The progesterone receptor (PR) is transiently expressed in the rat cortex during development and its expression is initiated in the developmentally critical layer, the subplate. As subplate neurons pioneer thalamocortical and corticofugal connectivity, the expression of PR in this layer suggests an important function for PR in cortical development. Using immunocytochemistry for PR, the present study determined the precise ontogeny of PR expression in subplate neurons. The number of cells containing PR immunoreactivity (PRir) within the subplate was quantified from embryonic day (E) 17 through postnatal day (P) 14. The subplate was positively identified by the marker calretinin and by BrDU birthdating. The results demonstrate that PRir is undetectable in fetal cortex on E17, but is first observed in the subplate on E18. The number of PRir cells peaks on P2 and then steadily declines, until PRir is once again not detectable in subplate by P14. This developmental window of PR expression within the subplate coincides with establishment of early cortical circuitry and the gradual demise of subplate cells, suggesting that PR may play a critical role in mediating these fundamental developmental processes.

Keywords: apoptosis, developing cortex, immunocytochemistry, steroid receptors, thalamocortical

Introduction

The mammalian neocortex is a complex laminated structure, divided into 6 layers in the adult, which are anatomically and functionally distinct (Uylings 2000). The mechanisms by which these layers are formed and the factors necessary to ensure proper afferent and efferent connectivity are not yet fully elucidated. However, growing evidence suggests that steroid hormone receptors, as powerful transcription factors, may play an important role in normal cortical development.

The subplate is one of the earliest formed layers of developing neocortex in mammalian brain and is necessary for appropriate cortical formation as demonstrated by developmental ablation studies (Ghosh and Shatz 1993; Lein et al. 1999; Grossberg and Seitz 2003; Kanold et al. 2003; Kanold and Shatz 2006). Subplate neurons are the first cortical neurons to become postmitotic (Wood et al. 1992; Allendoerfer and Shatz 1994) and their spontaneous activity in late embryonic and early postnatal development is important for establishing early synaptic circuitry and cortical columns. Subplate neurons are among the first cortical neurons to receive synaptic contacts from thalamus and send out "pioneer" axons that may serve as a scaffold for axons arising from neurons of more superficial layers (Konig and Marty 1981; De Carlos and O'leary 1992; McConnell et al. 1994).

The mechanisms by which subplate neurons ensure normal corticofugal and thalamocortical connections are still largely unknown. However, recent evidence suggests that steroid hormone receptors are expressed within the developing cortex (Miranda and Toran-Allerand 1992; Nunez et al. 2003) and may influence fundamental processes of cortical development such as apoptosis (Wade et al. 1999; Nunez et al. 2000), neurite outgrowth (Shughrue and Dorsa 1994), synapse formation (Hu et al. 2007), myelination (for review, see Schumacher et al. 2007), neuronal migration, and cell differentiation (for review, see Keyser 1983).

We have recently demonstrated that progesterone receptors (PRs) are expressed in the developing rat cortex in an anatomically and developmentally specific manner (Quadros et al. 2007; Lopez and Wagner 2009). PR immunoreactivity (PRir) is first detected in subplate on E18, then in layer 5 by P2, and in layers 2/3 by P7 and is expressed in non-γ-aminobutyric acidergic neurons within these layers (Lopez and Wagner 2009). Given the importance of subplate neurons to normal cortical development and the ability of steroid receptors to regulate fundamental processes of neural development, the present study examined the precise ontogeny of PR expression within the subplate. Subplate was positively identified using the marker calretinin and by BrDU birthdating. Results demonstrate that within subplate PR expression is transient and is initiated shortly after the layer is formed, thereby implicating PR in establishing cortical connectivity.

Materials and Methods

Animals

All animal procedures used in these experiments were approved by the Institutional Animal Care and Use Committee at the University at Albany, SUNY. All animals were housed in a temperature- and light-controlled room (10-h light, 14-h dark; lights on at 06:00 AM) with food and water available ad libitum. In all, 26 mated female Sprague-Dawley rats (60-80 days of age; Taconic Laboratories, Germantown, NY) were used to generate tissue for these experiments. For prenatal ages, litters were generated from 3 pregnant dams except for E17 age group, for which we used 4 pregnant dams. For postnatal ages, litters were generated from 13 additional dams and each postnatal age group had litters from at least 3 dams. The day copulatory plugs were found was designated as E1. Separately, 3 time-mated Sprague-Dawley females were used to generate litters for 5-bromo-2′-deoxyuridine (bromodeoxyuridine (BrDU) labeling of subplate neurons. Pregnant females were singly housed in plastic tubs with bedding and given food and water ad libitum. The day of birth was designated as P1.

Only the brains of male fetuses and pups were used for processing and analysis of PRir as previously published findings have reported no sex differences in subplate PR expression within primary motor cortex, somatosensory cortex, and binocular region of primary visual cortex (Lopez and Wagner 2009). Additionally, neither castration nor aromatase inhibition affected subplate PR levels in male pups (Jahagirdar et al. 2009).
To be consistent, BrDU immunoreactivity (BrDUir) was also carried out in male pups. Prenatally, fetuses were sexed as males by detecting the sry gene utilizing polymerase chain reaction (PCR) as described below and postnatal pups were sexed visually on the basis of their ano-genital distance.

**Tissue Preparation**

On the morning of E17, E18, E20, and E22, fetal brains were collected by performing cesarean sections on pregnant females. Briefly, pregnant females were administered a lethal dose of pentobarbital sodium (intraperitoneal [i.p.], Nembutal; Henry Schein, Melville, NY). 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 and then rapidly killed by decapitation. A small piece tail tissue was frozen at −20 °C for sry genotyping and the brain was removed and immersion fixed in 5% acrolein in 0.1 M phosphate buffer (PB; pH 7.6) overnight. For E17 and E18 pups, whole heads were immersion fixed in acrolein. Other pregnant females were allowed to deliver normally. Male neonates were anesthetized by hypothermia (P1, P2, P5) or given a lethal dose of pentobarbital sodium (P7, P10, and P14) and rapidly killed by decapitation. Brains were removed and immersion fixed as above. All brains were postfixed in 30% sucrose in 0.1 M PB (pH 7.6) for at least 24 h, until sectioned at 50 μm on a freezing rotary microtome in the coronal plane. In total, 3 additional P1 brains and 3 additional P5 brains were sectioned at 50 μm on rotary microtome in sagittal plane. The sections were stored in cryoprotectant (30% sucrose, 0.1% polyvinyl pyrrolidone-40 in ethylene glycol, and 0.1 M PB) at −20 °C until immunocytochemical processing as described below.

BrDU, which is incorporated into DNA in place of thymidine during the S-phase of cycling cells, was used to identify subplate cells, which are known to be generated in rat on E12 (≈E13, using E1 as the day of insemination) (Bayer and Altman 1990). Three time-mated Sprague-Dawley rats were injected i.p. with BrDU (50 mg/kg dissolved in 7 mM NaOH and 0.9% saline; Sigma-Aldrich, St, Louis, MO) on E13. Dams were allowed to deliver normally and fetal brains were collected at P2 and fixed as described above, until processed for immunocytochemistry for BrDU, according to previously published protocols (Al-Shamma and De Vries 1996).

**Sry Genotyping**

Genomic DNA was extracted from the frozen tails of embryonic pups by incubating the tails overnight in shaking water bath at 60 °C in a mixture of lysis buffer and proteinase K (10 μg/μl; Applied Biosystems, Foster City, CA). Genomic DNA was isolated and purified by a series of phenol-chloroform extractions precipitation with 100% ethanol and 3 F Chromatography. The DNA was resuspended in Tris-EDTA buffer and precipitated with 100% ethanol and 3 F. Genomic DNA was isolated and purified by a series of phenol-chloroform extractions precipitation with 100% ethanol and 3 F. Genomic DNA was extracted from the frozen tails of embryonic pups. Genomic DNA was isolated and purified by a series of phenol-chloroform extractions precipitation with 100% ethanol and 3 F. Genomic DNA was extracted from the frozen tails of embryonic pups. Genomic DNA was isolated and purified by a series of phenol-chloroform extractions precipitation with 100% ethanol and 3 F.

**PR Immunocytochemistry and Nissl Staining**

PR immunocytochemistry was performed as previously published (Quadros et al. 2007) using PR antisera (DAKO, Glostrup, Denmark), which is a rabbit polyclonal antibody directed against the DNA-binding domain of the human PR and detects both the A and B isoforms (Träsch and Wotz 1990). Briefly, sections were rinsed in 0.05 M Tris-buffered saline (TBS; pH 7.6) 3 times for 5 min to remove any residual cryoprotectant solution. Sections were then incubated in 1% sodium borohydride in TBS for 10 min, rinsed in TBS 5 times for 5 min each and then incubated in TBS containing 20% normal goat serum (NGS), 1% H2O2, and 1% bovine serum albumin for 20 min to block any nonspecific binding. PR antisera was diluted to 1:500 in TBS containing 2% NGS and 0.3% Triton X-100 for 72 h at 4 °C. Following 3 rinses (5 min each) in TBS containing 2% NGS, 0.3% Triton X-100, the sections were incubated for 90 min in biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA). After 2 rinses (5 min each) in TBS containing 2% NGS and 0.3% Triton X-100 and 2 rinses (5 min each) in TBS, the sections were incubated in AB reagent (Vectastain Elite Kit; Vector laboratories) for 60 min. Following 3 rinses (5 min each) in TBS, the sections were incubated in TBS containing 0.05% dianisobenzidine, 0.75 mM nickel ammonium sulfate, 0.15% β-glucose, 0.04% ammonium chloride, and 0.005% glucose oxidase for approximately 8–10 min. The sections were then rinsed 3 times (5 min each) in TBS and mounted on gelatin-coated microscope slides and allowed to dry. Following dehydration in increasing concentration of series of alcohol and delipidation, slides were coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). For Nissl staining, sections from 2 animals at each postnatal age, which were initially processed for PRir, were mounted on gelatin-coated microscope slides, stained with cresyl violet and then coverslipped with Permuent.

**Calretinin Immunocytochemistry**

Calretinin immunoreactivity has been previously documented within the subplate, marginal zone, and the cortical plate. Calretinin labeling is the most prominent in the subplate prenatally from E17 to E20 and postnatally from P0 to P3 (Fonseca et al. 1995). However, postnatally, calretinin staining markedly increases in other cortical layers. Hence, to properly delineate subplate layer, using calretinin as a marker, E20, a prenatal time point, was chosen. Calretinin immunocytochemistry was performed on brain sections from 3 E20 animals using affinity-purified anti-calretinin (1:500 for 72 h at 4 °C) developed in rabbit using synthetic peptide corresponding to the C-terminal region of rat calretinin (amino acids 194–209) (Sigma, St, Louis, MI, catalogue number C 7479). This sequence is specific for calretinin and is not found in other calcium-binding proteins like calbindin and parvalbumin. All the steps involved in calretinin immunocytochemistry are similar to those in PR immunocytochemistry except that calretinin-ir was visualized using the chromogen Vector SG (Vector SG Substrate Kit for Peroxidase; Vector Laboratories), which produced a blue-gray reaction product. Sections were mounted and coverslipped with Permament.

**Immunohistochemical Specificity**

PR antibody specificity within rat isocortex has been reported in detail by our laboratory previously in Lopez and Wagner (2009). Nuclear immunoreactivity was completely eliminated in all cortical areas when sections were incubated with PR antisera that had been preabsorbed with the antigen peptide (amino acids 533–547) or with human PRa and PRb. It was also demonstrated, using this antisera, that PRir is absent in brain sections from PR knockout mice (Veronica Lopez, Keith Lance Gonzales, and CKW, unpublished observation). In the present study, omission of primary antisera abolished nuclear PR staining (Fig. 1). The specificity of the BrDU antisera has been tested previously by Al-Shamma and De Vries (1996), reporting a lack of nuclear staining in
brain sections from animals not exposed to BrDU. Specificity of the anti-calreitin antisera has been previously reported by Irintchev et al. (2005) and by Yau et al. (2003).

Analyses of PRir
Two prenatal (Paxinos et al. 1994; Altman and Bayer 1995) and one adult (Paxinos and Watson 1998) rat brain atlases were used as points of reference to select a representative coronal brain section, at the level of somatosensory cortex, which was then matched across groups. The number of PRir nuclei (dark brown or intense black) within the entire dorsal-ventral extent of the subplate was counted bilaterally in each representative section at all ages (Fig. 2). Animals that did not have intact bilateral sections were omitted from the analyses. Raw cell counts were corrected using the Abercrombie Correction method as in Guillery (2002) and as previously published by our laboratory (Quadros et al. 2007). Briefly, raw cell counts for each animal at each age were multiplied by the correction factor determined by using the formula \( \frac{T}{T+h} \), where \( T \) = section thickness and \( h \) = mean nuclear diameter. For each age, mean nuclear diameter was determined using 3 randomly selected animals. From each animal, the diameter of 5 randomly selected PRir nuclei was measured from each hemisphere using NIH Image, for a total of 30 nuclei, to generate an average nuclear diameter for each age group. Using one-way analysis of variance (ANOVA), it was determined that there were not significant changes in the size of counted PRir nuclei across ages (E18 = 6.9 µm, ±0.22; E20 = 7.1 µm, ±0.14; E22 = 7.0 µm, ±0.08; P1 = 6.7 µm, ±0.12; P2 = 6.5 µm, ±0.10; P5 = 7.3 µm, ±0.23; P7 = 7.4 µm, ±0.10; P10 = 7.4 µm, ±0.12; F = 2.206). The numbers of PRir cells (corrected bilateral cell counts) were compared across ages using one-way ANOVA. Preplanned post hoc comparisons were conducted using Student-Newman-Keuls (\( P < 0.05 \)).

Results
PRir was observed within the subplate from just lateral to the cingulate cortex and throughout the ventral extent of the subplate (Fig. 2). Examination of PRir in sagittal sections revealed that PRir is found within the subplate throughout the rostrocaudal extent of developing cortex, from frontal cortex through visual cortex (Fig. 3). The corrected bilateral cell counts for PRir nuclei, within subplate of the chosen section, prenatally (E17, E18, E20, E22) and postnatally (P1, P2, P5, P7, P10, and P14) are depicted in Figure 4. There were significant differences in PR staining across ages (\( F = 124.656; P < 0.001 \)). PRir was first detected within a few cells of the subplate on E18. On E20, the number of PRir nuclei increases significantly compared with E18 (\( P < 0.001 \)). Subplate PR expression significantly increased by E20 and P1 reaching peak values on P2. The number of PRir nuclei on P2 is significantly higher than all other ages examined (\( P < 0.001 \)). After P2, the number of PRir nuclei significantly declined through P10 (P2 > P5 > P7 > P10; \( P < 0.001 \)). PRir was no longer detectable by P14. It is possible that the decrease in PRir cell number between P5 and P14 could be attributable to sampling cell number in a section through a structure that is expanding in volume across ages as the cortex grows. However, as PRir is completely abolished by P14 from the subplate (PRir cell number goes to zero), the decrease in PRir cells counted within the subplate must represent a true decline in the total number of PR-expressing cells. Examples of PRir in the subplate at P2 and P7 are shown in Figure 5.

Nissl-stained sections were utilized to identify the subplate layer cytoarchitecturally, and Nissl-PR-stained sections

Figure 1. Immunocytochemical controls for PR polyclonal antisera. Alternate representative coronal sections of P2 brain were incubated (A) in the presence of the primary antisera or (B) with primary antisera omitted. Omission of primary antisera abolished all immunoreactivity within the subplate. Bar = 100 µm.

Figure 2. The distribution of PRir nuclei, within the entire unilateral subplate region within a representative coronal section at the level of somatosensory cortex of P2 male. Black dots represent individual PRir nuclei.
confirmed that PR staining was, indeed, within the subplate (data not shown). That the expression of PR is, indeed, within the subplate was confirmed further by the incorporation of BrDU on E13, the known date of subplate cell genesis (Bayer and Altman 1990) and by the visualization of the calcium-binding protein, calretinin immunoreactivity, a reliable marker of subplate (Fonseca et al. 1995). In the present study, calretinin immunoreactivity was clearly present in the subplate at E20 but less evident at older ages. PRir nuclei, calretinin-immunoreactive cells, and BrDU-labeled cells were all located within the same cortical layer, indicating that PRir is, indeed, expressed in cells of the cortical subplate (Fig. 6).

Discussion

During development, steroid receptors are expressed in brain areas associated with cognitive function, in addition to brain regions with reproductive or neuroendocrine function. For example, estrogen and androgen receptors are expressed in the rodent neocortex during perinatal development (Miranda and Toran-Allerand 1992; Yokosuka et al. 1995; Nunez et al. 2003). Likewise, the present results demonstrate that PR is expressed within subplate, beginning as early as E18, reaching peak levels on P2 and decreasing significantly thereafter, until PR is no longer detectable by P14. PR expression within the subplate proper was confirmed by the expression of calretinin, an early marker for subplate cells, and identifying subplate cells by their E13 birthdate using BrdU. The transient expression of PR within a developmentally critical layer of subplate suggests that PR plays a fundamental role in the development of cortex. These findings are consistent with previous studies by Kato et al. (1984) and Hagihara et al. (1992) that document the presence of PR and its mRNA in the developing cortex. However, the use of immunocytochemistry in the present study affords the cellular level resolution necessary to identify PR within specific lamina, namely the subplate. This information is integral to generating hypotheses regarding a function for PR in cortical development.

The onset of PR expression at E18 within the subplate coincides with the arrival of thalamocortical afferents within this layer in the rat (Molnar et al. 1998). Furthermore, PR expression steadily increases at a time when thalamic axons establish transient synaptic connections with subplate neurons during a temporary “waiting” period. Presumably thalamic axons receive chemotrophic signals from postsynaptic subplate cells regarding their final target destination. Indeed, thalamocortical connections are aberrant in fetal and neonatal cats in which the subplate has been ablated (Ghosh and Shatz 1993). The timing of PR expression in subplate is consistent with the idea that PR may play a role in thalamic-subplate synaptogenesis and subsequent target finding by thalamic axons as they innervate the appropriate cortical layers.
Steroid hormones are known to profoundly affect connectivity within the brain by 1) regulating the expression of trophic factors that act as guidance cues to the growth cones of the growing axons and 2) altering synaptogenesis either by increasing dendritic spines or facilitating axonal transport (for review, see Simerly 2002, 2005). There is evidence to support the role of progesterone in mediating each of the developmental processes involved in establishing connectivity. In a rat model of spinal cord injury, progesterone treatment upregulated brain-derived neurotrophic factor expression within the ventral horn motoneurons, presumably providing some form of neuroprotection (Gonzalez et al. 2005). Progesterone treatment also restores axonal transport and muscle strength in a genetic model of motoneuron disease—the Wobbler mouse (Gonzalez Deniselle et al. 2005). Additionally, in cerebellar Purkinje cells, progesterone promotes dendritic growth, the genesis of dendritic spines and synaptogenesis through a PR-dependent mechanism (Sakamoto et al. 2003). Taken together, these data provide a strong rationale for the idea that PR expression within the subplate may regulate the expression of key chemotrophic molecules that serve as postsynaptic guidance cues for thalamic afferents, thereby mediating proper thalamocortical connectivity.

The fate of subplate cells in rats remains controversial, but it has been demonstrated that between P2 and P20, 50% of subplate neurons are eliminated and by P20 most BrDU-labeled subplate cells appear pyknotic, suggesting that the cells are lost by apoptosis (Ferrer et al. 1990; Robertson et al. 2000). However, as subplate cells are not completely eliminated, it is likely that some subplate neurons persist into adulthood as a distinct layer (Woo et al. 1991; Valverde et al. 1995; Robertson et al. 2000).

The decline in the number of PR-expressing subplate neurons observed in the present study, which occurs between P5 and P14, may reflect the demise of a specific population of subplate neurons, initiated by a PR-dependent mechanism. Interestingly, progesterone can prevent or promote cell death (depending on the tissue in which its receptors are expressed), by altering expression of proapoptotic and anti-apoptotic regulatory proteins like bax and bcl-2, respectively (Amezcue et al. 1999, 2000; Bozdogan et al. 2002; Jacobsen et al. 2005; Hofling et al. 2008; Yang et al. 2008). Given that PR appears to be expressed in the majority of subplate neurons, it seems more likely that PR promotes cell death in this transient structure. The pruning of pioneer neurons may be extremely critical for elimination of cortical circuits that, if permitted to persist into adulthood, would continue to be foci of repetitive electrical discharges. This has lead researchers to suggest that any anomalies in normal regressive processes, like apoptosis, within the oldest layers of developing cortex, may give rise to epileptic foci (Luhmann et al. 2003). Given the immense clinical relevance of such an outcome, the potential role of PR in mediating apoptosis within the subplate needs to be investigated further.

To date, not much is known about the role of PR within the developing cortex. However, findings from the present study lead to interesting, exciting, and testable hypotheses about the role of PR in 1) establishing early thalamocortical connectivity and 2) mediating apoptosis within the subplate. Interestingly, synaptic connectivity and apoptosis are considered to be important neural substrates for mediating cognitive processes. Thus, progesterone and its receptor may play an important role in the normal development of cognitive function. This is clinically very relevant as the practice of administering progesterone to pregnant women (for preventing premature births) has increased dramatically in recent years, and surprisingly, the effects of such an exposure on the neonatal cortical development are not well characterized (Ness et al. 2006). Lastly, the expression of PR within the subplate neurons, documented in the present study, to span all functional cortical regions and without an obvious overlap between the adjacent cortical layer VI or the white matter, from E18 to P10, cleanly delineates this layer and makes it a valuable marker of the subplate cells.

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**Notes**

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