Sexual differentiation of the human and rodent forebrain
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CHAPTER SIX

EFFECTS OF PROGESTIN RECEPTOR ANTAGONISTS ON APOPTOSIS IN THE DEVELOPING MEDIAL PREOPTIC AREA IN THE RAT BRAIN

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The sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rat brain is much larger in males than in females and is one of the best examples of involvement of testosterone-dependent apoptosis during sexual differentiation. The incidence of apoptosis in the developing SDN-POA is higher in females than in males, which is significantly attenuated with testosterone or its estrogenic metabolite estradiol on the day of birth. The mechanism(s) that underlie this protective effect are not known. Progesterin receptors (PR) in SDN-POA may be important in this process, because ligand-bound PRs prevent apoptosis in endometrial cells derived from the uterus, while PR antagonists: ZK 98,299 or RU 486 negate this protective effect. Indeed, the number of PR containing SDN-POA cells is much higher in males than in females. Therefore, we hypothesized that testosterone-derived estradiol protect male SDN-POA cells by increasing PR expression during early development. This was tested by comparing the incidence of apoptosis and the volume of the SDN-POA between male and female rat pups on postnatal day (PN) 8, which were injected daily until PN 7 with vehicle, ZK 98,299 or RU 486. In vehicle-treated animals, the incidence of apoptosis was higher in females than in males and the SDN-POA volume was larger in males than in females. Although PR antagonist treatment did not significantly affect the incidence of apoptosis or SDN-POA volume, post hoc tests showed that neither the incidence of apoptosis nor the SDN-POA volume significantly differed between males and females. The absence of any sex differences in the incidence of apoptosis or SDN-POA between PR antagonist-treated males and females indicate that postnatal treatment with ZK 98,299 or RU 486 affects sexual differentiation of the SDN-POA in an unexpected fashion.

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**INTRODUCTION**

Sexually dimorphic levels of circulating testosterone have profound permanent effects on rat brain development, which are clearly illustrated in the sexually dimorphic nucleus of the preoptic area (SDN-POA) of the medial preoptic nucleus (MPN). The SDN-POA in the rat brain is several times larger and contains more neurons in males than in females (Gorski *et al.*, 1978; Simerly *et al.*, 1985; Davis *et al.*, 1996). Lesion studies suggest that the SDN-POA may be involved in certain aspects of male sexual behavior, such as control of penile erection, intromission, mounting and sexual orientation (MacLean and Ploog, 1962; Turkenburg *et al.*, 1988; De Jong *et al.*, 1989; Paredes and Baum, 1995; Meisel and Sachs, 1994). Because SDN-POA is also highly interconnected with the anteroverventricular nucleus, arcuate nucleus and paraventricular nucleus, which are involved in the cyclic release of gonadotropins, it may also be involved in female sexual behavior (Simerly and Swanson, 1986; Simerly and Swanson, 1988; De Vries and Simerly, 2002).

Testosterone is first converted by aromatase into estradiol, before it facilitates the sex-dependent development of the rat SDN-POA. Indeed, SDN-POA volume is increased in both males and females after neonatal testosterone and/or estradiol treatment, an effect which can not be mimicked by the non-aromatizable androgenic metabolite of testosterone, dihydrotestosterone (Gorski *et al.*, 1978; Döhler *et al.*, 1982; Döhler *et al.*, 1984; Jacobson *et al.*, 1984; Davis *et al.*, 1996). Moreover, the increase in SDN-POA volume caused by neonatal testosterone can be prevented by aromatase inhibitors or antiestrogens (Döhler *et al.*, 1986; Houtsmuller *et al.*, 1994). Several studies showed that testosterone or its metabolite estradiol attenuate the incidence of apoptosis in the developing postnatal rat SDN-POA, which is higher in females than in males (Arai *et al.*, 1995; Davis *et al.*, 1995; Chung *et al.*, 2000). Perinatal testosterone or estradiol treatment markedly decreased the incidence of apoptosis in the female SDN-POA to that found in the male SDN-POA, whereas neonatal castration increased the incidence of apoptosis in the male SDN-POA to that found in the female SDN-POA (Arai *et al.*, 1996; Davis *et al.*, 1995; Chung *et al.*, 2000). These data suggest that male SDN-POA cells are protected against apoptosis by the presence of testosterone-derived estradiol during early perinatal development.

Recent studies showed that the number of progestin receptor immunoreactive (PR-IR) cells in the perinatal rat SDN-POA are much higher in males than in females.
Effects of PR Antagonists

(Wagner et al., 1998). This sex difference in the number of PR expressing SDN-POA cells depends on the presence of perinatal levels of testosterone or estrogens (Wagner et al., 1998; Wagner et al., 2001). In vitro studies showed that ligand-activated PRs protected endometrial cells derived from the uterus against apoptosis, which was negated by treatment with PR antagonists: ZK 98,299 (onapristone) or RU 486 (mifepristone) (Pecci et al., 1997). Furthermore, PRs have been shown to induce overexpression of Bcl-XL, which is an apoptosis inhibiting factor (Pecci et al., 1997; Tsujimoto and Shimizu, 2000). Therefore, we hypothesized that testosterone-derived estradiol may protect male SDN-POA cells by increasing PR expression during early development. This hypothesis was tested by blocking PRs in male and female rat pups with PR antagonists: ZK 98,299 or RU 486 from postnatal day (PN) 1 (is day of birth) until PN 7. The incidence of apoptosis and SDN-POA volume was compared between males and females on PN 8 for significant differences.

MATERIALS AND METHODS

Animals

Sprague-Dawley rat pups were born of dams (Taconic Farms, Germantown) mated in our own animal facilities in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. Rats were kept at a 10 h light: 14 dark cycle throughout the study and fed ad libitum. Rat pups were sexed by examining the anogenital distance on postnatal day (PN) 1 (day of birth). Litters from all dams were culled to six pups (3 males and 3 females). For our studies we used steroidal PR antagonists: ZK 98,299 and RU 486 in dosages, which have been shown to be effective in blocking female sexual behavior (Auger et al., 1997). The antagonistic effects of ZK 98,299 are conveyed in a different manner than that of RU 486. ZK 98,299 is a type I PR antagonist, which promotes conformational change in PR distinct from the type II PR antagonist RU 486 (Henderson, 1987; Clemm et al., 1995; Allan et al., 1996). Moreover, RU 486 stimulated phosphorylation at hormone-dependent sites on PRs, while ZK 98,299 does not (Beck et al., 1996). Male and female pups were treated daily until PN 7 with a subcutaneous injections of: ZK 98,299 (Schering, 8 mg/0.01cc/g body weight, n = 6 males, n = 6 females) or RU 486 (Sigma, 20 mg/0.01cc/g body weight, n = 9 males, n = 9 females) or an equal volume of vehicle (sesame oil, n = 9 males, n= 8 females) and sacrificed on PN 8.
Brain tissue processing and cresyl violet staining

Animals were anesthetized with choral hydrate/pentobarbital and decapitated. The brain was removed from the skull and immersion-fixed in 5% acrolein made in 0.1 M phosphate buffer (PB) pH 7.4. overnight and stored in 0.05 M Tris-buffered saline (TBS), pH 7.6 at 4°C. Brains were dehydrated in increasing grades of ethanol followed by toluene and embedded in paraffin wax. Transverse serial sections (15 μm) were made with a rotary microtome and mounted on gelatine-coated glass slides. The sections were deparaffinized using HemoD (Fischer Scientific), rehydrated with decreasing grades of ethanol followed by Rho-purified H2O, stained with cresyl violet, dehydrated and coverslipped using permount (Fischer Scientific).

Estimation of total volume and incidence of apoptosis

The volume of the MPNc and the incidence of apoptosis were estimated as described earlier in Chung et al., 2000. The boundaries of the SDN-POA were identified according to the atlas of the developing rat brain by Alvarez-Bolado and Swanson (1996) using bright field microscopy (BH-2 microscope; Olympus, Lake Success, NY) with a 10X objective. Digital images from every section through the SDN-POA were taken with a CCD72 camera (Dage; MTI. Michigan City, IN) attached to a Quick Capture frame grabber board (Data Translation, Marlboro, MA) in a Macintosh IIFx computer. The Scion IMAGE program v.1.57 developed by Dr. Rasband at the National Institutes of Health was used to measure the bilateral cross-sectional areas through the SDN-POA. The total SDN-POA volume was calculated by multiplying the sum of all the cross-sectional areas with 15 μm (i.e, thickness of each section) (Gundersen et al., 1988).

Apoptotic nuclei identified by intense staining, condensation, and often fragmentation of nuclear material, were counted in every other section with a 40X objective (Fig. 1). The total bilateral number of apoptotic nuclei in the SDN-POA was estimated by multiplying the total number of apoptotic cell nuclei counted in all sections through the SDN-POA by two (Königsmark and Murphy, 1970; Collado et al., 1998, Chung et al., 2000). The term “incidence of apoptosis” was used to describe the total number of apoptotic nuclei per μm³.

Statistical analysis

The data were analyzed for significant differences in the incidence of apoptosis
and SDN-POA volume using a two-way analysis of variance (ANOVA) with treatment and sex as between-subject variables. Student-Newman-Keuls tests were used for post hoc analysis. Differences were considered significant if \( p < 0.05 \). All measurements were conducted without knowledge of sex and treatment.

**RESULTS**

**Vehicle vs. ZK 98,299 effects**

Overall, the incidence of apoptosis in the SDN-POA was significantly higher in vehicle-treated females than in vehicle-treated males \( [F(1, 28) = 5.7, p < 0.05; \text{Fig. 2 A}] \). However, the incidence of apoptosis in the SDN-POA was not significantly affected by ZK 98,299 treatment, nor was there a significant interaction with sex. In addition, post hoc analysis showed that the incidence of apoptosis in ZK 98,299-treated males did not significantly differ from ZK 98,299-treated females.

Overall, the volume of the SDN-POA was significantly higher in vehicle-treated males than in vehicle-treated females \( [F(1, 28) = 6.6, p < 0.05; \text{Fig. 2 B}] \). However, the volume of the SDN-POA was not significantly affected by ZK 98,299 treatment, nor was there a significant interaction with sex. In addition, post hoc analysis showed that the SDN-POA volume in ZK 98,299-treated males did not significantly differ from ZK 98,299-treated females.

**Vehicle vs. RU 486 effects.**

Overall, the incidence of apoptosis in the SDN-POA was significantly higher in vehicle-treated females than in vehicle-treated males \( [F(1, 34) = 7.7, p < 0.05; \text{Fig. 2 C}] \). However, the incidence of apoptosis in the SDN-POA was not significantly affected by RU 486 treatment, nor was there a significant interaction with sex. In addition, post hoc analysis showed that the incidence of apoptosis in RU 486-treated males did not significantly differ from RU 486-treated females.

Overall, the volume of the SDN-POA was significantly higher in vehicle-treated males than in vehicle-treated females \( [F(1, 34) = 15.7, p < 0.001; \text{Fig. 2 D}] \). However, the volume of the SDN-POA was not significantly affected by RU 486 treatment, nor was there a significant interaction with sex. In addition, post hoc analysis showed that the SDN-POA volume in RU 486-treated males did not significantly differ from RU 486-treated females.
Figure 1. Effects of PR antagonists on the incidence of apoptosis in the SDN-POA on postnatal day 8. Note that the sex difference in apoptosis is not present in animals treated with A) ZK 98,299 or B) RU 486.

DISCUSSION

Sexual differentiation of the SDN-POA in the rat brain depends on testosterone-derived estradiol regulated apoptosis (Arai et al., 1996; Davis et al., 1996; Chung et al., 2000). In agreement with these earlier studies, we showed that the incidence of apoptosis is higher in vehicle-treated females than in vehicle-treated males, which was also reflected by the smaller SDN-POA volume found in females as compared to males. Even though statistical analysis showed no significant effects of PR antagonist on the incidence of apoptosis or volume of the SDN-POA, neither the incidence of apoptosis nor SDN-POA volume differed between male and female animals treated with PR antagonists. The absence of any sex differences in the incidence of apoptosis or SDN-POA between PR antagonist-treated males and females suggests
that postnatal treatment with ZK 98,299 or RU 486 could have affected sexual differentiation of the SDN-POA, albeit in an unexpected fashion.

Figure 2 Effects of PR antagonists on SDN-POA volume on postnatal day 8. Note the sex difference in SDN-POA volume is not present in animals treated with A) ZK 98,299 or B) RU 486.

Cresyl violet staining was used to reveal the typical darkly stained and condensed profiles of apoptotic nuclei instead of the terminal deoxynucleotidyl nick-end labeling (TUNEL), which was used in earlier studies studying sexual differentiation (Arai et al., 1996; Davis et al., 1996; Park et al., 1998; Chung et al., 2000). Because cresyl violet does not exclusively stain apoptotic nuclei, some apoptotic cells may theoretically be masked by cresyl violet staining in neighboring non-apoptotic cells. However, this does not seem to be a major problem because many studies showed that the number of apoptotic cells detected with cresyl violet or a similar histological staining strongly correlates with the number of apoptotic cells as visualized by the TUNEL
method (e.g., Rabacchi et al., 1994; Bonfanti et al., 1996; Park et al., 1998). Visualization of apoptotic cells using TUNEL relies on the presence of condensed and fragmented cell nuclei and is therefore not a specific marker for apoptosis, but a marker for DNA damage (Wijsman et al., 1994; Lucassen et al., 1996).

Perinatal activation of the hypothalamus-pituitary-adrenal (HPA) axis decreased the sex difference magnitude of the SDN-POA in the rat brain (Anderson et al., 1985). Prenatal immobilization or nutritional stress on pregnant female rats attenuated the SDN-POA sex difference between male and female offspring. Stress in pregnant female rats reduced the volume of the SDN-POA in the male offspring by about 50% as compared to control males, while not affecting the SDN-POA volume in the female offspring (Anderson et al., 1985). The absence of sex differences in the incidence of apoptosis or SDN-POA volume in ZK 98,299 or RU 486–treated animals may be due to the stress induced by the daily injections. However, activation of the HPA axis does not seem to be involved, because both the incidence of apoptosis and SDN-POA volume in animals injected daily with vehicle remained significantly different between males and females.

Earlier pharmacological studies showed that both ZK 98,299 and RU 486 can act as glucocorticoid receptor (GR) antagonists because of their affinity for GRs, albeit lower than that for PRs (Henderson, 1987). Therefore, both ZK 98,299 and RU 486 may have had an effect on the incidence of apoptosis and/or SDN-POA volume through GRs. Indeed, removal of the adrenals, the primary source of corticosteroids, in adulthood induced apoptosis in the hippocampus (Sloviter et al., 1993), which was prevented by corticosteroid replacement immediately after adrenalectomy (Hu et al., 1997). However, this is not a likely scenario because the neonatal rat pup is hyporesponsive to corticosteroids (De Kloet et al., 1988; Vasquez et al., 1998). Moreover, the expression of GR mRNA are low in the neonatal rat hypothalamus, except in the paraventricular nucleus, supraoptic nucleus and suprachiasmatic nucleus (Rosenfeld et al., 1988; Van Eekelen et al., 1991; Yi et al., 1994).

PR antagonists were expected to mainly affect the incidence of apoptosis in the male SDN-POA, because males have far more PR-IR SDN-POA cells than females during perinatal development. However, PR antagonist-treated animals showed an absence of sex differences in the incidence of apoptosis and SDN-POA, which seems to be primarily caused by a subtle decrease in the incidence of apoptosis and a subtle increase in the volume of the female SDN-POA, whereas no changes in either para-
meters were observed in males (see Fig. 2). These results may be explained by the partial estrogenic effects that are exerted by progestin antagonists (Jeng et al., 1993; Bigsby et al., 1994; Dibbs et al., 1995). For instance, RU 486 mimicked estrogen-induced increase of oxytocin receptors in uteri of ovariectomized rats, which was effectively inhibited by simultaneous treatment with the pure antiestrogen ICI 182, 780 (Dibbs et al., 1995). Moreover, in absence of estradiol in vitro RU 486 treatment significantly increased the expression of an estrogen response element-containing reporter gene by binding to estrogen receptors (ER: Dibbs et al., 1995). Similar estrogenic effects have been attributed to ZK 98,299 (Bigsby et al., 1994). Activation of ERs by testosterone-derived estradiol has been presumed to convey the protective effects of testosterone, therefore, it is possible that the partial estrogenic effects of both ZK 98,299 and RU 486 may have decreased the incidence of apoptosis in the female SDN-POA. Indeed, both males and females contain high levels of mRNA and protein for ERs in the SDN-POA (DonCarlos and Handa, 1994; Yokosuka et al., 1997), which may have interacted with the PR antagonists. The estrogenic effects of PR antagonists may have had little or no effect on the male SDN-POA, because the male animals were not castrated, and therefore, contained circulating levels of testosterone already protecting SDN-POA cells against apoptosis. Alternatively, PR antagonists may affect circulating serum levels of testosterone or estradiol, which in turn could attenuate the magnitude of the sex difference in the incidence of apoptosis and SDN-POA volume. Serum levels of testosterone and estradiol were increased in rats and in pregnant or post-menopausal women treated with RU 486 (Lamberts et al., 1991; Wang et al., 1994; Ruiz et al., 1997; Heikinheimo et al., 2000). These subtle effects of either PR antagonists may be the basis for the absence of sex differences in apoptosis and SDN-POA volume in animals treated with either ZK 98,299 or RU 486.

Progestin receptors were hypothesized to mediate the protective effects of testosterone or its metabolite estradiol during the sexually dimorphic development of the rat SDN-POA. However, since neither ZK 98,299 nor RU 486 postnatal treatment significantly affected the incidence of apoptosis or SDN-POA volume, it is possible that testosterone or its metabolite estradiol may not act through PR to protect SDN-POA cells against apoptosis. This could be the case, since neonatal treatment with progesterone does not markedly change the size of the SDN-POA (Gorski et al., 1978). Moreover, aromatase inhibitors and estradiol antagonist in early development
were very effective in preventing the sexually dimorphic organization of the SDN-POA (Döhler et al., 1984; 1986; Houtsmuller et al., 1994). Therefore, our results reiterate the idea that the sex-dependent organization of the rat SDN-POA is mainly regulated by direct effects of testosterone or its metabolite estradiol (Arai et al., 1996; Davis et al., 1996; Chung et al., 2000), which act independent from PRs. Alternatively, PR antagonist treatment given only during early postnatal development may not have been sufficient to prevent possible PR effects on prenatal SDN-POA development. Studies from this laboratory showed that the number of PR-IR cells in the SDN-POA is already sexually dimorphic from embryonic day 19 onwards coinciding with the peak in testosterone levels found only in male rat fetuses (Wagner et al., 2000). Earlier studies showed that the increase of the female SDN-POA volume comparable to that found in males requires the presence of both pre- and postnatal testosterone or its metabolite estradiol (Döhler et al., 1984; Tarttelin and Gorski, 1988). Therefore, in order to affect sexual differentiation of the SDN-POA, both prenatal and postnatal treatment with ZK 98,299 or RU 486 may be required. However, this idea cannot be verified directly using PR antagonists, because PR binding by its ligand progesterone is essential for the maintenance of pregnancy.

Although the present study did not find any marked effects of PR antagonists during postnatal SDN-POA development in relation to apoptosis and volumetric changes, PR antagonists do seem to affect the regulation of behavioral processes, such as sexual and fearful behaviors in adulthood (Lonstein et al., 2001). RU 486 treatment in first ten postnatal days affected behavioral functions. Male sexual behavior was decreased by RU 486 treatment, while having no effect on female sexual behavior. Fear response in both males and females treated with RU 486 in neonatal development was also reduced. Therefore, antiprogestins during early postnatal development can have influenced the organization of the developing rat brain, which may not be reflected by the incidence of apoptosis or SDN-POA volume. For instance, marked changes may have occurred in the number of synaptic contacts, dendritic field and/or neurochemical phenotype, which are not reflected by the number of apoptotic cells or SDN-POA volume. The absence of any sex differences in the incidence of apoptosis or SDN-POA between PR antagonist-treated males and females indicate that postnatal treatment with ZK 98,299 or RU 486 affects sexual differentiation of the SDN-POA in an unexpected fashion.
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