Progesterone Receptor Expression in the Developing Mesocortical Dopamine Pathway: Importance for Complex Cognitive Behavior in Adulthood

Jari Willing^{a–c} Christine K. Wagner^{a, b}

^aDepartment of Psychology, and ^bCenter for Neuroscience Research, University at Albany, SUNY, Albany, N.Y., and ^cBehavioral Neuroscience Division, Department of Psychology, University of Illinois at Urbana-Champaign, Champaign, Ill., USA

Key Words

Progesterone receptor · Dopamine · Development · Cognitive flexibility

Abstract

Background: Numerous psychiatric and behavioral disorders such as autism, attention deficit disorder and schizophrenia may involve disruptions in the development of the mesocortical dopamine pathway, consisting of dopaminergic projections from the midbrain ventral tegmental area (VTA) to the medial prefrontal cortex (mPFC). Nuclear steroid hormone receptors are powerful transcription factors and can profoundly and permanently alter fundamental processes of neural development. Nuclear progesterone receptor (PR) is transiently expressed in both the VTA and the PFC of rodents during perinatal life, suggesting that PR may regulate the normal development of this important behavioral circuit. **Methods and Results:** Here, we demonstrate that virtually all PR-immunoreactive (PR-ir) cells in the VTA also express tyrosine hydroxylase immunoreactivity (TH-ir). In addition, retrograde tract tracing reveals that many PR-ir cells in the VTA project to the mPFC. Administration of a PR antago-

nist to rats during the neonatal period decreased TH-ir fiber density in the prelimbic mPFC of juveniles (postnatal day 25) and decreased levels of TH-ir in the VTA of adults. Neonatal treatment with a PR antagonist impaired adult performance on a passive inhibitory avoidance task and an attentional set-shifting task, measures of behavioral inhibition/impulsivity and cognitive flexibility, respectively. TH-ir levels in the VTA were reduced and cognitive flexibility was impaired in PR knockout mice as well. **Conclusions:** These findings provide novel insights into a potential role for PR in the developmental etiology of behavioral disorders that involve impairments in complex cognitive behaviors and have implications for the use of synthetic progestins in humans during critical neurodevelopmental periods.

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Introduction

The mesocortical dopamine pathway consists of dopaminergic projections from the midbrain ventral tegmental area (VTA; A10) to cortical areas, especially the prefrontal cortex (PFC). Dopamine release in the PFC is crit-

ical for 'executive functions' such as attention, working memory, behavioral inhibition and cognitive flexibility. Even slight perturbations during critical periods of development can cause permanent alterations in the function of this pathway and may underlie some of the cognitive deficits in behavioral disorders such as schizophrenia [1, 2] and attention deficit disorder [3–7].

The mesocortical dopamine pathway may be sensitive to steroid hormone action during development. Nuclear steroid hormone receptors are powerful transcription factors and regulate fundamental processes of neural development. Progesterone receptor (PR) is expressed throughout the perinatal rat brain including brain regions associated with cognitive function [8–10]. PR immunoreactivity (PR-ir) and PR mRNA are transiently expressed in the subplate and pyramidal cell layers of all functional regions (frontal, motor, somatosensory, auditory and visual) of the fetal and neonatal cortex, including the PFC [10, 11]. Preliminary data also suggest that PR is transiently expressed in the neonatal medial PFC (mPFC). In addition, PR is expressed during perinatal life in several midbrain nuclei including the VTA [8, 12]. The timing of PR expression in the VTA and PFC corresponds to periods of significant dendritic maturation of mPFC neurons and synaptogenesis for dopaminergic axons within the frontal cortex [13–15], suggesting that PR activity may be important for establishing functional connectivity. Consistent with this idea, progesterone treatment during postnatal life increased the number of midbrain neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of dopamine [16], and altered levels of dopamine and its metabolites in the PFC in adulthood [17].

The present study characterizes the full ontogeny and phenotype of PR-ir cells in the mesocortical pathway and tests the hypothesis that PR activity during perinatal life plays a critical role in the development of dopaminergic innervation of the mPFC and in subsequent mPFC-dependent cognitive behaviors in adulthood. Results demonstrate that PR is expressed in dopaminergic cells of the VTA and within VTA cells that project to the mPFC in neonates, and that inhibition of PR activity decreases dopaminergic innervation of the prelimbic (PL) mPFC, decreases TH expression in the VTA and impairs cognitive flexibility and behavioral inhibition in adulthood. These results provide valuable insight into the normal development and potential susceptibilities of this critical behavioral circuit and further elucidate the importance of steroid hormones and their receptors in neural development.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University at Albany, SUNY. Pregnant female Sprague-Dawley rats were purchased from Taconic Laboratories (Germantown, N.Y., USA) at gestational day 16 and were allowed to deliver pups normally. The day of birth was designated as postnatal day 1 (P1). The mice used in this study were of a mixed background (C129SvEv × C57Bl) and either homozygous for an insertional mutation in the PR gene (PRKO) or homozygous for the wild-type PR gene (WT). They were bred at the University of Albany, SUNY, by crossing heterozygous males and females. PRKO mice were created by the insertion of a neomycin resistance gene and a lacZ reporter gene into exon 1, thereby inhibiting the synthesis of functional PR transcripts and preventing the expression of both PR_A and PR_B isoforms of the receptor protein [18]. All animals were housed on a reverse 12-hour light/12-hour dark cycle at a constant temperature of 25 ± 2 °C with food and water available ad libitum.

RU 486 Administration

Male rat pups were treated daily with the PR antagonist RU 486 (20 mg/kg, s.c., in sesame oil [19, 20]; Mifepristone; Sigma-Aldrich, St. Louis, Mo., USA) or an equal volume of the oil vehicle alone from P1 to P14, the period spanning peak PR expression in the VTA and PFC.

Genotype Determination

From mice, ear tissue was collected by ear punch for genotype determination. Tissue was first lysed using proteinase K in a water bath heated to 55 °C overnight, after which total genomic DNA was purified and amplified using the Qiagen DNeasy Blood and Tissue kit. PCR was performed on purified DNA using three separate primers for PR and neomycin (PR: 5'-TAGACAGTGTCTTAGACTCGTTGTTG-3'; 5'-GATGGGCACATGGATGAAAC-3'; neomycin: 5'-GCATGCTCCAGACTGCCTTGGGAAA-3'). Using gel electrophoresis, the genotype was determined by the molecular weight of the extracted PCR product. Treatment groups were counterbalanced by litter, such that WT and PRKO mice from at least 5 different litters were represented in each group to avoid possible litter effects.

Tissue Collection

All rats and mice in these experiments were sexed using anogenital distance as a marker. Neonatal rats were anesthetized by hypothermia, while juvenile and adult rats and mice were administered a lethal injection of sodium pentobarbital (Sleepaway; Zoetis, Florham Park, N.J., USA). All animals were euthanized by decapitation, and the brains were rapidly removed and immersed in 5% acrolein in 0.1 M phosphate buffer (pH 7.6) for 6 h, then cryoprotected in 30% sucrose in 0.1 M phosphate buffer for 72 h. The brains were sectioned in the coronal plane at 50 µm on a freezing microtome and stored in cryoprotectant (30% sucrose, 0.1% polyvinylpyrrolidone-40 in ethylene glycol and 0.1 M phosphate buffer) until immunocytochemical processing.

PR Immunocytochemistry

Free-floating sections were rinsed in 0.05 M Tris-buffered saline (TBS), incubated in 1% sodium borohydride in TBS for 10 min, and blocked in TBS containing 20% normal goat serum, 1%

hydrogen peroxide and 1% bovine serum albumin for 30 min. The sections were then incubated in primary antisera against PR (rabbit polyclonal; DAKO, Carpinteria, Calif., USA; 1:1,000 in TTG, i.e. TBS containing 0.3% Triton X-100 and 2% normal goat serum) for 72 h. These rabbit polyclonal antisera were raised against the following sequence of the PR protein: amino acids 533–547, which is the DNA-binding domain of the human PR protein and is contained in both A and B isoforms of the receptor [21]. The specificity of this antiserum has previously been demonstrated by our laboratory [8, 10]. Nuclear immunoreactivity is completely eliminated by preabsorption of the PR antiserum overnight with either the antigen peptide or a 10-fold molar excess of human PR_A and PR_B proteins [20, 22] or when the primary antiserum was omitted. In addition, all immunoreactivity is absent in PRKO mice [10].

Following primary antibody incubation, sections were rinsed 3 times in TTG (5 min each) and placed in a solution containing 5 µg/ml goat anti-rabbit biotinylated secondary antibody in TTG for 90 min at room temperature, followed by incubation in Avidin-Biotin Complex in TBS (Vectastain Elite Kit; Vector Laboratories Inc., Burlingame, Calif., USA) for 1 h. The sections were then rinsed 3 times in TBS, then incubated in TBS containing 0.05% diaminobenzidine, 0.75 nM nickel ammonium sulfate, 0.15% β-D-glucose, 0.04% ammonium chloride and 0.001% glucose oxidase for 10 min. After being rinsed in TBS, the sections were mounted on gelatin-coated slides and coverslipped with Permount (Fisher Scientific, Pittsburgh, Pa., USA).

TH Immunocytochemistry

The immunocytochemical procedures were the same as described above, except that tissue was incubated in TH antisera (rabbit polyclonal; Millipore, Billerica, Mass., USA) for 48 h at 1:50,000 for TH-ir cell bodies in the VTA, and at 1:1,000 for TH-ir fibers in the mPFC.

Double Immunocytochemistry

For the colocalization of PR-ir and TH-ir and the colocalization of PR-ir and FluoroGold (FG), free-floating sections were incubated in 1% sodium borohydride in TBS for 10 min, then blocked in 20% normal goat serum, 1% bovine serum albumin and 1% hydrogen peroxide in TBS for 30 min. The sections were incubated in both antisera against PR (1:500, rabbit polyclonal; DAKO) and antisera against TH (1:50,000, mouse monoclonal; Millipore) or antisera against PR (1:500) and antisera against FG (1:500, guinea pig monoclonal; Protos Biotech, New York, N.Y., USA) diluted in TTG for 72 h. For adequate visualization of PR, the immunohistochemical signal was enhanced using the Tyramide Signal Amplification method (PerkinElmer Inc., Waltham, Mass., USA). Briefly, after primary antibody incubation, the tissue was incubated in biotinylated goat-anti-rabbit secondary antibody (5 µg/ml) in TTG for 90 min at room temperature, followed by streptavidin-horseradish peroxidase and biotinyl tyramide solutions. Finally, tissue was incubated for 1 h in a solution containing streptavidin-conjugated fluorophore to detect PR (1:200, Alexa Fluor 488; Invitrogen, Carlsbad, Calif., USA), along with a goat-anti-mouse, fluorescently tagged secondary antibody for the detection of TH, or goat-anti-guinea pig for FG (1:200, Alexa Fluor 568), both diluted in TBS. The sections were then mounted on slides and coverslipped with ProLong Gold Antifade reagent (Invitrogen).

Behavioral Testing

Male rats were raised/treated as described above. At P90, all experimental animals underwent daily pretest handling (1 min/day) for 1 week prior to testing. One cohort of rats was tested on the inhibitory avoidance task. To test the effects of RU 486 on cognitive flexibility, an additional cohort of rats was tested on the attentional set-shifting task. Last, a cohort of adult WT and PRKO mice was used to test cognitive flexibility in the adapted water maze task [23]. For these mice, testing commenced at P60. All testing procedures were conducted by an experimenter blinded to treatment group.

Experiment 1: Ontogeny of PR Expression in the Rat VTA and mPFC

For VTA ontogeny, caesarean sections were performed to collect fetal tissue on embryonic day 16 (E16), E18 and E20 (3 litters per age group). Briefly, pregnant females were given a lethal dose of sodium pentobarbital. The abdominal cavity was opened, and the uterine horns were removed and placed on ice. Following removal from the uterine horn, each fetus was placed on ice prior to sacrifice by decapitation. Neonatal brains were collected at P1, P7, P14 and P28 and fixed in 5% acrolein. All tissue was fixed, cut and processed for immunocytochemistry for PR as described above. For mPFC ontogeny, animals were sacrificed at P2, P6, P10, P15, P20, P25 and P30. For both VTA and mPFC ontogeny, tissue from 5 males and 5 females was collected for each age group. For both VTA and mPFC ontogeny, the exact dates were chosen based on previous studies from our laboratory [9, 11].

A representative, anatomically matched section from the VTA and mPFC of each animal was selected for image analysis. Microscopic images were captured with a Nikon Eclipse E600 microscope fitted with a SPOT Insight camera connected to a Dell Inspiron computer. Scion Image software (Scion Corp., Frederick, Md., USA) was used to analyze the captured images. The relative amount of PR-ir was determined as described previously [20, 24] by measuring the area (in µm²) covered by 'thresholded' pixels [i.e. those pixels with a gray level higher than a defined threshold density (specific immunoreactive staining)]. The threshold was determined as a constant function of the background optical density defined as the mean optical density 3–5 SDs higher than the mean background density. The mean background density was measured in a region devoid of PR-ir immediately adjacent to the analyzed region containing PR-ir. Statistical analyses were performed for each region using a two-way ANOVA (sex × age), followed by pre-planned post hoc comparisons using the Student-Newman-Keuls test ($p < 0.05$).

Experiment 2: Characterization of the Phenotype of PR-ir Cells in the Neonatal Rat VTA

Experiment 2a: PR Expression in Dopaminergic Cells of the VTA during Development. Tissue was collected from 5 male and 5 female rats at P1 and P7, and processed for double immunocytochemistry for PR-ir and TH-ir as described above. Analysis of the coexpression of PR and TH was conducted using a Zeiss LSM 510 confocal microscope. Images were taken sequentially at ×63 magnification using an argon laser for 488-nm wavelengths (for PR detection) and a HeNe laser for 568-nm wavelengths (for TH detection). The z-stack consisted of 21 sequential images at 1 µm each. For each animal, 3 matching representative brain sections through the VTA were investigated. For each of the 3 sections per

animal, 10 cells which were immunoreactive for PR were sampled at $\times 63$ magnification from 2 different sites per section, i.e. from the parabrachial and paranigral subregions of the VTA, such that approximately 30 cells from each animal were considered for this analysis. From this sample, the percentage of cells that was immunoreactive for both PR and TH was estimated.

Experiment 2b: PR Expression in VTA Cells Projecting to the mPFC. Male and female pups ($n = 20$) were anesthetized by hypothermia on P3, and their heads were secured using a stereotaxic apparatus adapted for neonatal rats (David Kopf Instruments, Tujunga, Calif., USA). Using neonatal rat stereotaxic coordinates (1.5–2.5 mm ventral, 2.0 mm rostral to the bregma) [25], a 1.0- μ l Hamilton syringe needle containing FG (hydroxystilbamidine: 0.2 μ l of 2% FG in sterile water) was lowered through the skull and into the frontal cortex targeting the PL and infralimbic (IL) mPFC. FG was injected using a microinfusion pump (World Precision Instruments, Sarasota, Fla., USA) at 0.005 μ l/s. Following recovery from anesthesia, the pups were returned to their home cages for a 3-day survival period, after which brain tissue was collected at P6, as described above, and immersion fixed in 5% acrolein. FG fluorescence was analyzed on sections through the mPFC to determine the accuracy and the extent of the FG injections. Only animals whose FG injections were limited to the PL and IL mPFC were included for this analysis. While all injections hit the mPFC, a total of 7 animals were excluded from the study due to the fact that the FG infiltration tissue was outside of the PL/IL region. In addition, as a control for FG specificity, 2 animals intentionally received injections into the striatum. In these animals, it was confirmed that FG-labeled cells were observable in the substantia nigra and not in the VTA. Analysis of PR and FG colocalization was conducted on the remaining 11 animals (6 male, 5 female). Sections through the VTA were immunocytochemically processed for FG and PR-ir as described above. Three representative matching sections from the VTA were chosen for each animal. Confocal images at $\times 63$ magnification were taken sequentially using an argon laser for 488-nm wavelengths (for PR detection) and a HeNe laser for 568-nm wavelengths (for FG detection). Analysis was limited to regions of the VTA that contained both PR and FG staining. From the 3 sections used, approximately 10 PR-ir cells per section were randomly selected for a total of 30 cells per animal. A semiquantitative assessment of the percentage of PR-ir cells in the VTA that were colocalized with FG was conducted.

Experiment 3: PR Activity during Development and Levels of TH in the VTA

Because no sex differences were observed in the expression of PR or in the phenotype of PR-ir cells, the following studies were conducted on male rats treated with sesame oil or RU 486 as well as on male WT and PRKO mice. Brain tissue was collected from male rats (RU 486 and oil treated) at P25 and P90 and from male mice (WT and PRKO) at P25 and P70 ($n = 9$ or 10 per group). P25 represents the developmental period prior to significant synaptic pruning in the mPFC, and P70 (in mice) and P90 (in rats) represents the adult stage of full maturity of the mPFC [26–29]. Tissue was processed for TH immunocytochemistry as described above. The density of TH-expressing cells was determined in 1 matching representative section from each animal. Labeled TH-ir cells were counted in 1 representative section containing the parabrachial and paranigral VTA using a 10×10 grid reticule attached to the eyepiece of a Nikon Eclipse E600 microscope. This grid was large enough that all TH-ir cells within the representative section were counted.

Additionally, using Scion Image software, TH optical density was measured in the VTA to determine relative levels of TH expression as opposed to the number of cells expressing TH. Using Scion Image software, the VTA from 1 representative, anatomically matched section per animal was digitally traced, defined by borders outlined in Paxinos and Watson [30]. Using a $\times 20$ objective, photographs were acquired as described in experiment 1. The optical density scale used to measure relative TH immunoreactivity was inverted so that 0 was white and 254 was black (i.e., a higher number indicating higher relative levels of TH-ir). *t* tests were used for the experiments using rats and mice for TH cell counts and TH optical density.

Experiment 4: PR Activity during Development and Dopaminergic Innervation of the mPFC

Sections containing PFC were collected/processed from animals used in experiment 3 and processed for TH immunocytochemistry as described above. Analysis of TH fiber density in the mPFC was accomplished using previously established methods [31]. Briefly, representative, anatomically matched sections through the mPFC were used to capture photographs in darkfield illumination using a Model 1.3.0 SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, Mich., USA) attached to a Nikon E600 microscope using a $\times 20$ objective. The density of TH-ir fibers (area covered by thresholded pixels per square unit area) was quantified using Scion Image software as described previously. Thresholded pixels were distinguished as being above a predetermined background level that was devoid of TH staining. Separate photographs were acquired from layers I–II and from layers V–VI. A separation of these layers is identifiable based on TH-ir fiber orientation [32, 33]. The PL mPFC was identified based on proximity to the forceps minor of the corpus callosum, easily identifiable by its white matter composition [30]. Treatment groups were compared using separate Student *t* tests for layers II and V of each mPFC subregion.

Experiment 5: PR Activity during Neonatal Life in Rats and Behavioral Inhibition in Adulthood

Neonatal rats were treated as described above with either RU 486 or sesame oil ($n = 9$ per group). In adulthood, the animals underwent a 2-trial passive inhibitory avoidance task. This task measures both long-term memory of negative consequences and behavioral inhibition and may be influenced by activity in the mPFC [34–36]. In trial 1, rats were placed into a chamber lit by ambient room light, with access to an adjoining, enclosed, dark chamber, which was accessible via the opening of an electronic door after a 30-second habituation period. One second after the rats entered the dark chamber, they received a mild, but aversive, inescapable foot shock (0.5 mA, 3 s). In trial 2, 48 h later, the rats were placed back into the lit chamber, and the latency to reenter the dark chamber was measured, with a maximum latency of 900 s. A shorter latency to reenter the dark chamber is indicative of impaired memory and/or a deficit in behavioral inhibition. A two-way ANOVA (day $\times$ treatment) was used to assess performance across days, followed by a nonparametric Mann-Whitney *U* test to compare latencies on the second day of the task.

Experiment 6: Inhibition of PR Activity during Neonatal Life and Cognitive Flexibility in Adulthood

Attentional Set-Shifting Task in Rats. Neonatal rats were treated as described above with RU 486 or sesame oil ($n = 9$ per group)

and tested at P90 on the attentional set-shifting task, which assesses cognitive flexibility in rats. Prior to testing, food was restricted to 15 g/day, maintaining the body weight at 80–90% of the free-feeding weight to ensure motivation for a food reward. The animals were handled and weighed daily to ensure that their body weights did not drop below 80% of the free-feeding weight. The testing apparatus is an adaptation of a plus maze, in which the arms of the maze are painted either white or black and the floors have either a rough or a smooth texture. All experimental procedures are identical to those described in Stefani and Moghaddam [37].

Pretesting Procedures. All animals underwent extensive pretest handling and habituation to the apparatus for 11 days prior to testing. Food restriction was continued throughout the testing procedure. On days 1–5, each animal was handled by the experimenter for 1 min, then given 3–4 sucrose pellets (45 mg, Dustless Precision Pellets; Bio-Serv, Frenchtown, N.J., USA) in their home cages. On days 6–8, the rats underwent maze habituation in which all 4 arms of the maze were baited with sucrose pellets. The habituation trials lasted until all pellets were eaten or a maximum of 5 min. On days 9–11, 1 arm of the maze was blocked, and the animals were trained to make an ‘arm choice’ by leaving the start arm and entering 1 of 2 open arms of the maze. For these days, there were 8 consecutive training trials in which 50% of their choices were rewarded in a random manner.

Testing Procedures. Testing was conducted on 2 consecutive days, consisting of multiple trials on each day (‘sets’ 1 and 2). For both sets, the maze was arranged as it was in the training trials such that animals placed in alternating start arms always had an option of turning right or left into 1 of 2 target arms. In set 1, the animals were trained to enter baited arms using 1 specific cue (based on arm color or texture) to earn a food reward. Set 1 ended when a performance criterion of 8 consecutive correct trials was met. Animals unable to reach this criterion within 120 trials were excluded from the study. In set 2 (24 h later), the ‘rule’ or contingency for the reward was changed to a different sensory modality (e.g. food reward switched from light arms to rough arms). In set 2, all animals completed 80 trials regardless of performance. This extradimensional shift (from one sensory domain to another) is dependent on dopaminergic activity in the mPFC [38–42]. The number of trials to achieve the criterion on test day 1, the percentage of trials with correct choices across trial ‘blocks’ (8 consecutive trials per block) on test day 2 and the number of perseverative errors (i.e. incorrect responses based on the reward contingency from test day 1) were analyzed. A perseveration error was defined as making a choice indicative of the first rule association after the rule has changed in set 2. ‘Omission’ errors, incorrect choices that are not representative of either rule 1 or rule 2, were also counted. Because of the cap on the maximum number of trials, trials to criterion in set 1 were analyzed using the Mann-Whitney U test. A two-way ANOVA (treatment $\times$ trial block) was used to analyze the percentage of correct choices across set 2. Additionally, separate two-way ANOVAs were conducted for the first half (trial blocks 1–5) and the second half (trial blocks 1–6). Student’s *t* tests were used to compare the number of perseveration and omission errors between groups on set 2.

Water Maze Task in Adult WT and PRKO Mice. WT and PRKO mice (*n* = 10 per group) were tested on a water maze task at P60. Procedures for this adapted water maze test were adopted from Karatsoreos et al. [23] to assess cognitive flexibility in mice. Male WT and PRKO mice were housed with littermates throughout the

duration of testing, and all tests were conducted during the animals’ dark phase to enhance the activity level. Testing was conducted in a plastic tank filled with water (26°C) made opaque with powdered milk. Trials were recorded using ANY-maze behavior-tracking software (Stoelting, Wood Dale, Ill., USA) by an experimenter blinded to treatment group. The task occurred over 5 consecutive days (4 trials per day). In each trial, the latency to reach a ‘safety’ platform was recorded, with a maximum latency of 60 s. On day 1, to assess swimming ability and motivation, animals were placed into the maze with a visible platform. On days 2 and 3, the platform remained in the same location in the maze but was submerged below the water surface and was no longer visible, requiring spatial memory of the location of the platform. Latency to reach the platform on days 2 and 3 assesses long-term spatial memory. On day 4, the location of the platform was moved to an opposite quadrant of the maze, requiring the animals to learn this new location. On day 5, the platform was in the same location as on day 4. Latency to reach the platform on days 4 and 5 represents cognitive flexibility, the ability to learn the position of the new platform location while inhibiting the tendency to revert back to the first location of the safety platform. On days 4 and 5, the number of entries into the ‘old quadrant’ (the quadrant where the platform was located on days 1–3) was recorded as perseveration errors [23]. Swim speed was also recorded for all trials as a control to ensure that differences in latency were indicative of differences in cognition and not of motor abnormalities. Differences in the latency to reach the platform across days were analyzed using two-way repeated-measures ANOVA (genotype $\times$ day). Student’s *t* tests were used for between-group comparisons of swim speed (in cm/s) and entries into the old quadrant.

Results

Experiment 1: PR Is Transiently Expressed in the Rat VTA and mPFC during Neonatal Life

Preliminary evidence suggested that PR was expressed in both the VTA and the mPFC during development in the rat. In the first experiment, we sought to determine the developmental window during which PR-ir cells are present in the VTA and mPFC.

Ventral Tegmental Area

PR-ir was not observed at E16, E18 or E21. However, PR-ir was present within the parabrachial and paranigral VTA on P1, P7 and P14 but absent by P28 (fig. 1a). For the relative amount of PR-ir in the VTA, a two-way ANOVA (age $\times$ sex) revealed a significant main effect of age ($F_{2, 29} = 21.988$, $p < 0.001$) but no significant effect of sex ($p = 0.923$) and no significant interaction ($p = 0.780$). PR expression in the VTA was highest on P1 in males and females, and thereafter declined by P7 and P14 ($p < 0.05$) and was virtually absent by P28. Student-Newman-Keuls post hoc analysis revealed a significant difference between P1 and both P7 and P14 ($p < 0.05$).

Fig. 1. Transient expression of PR in the rat VTA and mPFC during development. The mean ($\pm$ SEM) relevant total amount of nuclear PR-ir in the VTA (**a**) and the PL mPFC (**b**) is shown. **a** Nuclear PR-ir was not detectable in the VTA prenatally (E16–E21), was transiently expressed from the day of birth (P1) through at least P14 and was absent by P28. PR-ir levels were highest on

P1, with a significant decline at P7 and P14. **b** In the mPFC, PR-ir was not detectable on P2, was expressed from P6 through P25 (peaking around P10) and was absent by P30. Postnatal ages with differing letters were significantly different from one another ($p < 0.05$). There was no significant effect of sex at any age in either region.

Medial Prefrontal Cortex

PR-ir was present in layers II and III of the mPFC. PR expression was first detected on P6, peaked on P10, then progressively declined, and was undetectable at P30 (fig. 1b). A two-way ANOVA (age $\times$ sex) revealed a main effect of age ($F_{4, 50} = 11.623$, $p < 0.001$), no effect of sex ($p = 0.512$) and no age-by-sex interaction ($p = 0.837$). The post hoc analysis revealed a significant difference between P10 and every other age examined. No PR-ir cells were detected in any other mPFC layer. PR-ir was not detected at P2 or P30.

Experiment 2: PR Is Expressed in Dopaminergic Cells and Cells Projecting to the mPFC in the Rat VTA

In order to determine if PR was expressed in dopaminergic cells of the VTA, we examined the colocalization of PR-ir and TH-ir in males and females on P1 and P7 using confocal microscopy. In both sexes, $>90\%$ of PR-ir cells also contained TH-ir at P1 and P7 in the parabrachial and paranigral subdivisions of the VTA (fig. 2). TH-ir was limited to the cytoplasm and dendrites, allowing for clear visualization of PR-ir in the nucleus of individual cells.

Because PR was expressed in dopaminergic projection neurons of the VTA, we next examined whether PR was expressed in VTA cells that project to the mPFC. The ret-

rograde tracer FG was injected into the PL and IL mPFC on P3, and confocal microscopy was used to examine the colocalization of FG and PR-ir in VTA on P6. FG injections that were limited to the PL/IL mPFC (fig. 3a) labeled many cells of the VTA (fig. 3b). FG injections that missed the PL/IL mPFC resulted in no FG labeling of the VTA. As an additional specificity control, FG injections intentionally made into the striatum labeled cells in the substantia nigra but not in the VTA. Approximately 50% of the FG-labeled cells in the VTA also expressed nuclear PR-ir (fig. 3c, d). It is important to note that in addition to the PR/FG colocalized cells, approximately 50% of the PR-ir cells were not immunoreactive for FG as well, and approximately 50–60% of the FG-ir cells did not express PR. Taken together, these results suggest that PR is transiently expressed during development in midbrain VTA cells of the mesocortical dopaminergic pathway to the mPFC.

Experiment 3: PR Activity during Development Regulates Levels of TH in the VTA

PR is transiently expressed in dopaminergic cells of the VTA during postnatal development. To determine whether PR activity may regulate dopamine synthesis later in life, we examined mean levels of TH-ir per cell in the VTA in rats treated with a PR antagonist during neonatal

Fig. 2. PR is expressed in dopaminergic cells of the rat VTA. Colocalization of PR-ir (green; nuclear) and TH-ir (red; cytoplasmic) in the neonatal VTA is shown. **a** P1 male. **b** P1 female. **c** P7 male. **d** P7 female. More than 90% of the PR-ir cells in the parabrachial VTA were dopaminergic.

Fig. 3. PR is expressed in VTA cells that project to the mPFC in neonatal rats also immunoreactive for PR. The retrograde tracer FG was injected into the PL and IL mPFC and labeled cells of the VTA that were immunoreactive for PR. **a** FG-ir within the IL and PL mPFC at the injection site. **b** Retrogradely labeled FG-ir cells within the parabrachial VTA (PB-VTA). **c, d** Colocalization of PR-ir (green; nuclear) and FG-ir (red; cytoplasmic) in the VTA of a male rat at P6.

Fig. 4. Inhibition of PR activity during development decreases TH levels in the adult VTA. Relative total amounts of TH-ir in the VTA of P25 (a) or P90 rats (b) following administration of the PR antagonist RU 486 (20 mg/kg) or the oil vehicle during neonatal life, or in the VTA of P25 (c) or P70 WT or PRKO mice (d) are shown. Data are presented as means $\pm$ SEM. * Significantly different from same-age counterparts at $p < 0.05$.

life as well as in both juvenile and adult PRKO mice. At P25, there was no effect of RU 486 treatment in rats, or genotype in mice, on relative levels of TH-ir in the VTA (optical density) (fig. 4a, c). In contrast, levels of TH-ir were significantly lower in the VTA of RU 486-treated rats ($t = 2.681$, $p = 0.017$) and PRKO mice ($t = 2.587$, $p = 0.012$) compared to controls in adulthood (P90 and P70, respectively) (fig. 4b, d). There was no effect of treatment or genotype on the number of TH-ir cells in the VTA at P25 or in adulthood for either rats or mice (data not shown). These results suggest that disruption of PR activity during early life alters the development of the VTA in such a way as to decrease dopamine synthesis sometime after, but not prior to, adolescence.

Experiment 4: PR Activity during Development Influences Dopaminergic Innervation of the mPFC

PR is transiently expressed in dopaminergic cells of the VTA during a period of dopaminergic synaptogenesis and pruning in the PFC. We therefore tested the hypoth-

esis that PR activity during neonatal life is important for dopaminergic innervation of the mPFC. Neonatal administration of RU 486 to rats significantly decreased the density of TH-ir fibers within layer II of the PL mPFC in rats at P25 ($t = 2.44$, $p = 0.024$) but not at P90 (fig. 5a, b). RU 486 had no significant effect on TH-ir fiber density in layer V at either age in rats (data not shown). In mice, there were no effects of genotype on TH-ir fiber density in either layer V or layer II of the P25 or P70 mouse mPFC (fig. 5c, d).

Experiment 5: Inhibition of PR Activity during Development Increases Impulsivity in Adulthood

The evidence thus far suggests that PR is transiently expressed in both the mPFC and in dopaminergic cells of the VTA that project to the mPFC, generating the hypothesis that PR activity during development may be important for cognitive behaviors in which the mesocortical dopamine pathway is critical. In the first behavioral experiment, we examined behavioral inhibition/impul-

Fig. 5. Inhibition of PR activity during development alters dopaminergic innervation of the mPFC in adolescent rats. Relative density of TH-ir fibers within layer II of the PL mPFC in rats at P25 (**a**) or P90 (**b**) following administration of the PR antagonist or the oil vehicle during neonatal life. There was a trend toward reduced TH-ir fiber density in the mPFC of PRKO mice at P25 (**c**); there were no significant differences in the mouse mPFC at either age (**c**, **d**). Data are presented as means $\pm$ SEM. * $p < 0.05$.

sivity using the active avoidance task in adult rats treated with the PR antagonist as neonates. A two-way ANOVA revealed a significant main effect of treatment ($F_{1, 17} = 4.938$, $p = 0.035$), a main effect of day ($F_{1, 17} = 59.165$, $p = 0.034$) and a significant treatment-by-day interaction ($F_{1, 17} = 4.942$, $p = 0.034$) (fig. 6). Two follow-up non-parametric Mann-Whitney rank-sum tests showed that there was no difference between groups on day 1 but there was a significant difference in latency on day 2 ($T = 38.0$, $p = 0.04$). Control rats approached the maximum latency, whereas RU 486-treated rats entered the dark chamber after about half the time. These results suggest that disruption of PR activity during development impairs the long-term memory for an aversive event and/or behavioral inhibition, the ability to inhibit a motivated behavior despite a previous, negative consequence. This may also be interpreted as an increase in impulsivity (i.e. engaging in a behavior without considering potential risks).

Experiment 6: Disruption of PR Activity during Development Impairs Cognitive Flexibility and Increases Perseveration in Adult Rats and Mice

To examine the role of PR activity in cognitive flexibility, which is dependent upon dopamine release in the mPFC, we utilized the attentional set-shifting task in adult rats treated neonatally with the PR antagonist, or we used an adapted water maze in adult PRKO mice.

Attentional Set-Shifting Task in Rats

In the initial rule-learning association trials (set 1), neonatal RU 486 administration increased the number of trials required to reach the criterion of 8 consecutive correct choices compared to controls ($t = -2.462$, $p = 0.026$) (fig. 7a), suggesting that RU 486-treated rats were slower to learn the initial rule. Additionally, 1 animal from the RU 486 group failed to reach the criterion within 120 trials and was excluded from the study. Nonetheless, all rats from both groups reached the criterion and therefore went into set 2 at similar levels of performance on the first

Fig. 6. Inhibition of PR activity during neonatal life decreases behavioral inhibition/increases impulsivity in adulthood. Neonatal administration of the PR antagonist RU 486 impaired performance on the inhibitory avoidance task compared to oil-treated controls. The latency to enter the dark chamber was not significantly different between the groups in trial 1. In trial 2, both groups had significantly longer latencies compared to trial 1, i.e., in effect, an avoidance of the dark chamber, indicating a memory for the aversive event in trial 1 in both groups. However, the latency to enter the dark chamber was significantly shorter for RU 486-treated rats in trial 2 compared to controls, suggesting an impaired ability to inhibit a motivated response or an increase in impulsivity. Data are presented as means $\pm$ SEM. * $p < 0.05$.

rule. RU 486-treated animals again required significantly more trials to reach the 8 consecutive correct choices in set 2 ($t = -2.275$, $p = 0.039$) (fig. 7b).

In set 2, the rule was changed to a different sensory modality (e.g. from light to rough arms baited, an extra-dimensional shift). Both groups performed at about baseline levels for trial blocks 1–5. However, at about trial block 6, control rats made the ‘shift’ to the new rule and achieved >90% correct responses by trial block 10. In contrast, RU 486-treated rats were slower to make the shift to the new rule and never performed quite as well as controls (fig. 7c). A two-way repeated-measures ANOVA of trial blocks 1–5 revealed no significant main effect of trial block, no significant effect of treatment and no significant interaction. However, in trial blocks 6–10, there was a significant main effect of trial block ($F_{9, 159} = 11.760$, $p < 0.001$) and treatment ($F_{1, 159} = 4.756$, $p = 0.031$) (fig. 7c), with no significant interaction between trial block and treatment.

The number of perseveration errors (incorrect responses made using the old rule) did not differ between the groups in trial blocks 1–5 (both groups displayed a high number of perseveration errors). However, in trial blocks 6–10, RU 486-treated rats continued to make perseveration errors, and they made significantly more perseveration errors than controls ($t = -2.152$, $p = 0.049$) (fig. 7f, g). In contrast, the number of omission errors (incorrect responses using neither the old nor the new rule) did not differ between the groups in either trial blocks 1–5 or 6–10 (fig. 7d, e). Taken together, these results suggest that disruption of PR activity during development impairs cognitive flexibility and increases perseverative behavior.

Water Maze Task in Adult WT and PRKO Mice

We next examined the role of PR in cognitive flexibility using PRKO mice in an adapted water maze. WT and PRKO mice did not differ in their ability to learn the placement of a hidden platform. However, when the location of the hidden platform was changed, PRKO mice were slower to shift to the new location, and they made significantly more perseveration errors. A two-way repeated-measures ANOVA revealed a significant interaction between genotype and test day ($F_{4, 100} = 2.770$, $p = 0.034$), a significant main effect of test day ($F_{4, 100} = 28.439$, $p < 0.001$) and no significant main effect of genotype (fig. 8a). Post hoc tests revealed that PRKO mice had a significantly longer latency to reach the platform on test day 4 ($p < 0.05$), the first day the platform was moved to the new quadrant. There were no significant effects of genotype on any other test day. In addition, PRKO mice committed significantly more perseveration errors (i.e. entries into the previously correct quadrant) on test days 4 ($t = 2.271$, $p = 0.036$) and 5 ($t = 2.221$, $p = 0.04$) (fig. 8c, d). There were no significant differences in swim speed between PRKO and WT mice across all test days (fig. 8b). The results for PRKO mice are consistent with the findings for rats and suggest that PR expression is important in cognitive flexibility. An absence of functional PR expression increases perseverative behavior.

Discussion

Fundamental processes of early neurodevelopment are critical for the proper maturation of mesocortical connectivity and for subsequent cognitive function [26, 27, 43]. The present results support the idea that the activity of PR, a powerful transcription factor, during neonatal

Fig. 7. Inhibition of PR activity during neonatal life impairs cognitive flexibility and increases perseverative errors in adult rats. **a, b** RU 486-treated rats required a significantly greater number of trials to reach 8 consecutive correct responses in set 1 (**a**) and in set 2 (**b**) compared to controls. **c** Percent correct responses in set 2 following the rule change for trial blocks 1–10 (8 trials each) in RU 486-treated rats or oil-treated controls. There was no significant difference in performance between the treatment groups in trial blocks 1–5. However, in trial blocks 6–10, RU 486-treated rats were slower to acquire the new rule (i.e. had significantly fewer correct

responses) and never reached the performance of controls. **d, e** There was no effect of treatment on the number of omission errors (i.e. errors made using neither the first nor the new rule) in either trial blocks 1–5 (**d**) or 6–10 (**e**). **f, g** Both groups made a high number of perseverative errors (i.e. errors made using the first rule) in trial blocks 1–5 (**f**). RU 486-treated rats continued to make a significantly greater number of perseverative errors in trial blocks 6–10 compared to controls (**g**). Data are presented as means $\pm$ SEM. * $p < 0.05$.

Fig. 8. Cognitive flexibility is impaired in PRKO mice. **a** There was no significant effect of genotype on the mean latency to find a visible platform (visible) or to learn the location of a hidden platform (hidden 1 and 2). However, PRKO mice had a significantly longer latency to find the hidden platform after it was moved to a new quadrant (switch 1), but they reduced their latency to the level of the controls during the second trial (switch 2), suggesting that PRKO mice were capable of learning the new location but were slower to make the change (i.e. reduced cognitive flexibility). **b** There was no significant difference in swim speed across all trials between PRKO and WT mice. **c, d** PRKO mice made a significantly greater number of perseveration errors (entering the quadrant in which the platform was previously hidden) on both switch 1 and switch 2. Data are presented as means $\pm$ SEM. * $p < 0.05$.

life plays a previously overlooked role in the normal development of the mesocortical dopaminergic pathway and in normal complex cognitive behaviors in adulthood. PR is transiently expressed during development in both the source cells and target cells of the mesocortical pathway. PR-ir is observed in the parabrachial and paranigral subregions of the VTA during the first 2 weeks of life, with peak levels of expression occurring just after birth, continuing through at least P14, but being absent by P28. The vast majority of PR-expressing cells in the neonatal VTA of rats are dopaminergic, coexpressing TH. Indeed, many of the VTA PR-ir cells project directly to the PL and IL mPFC. PR is also transiently expressed within layers II and III of the mPFC beginning at P6, with a peak in expression around P10, but being absent by P30. Inhibition

of PR activity during neonatal life significantly reduced TH-ir fiber density in layer II of the PL mPFC in preadolescent rats (with a similar trend in PRKO mice) and decreased TH-ir levels in the VTA of both rats and PRKO mice. Lastly, inhibition of PR activity during development altered behavioral inhibition/impulsivity in adult rats and impaired cognitive flexibility in rats and PRKO mice in adulthood.

To our knowledge, the present findings are the first to report the presence of steroid receptors in cortically projecting dopaminergic cells of the VTA during development, although these results are consistent with previous reports from our laboratory demonstrating developmentally transient expression of PR-ir in the VTA and substantia nigra, as well as in other areas of the midbrain and

hindbrain [9]. In adulthood, androgen receptor and estrogen receptor- β , but not estrogen receptor- α , were colocalized with TH in VTA [44, 45] and were found in VTA cells projecting to PFC [46]. However, the transient expression of PR in the VTA during an important period of dopamine cell maturation [27] identifies PR as a potential contributor to the proper development of dopaminergic efferents from the midbrain.

Neonatal inhibition of PR with RU 486, while not altering the number of dopaminergic cells in the VTA, decreased relative total levels of TH protein, suggesting overall reduced rates of dopamine synthesis. The timing of peak PR expression in dopaminergic VTA cells during the first postnatal week coincides with transient alterations in the number of TH-ir neurons in the VTA [27]. Interestingly, in both rats and mice, a decrease in TH levels was only observed in adulthood but not at P25, suggesting that PR activity in early life might influence further developmental processes in the mesocortical pathway that occur during adolescence. This is consistent with findings that estradiol exposure on the day of birth altered adult levels of dopamine in the VTA as measured by HPLC [47] and with reports that in undifferentiated embryonic cultured stem cells, progesterone increased the number of TH-positive cells and virtually all cells induced to become dopaminergic expressed PR [16]. In addition, progesterone increased promoter activity in the TH gene in cells transfected with the gene for PR_B, suggesting that ligand-bound PR_B can be a transcriptional activator of TH gene expression [48]. PR activity in the VTA during development may induce transient changes in TH gene expression, leading to long-term alterations in dopamine synthesis in the adult VTA.

PR was also transiently expressed in layers II and III of the mPFC during the period in which afferents arrive in the mPFC and form mature patterns of connectivity in superficial cortical layers, similar to the developmentally and anatomically specific pattern of PR expression in all other functional regions of the cortex [10, 11, 49]. RU 486 treatment neonatally decreased the density of TH-ir fibers in the PL mPFC at P25 but not in adulthood, suggesting that PR activity earlier in development may set the stage for subsequent maturation that occurs in the PFC during adolescence, potentially permanently altering subsequent dopaminergic activity. Indeed, previous work shows that neonatal progesterone administration increased the dopamine metabolites DOPAC and homovanillic acid in the adult PFC [17], indicating an increase in dopamine turnover.

There are several potential mechanisms by which PR activity might alter the 'attractiveness' of mPFC target cells to influence the development of mesocortical circuits. For example, it is generally accepted that the extension and branching of the dendritic arbor and the density of dendritic spines on target cells are integral to the process of synaptogenesis [50, 51], and steroid hormones can regulate dendritic structures in both the adult and the developing brain [52–55]. In developing pyramidal cells of the hippocampus, testosterone increased dendritic length and increased the number of dendritic branches [56]. In neonatal Purkinje cells, both progesterone and estradiol can promote dendritic outgrowth and increase the density of dendritic spines [55, 57, 58]. In developing cortical pyramidal cells, progesterone given to pregnant rats increased dendritic branching and increased the density of dendritic spines [59]. As another possibility, PR may regulate the expression of trophic factors in the developing cortex. Indeed, there is a wealth of evidence that progesterone and its metabolites can regulate brain-derived neurotrophic factor expression in numerous brain regions in adulthood [reviewed in 60], although little evidence exists regarding early development. However, in dissociated P3 cell cultures of the PFC, progesterone increased brain-derived neurotrophic factor expression, and this increase was attenuated by RU 486 and in PRKO mice [61]. PR regulation of the mPFC target cell phenotype might influence synaptogenesis amongst arriving afferents, thereby potentially altering the strength and spatial patterning of connectivity.

Consistent with alterations in the mesocortical dopamine circuitry, PR inhibition during neonatal life impaired complex cognitive behaviors that are dependent on dopaminergic activity in the mPFC [31, 62, 63]. First, RU 486 treatment during the first 2 weeks of life decreased behavioral inhibition and/or increased impulsivity. In a passive inhibitory avoidance task, RU 486-treated rats were quicker to enter the dark chamber compared to controls, despite having received an aversive foot shock in that chamber previously. Both groups were equally highly motivated to enter the dark chamber on the first trial, and RU 486-treated rats had a significantly longer latency to enter the dark chamber on the second trial compared to the first trial, suggesting that memory for the aversive stimuli was intact. Rather, the findings suggest that RU 486 treatment impaired the ability to inhibit a motivated response (i.e. increased impulsivity) even in light of negative consequences.

Neonatal RU 486 treatment also impaired cognitive flexibility and increased perseveration in the attentional

set-shifting task. RU 486-treated animals were slower than controls to make the shift to the new rule and made significantly more perseverative errors, in effect continuing to apply the old rule. Interestingly, RU 486-treated animals required more trials to reach the criterion of learning the initial rule, suggesting that PR expressed in the developing hippocampus [8] or the mesocortical pathway may play a role in adult working memory as well. Nonetheless, both groups were performing at equal levels by the end of testing on the first day and made equivalent errors using the first rule on the beginning of the second day, suggesting that RU 486 did not affect retention of the initial rule. RU 486 is a potent PR antagonist, but it also acts as an antagonist at glucocorticoid receptors, even if with lower affinity. However, glucocorticoid receptor expression is relatively low in the neonatal hippocampus and cortex [64, reviewed in 65], suggesting that the effects of RU 486 in the present studies were most likely attributable to inhibition of PR activity specifically. Additionally, PRKO mice showed a similar deficit in cognitive flexibility on an adapted water maze task and also made more perseverative errors compared to controls. These results provide a convergence of evidence, using both pharmacological and genetic approaches, suggesting that PR activity during development is important for the normal development of mPFC-dependent cognitive behaviors.

Interestingly, the deficits induced by developmental PR inhibition are in many ways similar to those seen in a

variety of human behavioral disorders. Disruptions of normal development can lead to permanent alterations in mesocortical structure and function which can manifest in behavioral disorders such as attention deficit/hyperactivity disorder and schizophrenia [1–7], both of which are associated with deficits in cognitive flexibility and impaired impulse control. The present results implicate PR in the normal development of the circuits underlying these behaviors and raise the possibility that perturbations in the progesterone exposure or in the levels of PR expression during critical developmental windows may increase susceptibility to these disorders. Furthermore, these findings have implications for the use of synthetic progestins for the prevention of premature birth in at-risk pregnant women [reviewed in 66] and the potential effects on development of the fetal brain.

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The authors declare no competing financial interests.

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