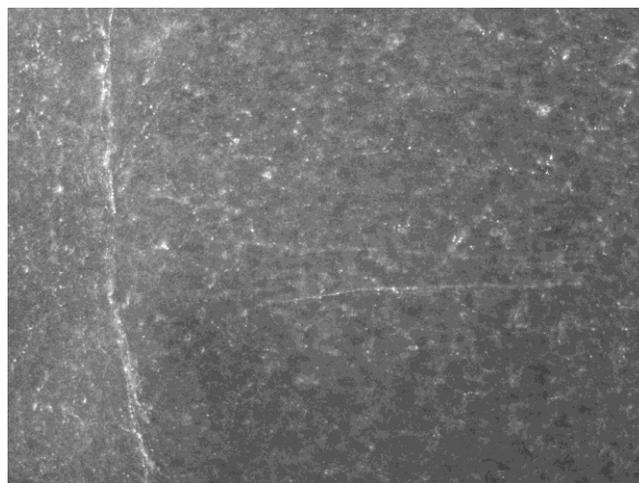
*, Significantly different from vehicle (p < 0.05)

Figure 2. Latency to enter the dark compartment in the inhibitory avoidance task.

*, Significantly different from vehicle (p < 0.05)

Appendix A

TH immunoreactivity in the prelimbic region of the medial prefrontal cortex in vehicle animal

(top) and RU486 treated animal (bottom)

Appendix B

PR immunoreactivity in the medial prefrontal cortex