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# 3α,5α-THP mediates progestins' effects to protect against adrenalectomy-induced cell death in the dentate gyrus of female and male rats

Madeline E. Rhodes[a], Cheryl M. McCormick[b], Cheryl A. Frye[a,c,d,*]

[a]*Department of Psychology, University at Albany-SUNY, Social Science 220, 1400 Washington Avenue, Albany, NY 12222, USA*
[b]*Department of Psychology, Bates College, Lewiston, ME 04240, USA*
[c]*Department of Biological Sciences, The University at Albany-SUNY, 1400 Washington Avenue, Albany, NY 12222, USA*
[d]*The Center for Neuroscience Research, The University at Albany-SUNY, 1400 Washington Avenue, Albany, NY 12222, USA*

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**Abstract**

Progestins have neuroprotective effects in several in vitro models of neurodegeneration and in vivo in seizure models. The extent to which progesterone's in vivo protective effects may generalize to models not involving seizure processes and whether progesterone's protective effects are modulated by its metabolites have not been comprehensively investigated. The present experiments investigated the effects of progesterone and its metabolites, dihydryoprogesterone (DHP) and 5α-pregnan-3α-ol-20-one (3α,5α-THP), to protect the hippocampus from damage induced by adrenalectomy (ADX). In Experiments 1 and 2, progesterone, DHP, or 3α,5α-THP administration (1 mg/kg sc) to female (Experiment 1) or male (Experiment 2) rats similarly reduced the total number of ADX-induced pyknotic cells in the dentate gyrus compared with vehicle administration. In Experiment 3, blocking progesterone's metabolism to 3α,5α-THP with coadministration of a 5α-reductase inhibitor, finasteride (10 mg/kg sc), in female rats attenuated progesterone's protective effects on cell death in the dentate gyrus. Together, these data suggest that progestins can protect against ADX-induced cell death and that the actions of the progesterone metabolite, 3α,5α-THP, may underlie these effects.


*Keywords:* Progesterone; Hippocampus; Nongenomic; Neurodegeneration; Cell death

## 1. Introduction

Progestins may have neuroprotective effects. In vitro, progesterone protects against glutamate toxicity (Nilsen and Brinton, 2002; Ogata et al., 1993) and increases antioxidant enzyme activity (Pajovic et al., 2003). In vivo, progesterone protects motoneurons following axotomy (Yu, 1989) and dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) toxicity (Callier et al., 2001) and prevents neuron loss in the brain following various insults (Asbury et al., 1998; Murphy et al., 2002; Roof et al., 1994; Stein and Hoffman, 2003). Notably, progesterone also decreases seizure activity and protects the brain from damage produced by seizures. In rodent models, progesterone reduces seizure susceptibility (Frye and Bayon, 1999; Frye et al., 2002; Frye and Scalise, 2000; Hoffman et al., 2003; Lonsdale and Burnham, 2003; Selye, 1942), and this effect is associated with decreased damage in the hippocampus (Frye and Bayon, 1999; Hoffman et al., 2003). Together these data suggest that progesterone can have neuroprotective effects in animal models of neurodegeneration.

Progesterone's protective effects may be due, in part, to the actions of its metabolites. Progesterone is produced by peripheral glands, such as the ovaries and adrenals, and is also synthesized in glial cells from cholesterol. Progesterone can be metabolized by 5α-reductase to dihydroprogesterone (DHP). DHP is then further reduced by 3α-hydroxysteroid dehydrogenase to 5α-pregnan-3α-ol-20-one (3α,5α-THP; Mellon and Griffin, 2002; Stoffel-Wagner, 2001, see Fig. 1).

* Corresponding author. Department of Psychology, University at Albany-SUNY, Social Science 220, 1400 Washington Avenue, Albany, NY 12222, USA. Tel.: +1-518-442-4836; fax: +1-518-442-4867.
*E-mail address:* cafrye@albany.edu (C.A. Frye).

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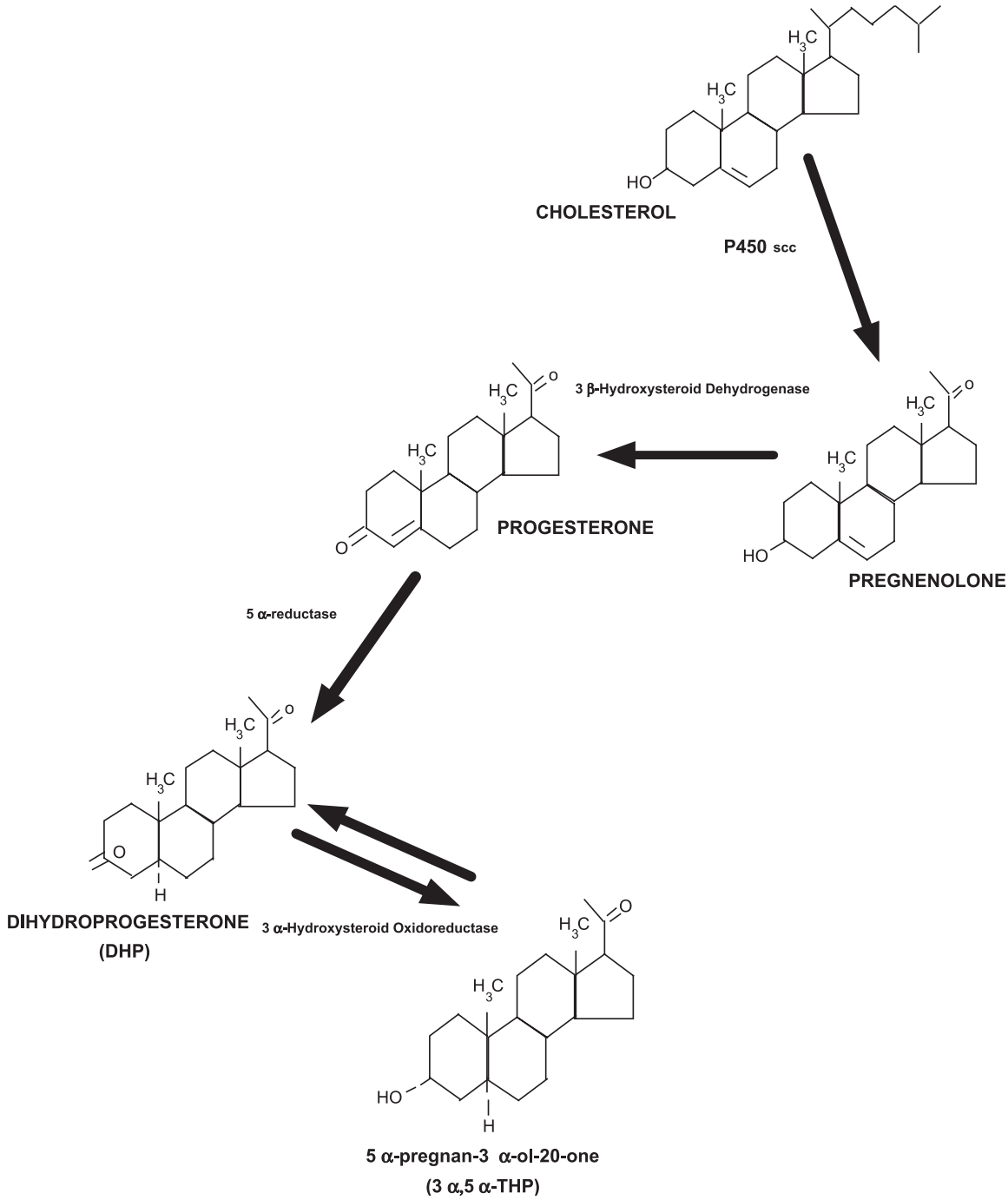


Fig. 1. In the brain, cholesterol can be converted to pregnenalone via actions of the P450 side-chain cleavage (P450scc) enzyme. Pregnenalone can serve as a prohormone for the production of progesterone via the actions of 3β-hydroxysteroid dehydrogenase. Progesterone is also derived from the ovaries and adrenals. In neurons, progesterone can be metabolized by 5α-reductase to DHP. 5α-pregnan-3α-ol-20-one (3α,5α-THP) is formed from DHP by the actions of 3α-hydroxysteroid oxidoreductase.

Evidence suggests that in seizure models, some of progesterone's neuroprotective effects may be mediated by its metabolite 3α,5α-THP. First, 3α,5α-THP is as effective as progesterone at decreasing seizures and reducing damage in the hippocampus induced by seizures (Frye, 1995; Frye et al., 2000; Frye and Muscatiello, 2001; Frye and Scalise, 2000; Leskiewicz et al., 1997). Second, withdrawal from endogenous or exogenous 3α,5α-THP increases seizure activity (Frye and Bayon, 1999; Reddy and Rogawski, 2001; Smith, 2002). Third, blocking progesterone's metabolism to 3α,5α-THP with a 5α-reductase inhibitor increases seizures of rats (Frye et al., 1998). Finally, 5α-reductase

knockout mice, which are unable to metabolize progesterone to 3α,5α-THP, do not have fewer seizures following progesterone administration, as is seen in wild-type mice administered with progesterone (Frye et al., 2002). Thus, 3α,5α-THP may underlie some of progesterone's protective effects in seizure models.

There is some evidence that 3α,5α-THP may prevent neuronal death following injury in other models of neurodegeneration as well. In vitro, 3α,5α-THP attenuates NMDA-induced excitotoxicity and apoptosis in human NT2 cells (Lockhart et al., 2002) and protects hippocampal pyramidal neurons from hypoxia (Frank and Sagratella, 2000). Although these data suggest that 3α,5α-THP may have protective effects in models of neurodegeneration that do not involve seizure processes, this has not been extensively investigated.

The adrenalectomy (ADX) model of neurodegeneration is a robust, reliable, noninvasive means of producing highly selective cell death. Removal of the primary source of glucocorticoids via ADX results in selective cell death in the granule layer of the dentate gyrus (Roy et al., 1990; Sapolsky et al., 1991; Sloviter et al., 1989). Degeneration is visible in the granule layer in as little as 2–4 days post-ADX, and, over longer periods, results in severe to complete loss of granule cells (Bye and Nichols, 1998; Conrad and Roy, 1995; Gould et al., 1990; Jaarsma et al., 1992; McCormick et al., 1997; Sloviter et al., 1989). Thus, ADX was utilized in the present experiments as a model to examine the effects of progestins on cell death in the hippocampus.

The purpose of the present studies was to further investigate the effects of progestins on ADX-induced cell loss in the hippocampus of female and male rats, both of which can respond to, and have substrates for, progestins actions (Guerra-Araiza et al., 2002). We hypothesized that if progesterone's protective effects are due to 3α,5α-THP, then (1) progesterone, DHP, and 3α,5α-THP should have similar effects to protect against ADX-induced cell death and (2) blocking progesterone's metabolism to 3α,5α-THP should attenuate the protective effects of progesterone.

## 2. Methods

These methods were preapproved by the Institutional Animal Care and Use Committee at the University at Albany-SUNY.

### 2.1. Animals and housing

Adult (60 days of age), female ($n=59$) or male ($n=60$) Long-Evans rats were bred and raised in the animal facility at SUNY-Albany from stock obtained from Taconic Farms (Germantown, NY). Rats were housed in groups in polycarbonate cages ($45 \times 24 \times 21$ cm) in a temperature-controlled room ($21 \pm 1$ °C) in the laboratory animal care facility. The rats were maintained on a 12:12-h reversed light cycle (lights off 8:00 am) with continuous access to Purina Rat Chow and tap water in their home cages.

### 2.2. Surgery

Surgery was performed under Rompun (12 mg/kg) and Ketaset (80 mg/kg) anesthesia. To control the endogenous secretion of steroids, all rats were ovariectomized or gonadectomized and adrenalectomized. Ovariectomy of female rats was performed 1 week prior to ADX. The gonadectomy of male rats was performed 4–6 weeks prior to ADX. The timing of ovariectomy or gonadectomy prior to ADX was based upon previous findings that this is the minimum time necessary (for females and males, respectively) for endogenous hormone levels to reach nadir and thereby minimize any confounds with exogenously administered hormones (Frye et al., 1996, 2004). All rats were then ADX, and hormone and/or metabolism inhibitor regimen were started the day of ADX (Day 1) and continued for 4 days (Days 2–5).

### 2.3. Procedure

#### 2.3.1. Hormone replacement—Experiments 1 and 2

Female (Experiment 1) and male (Experiment 2) rats were randomly assigned to receive subcutaneous vehicle (sesame oil, female $n=13$; male $n=12$), progesterone (1.0 mg/kg; female $n=14$; male $n=10$), DHP (1.0 mg/kg; female $n=12$; male $n=12$), or 3α,5α-THP (1.0 mg/kg; female $n=14$; male $n=13$).

#### 2.3.2. Hormone replacement and metabolism inhibition—Experiment 3

Female rats were randomly assigned to receive subcutaneous vehicle (female $n=13$), progesterone (1 mg/kg; female $n=14$), finasteride (10 mg/kg; female $n=10$), or finasteride + progesterone (female $n=10$).

### 2.4. Tissue collection and preparation

On the fifth day after ADX, rats were exposed to ether, and then blood for CORT measurement was collected. Rats were then exsanguinated with 0.9% saline and perfused with 10% formalin, and fixed brains were removed from the skull. Brains were placed in 10% formalin for 24 h and then switched to sucrose PBS until brains dropped. Brains were then sliced on a freezing microtome at 10 μm. Slices were stained with cresyl violet to determine the pyknotic cell counts. To reduce the possible effects of fixative on tissues (i.e. shrinking), all tissues were sliced and stained within 4 weeks of tissue collection. As well, brains were randomly chosen from each group and sliced so that no one experimental group remained in fixative for longer than any other.

### 2.5. Determination of pyknotic cells

Total pyknotic cell counts were determined as previously described (Frye and McCormick, 2000a,b). Cells that had a smaller volume, had multiple, densely-stained clumps of chromatin, a condensed shriveled nucleus, and blebbing of the membrane were considered pyknotic (Frye, 2001; Frye and McCormick, 2000a,b). Briefly, cresyl-violet-stained 10-μm sections from the anterior, middle, and posterior sections of the hippocampus were used to determine pyknotic cells in the granule cell layer of the dentate gyrus. See Fig. 2 for a schematic depiction of the anterior, middle, and posterior regions of the hippocampus that were counted. Counts were initially divided into anterior, middle, and posterior sections to examine whether there were differences in these areas. Every fourth section was counted in both hemispheres. Apoptotic cells were counted within a 100 × visual field in which the arch of the suprapyramidal blade was centered. Two investigators counted the number of apoptotic neurons; their interrater reliability was greater than 90%. Given that there were no differences across regions, the mean number of apoptotic cells in each of the three regions was added together as the index of total pyknotic cell numbers in the hippocampus.

### 2.6. Statistical analyses

One-way analyses of variance (ANOVAs) were used to examine effects of hormonal milieu on the number of pyknotic cells in the dentate gyrus. Where appropriate, ANOVAs were followed by Fisher's Least Significant Difference Post Hoc Tests to determine differences among groups. Alpha level for the determination of statistical significance was $P < .05$.

## 3. Results

### 3.1. Validation of ADX

After ADX, some rats continue to produce corticosterone (CORT) from accessory tissues and/or remnants of the adrenal cortex, which may prevent degeneration (Conrad and Roy, 1993). For this reason, several measures were taken into account when determining if rats were truly ADX. Water and saline intake were monitored following ADX. Across experiments, ADX and non-ADX rats drank similar amounts of water (ADX: 60 ± 7 ml; non-ADX: 59 ± 10 ml); however, ADX rats drank more saline (79 ± 8 ml) than did non-ADX rats (66 ± 9 ml). As well, CORT levels were measured in all rats according to previously published methods (Frye et al., 1996). Rats with basal levels of CORT (less than 2.0 μg/dl) at the time of perfusion were considered truly ADX. Across experiments, CORT levels of ADX rats were 0.2 ± 0.1 μg/dl, and for non-ADX rats, levels were 24.6 ± 10.8 μg/dl. Non-ADX rats have very little degeneration in the dentate gyrus compared to ADX rats (see Fig. 3 and also Frye, 2001).

### 3.2. Effects of progestins on cell death of female rats

There was an overall effect of progestin administration on the total number of pyknotic cells in the dentate gyrus [$F(3,51) = 10.53$, $P < .01$]. Female rats administered with

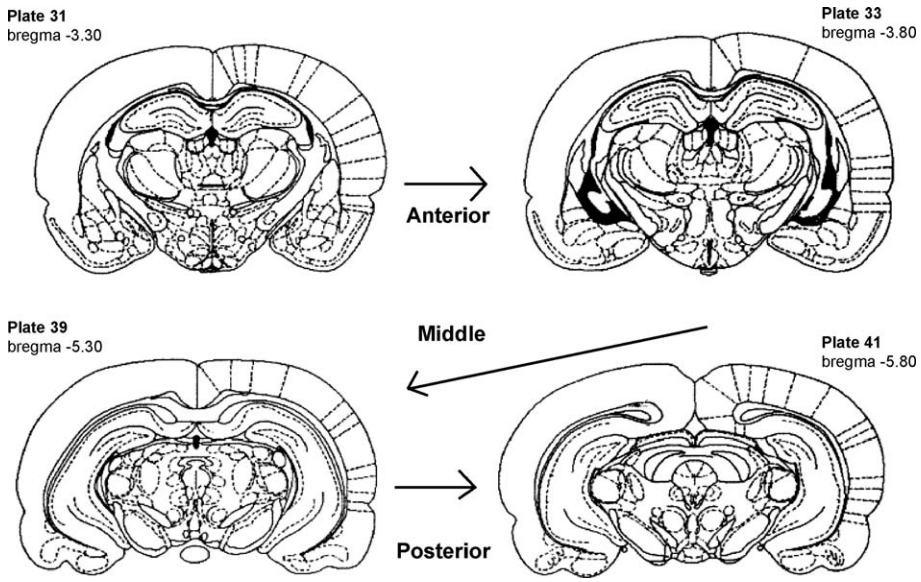


Fig. 2. Schematic illustrating the extent of the anterior (Plates 31–33, −3.30 to −3.80 from bregma; top; Paxinos and Watson, 1982), middle (Plates 33–39, −3.90 to −5.30 from bregma; diagonal), and posterior (Plates 39–41, −5.40 to −5.80 from bregma; bottom) sections of the hippocampus used when counting pyknotic cells in the dentate gyrus. There were no differences in counts across the anterior, middle, or posterior sections; thus, counts were averaged and reported as total counts.

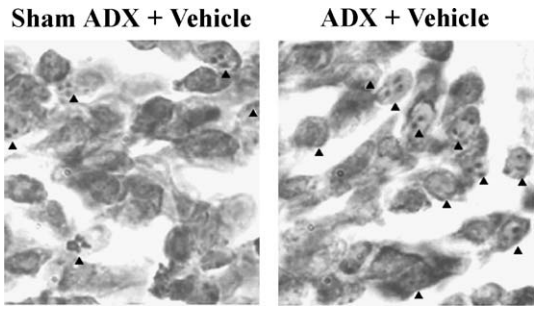


Fig. 3. Representative photomicrographs taken at 100 × of the dentate of sham (left) or ADX (right) of ovariectomized rats administered with vehicle. Note that there are more pyknotic cells (indicated by arrows) in the ADX compared with the sham ADX rats.

progesterone ($P<.01$), DHP ($P<.01$), or 3α,5α-THP ($P<.01$) had significantly fewer pyknotic cells in the dentate gyrus than did females administered vehicle (see Fig. 4).

### 3.3. Effects of progestins on cell death of male rats

There was a main effect of progestin administration on the total number of pyknotic cells in the dentate gyrus [$F(3,47)=3.66$, $P<.01$]. Male rats administered progesterone ($P<.01$), DHP ($P<.01$), or 3α,5α-THP ($P<.01$) had significantly fewer pyknotic cells in the dentate gyrus than did male rats administered vehicle (see Fig. 5).

NB: There were no differences in the effects of progestins to reduce cell death in the dentate gyrus of female or male rats. Thus, the subsequent experiment examined the effects of blocking progesterone's metabolism on cell death in the dentate gyrus of female rats.

### 3.4. Effects of blocking progesterone metabolism on cell death of female rats

Blocking progesterone's metabolism to 3α,5α-THP attenuated progesterone's ability to protect against cell death in the dentate gyrus of female rats [$F(3,43)=20.16$, $P<.01$]. Ovariectomized rats administered progesterone had decreased total pyknotic cells in the dentate gyrus compared with rats administered vehicle ($P<.01$). Rats administered the combination of finasteride and progesterone had similarly increased total pyknotic cells in the dentate gyrus as did rats administered vehicle ($P>0.05$), both were greater than levels seen in progesterone-administered rats ($P<.01$; see Fig. 6). Notably, finasteride administration alone increased the number of pyknotic cells in

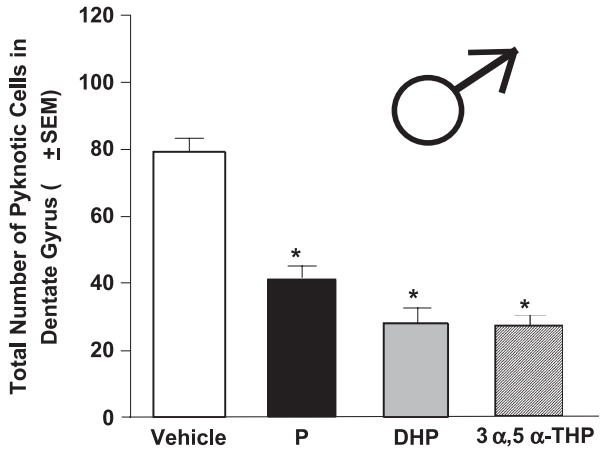


Fig. 5. Total number of pyknotic cells in the dentate gyrus of gonadectomized male rats administered with vehicle (open bar), progesterone (black bar), DHP (gray bar), or 3α,5α-THP (striped bar). * Significant difference from vehicle administration.

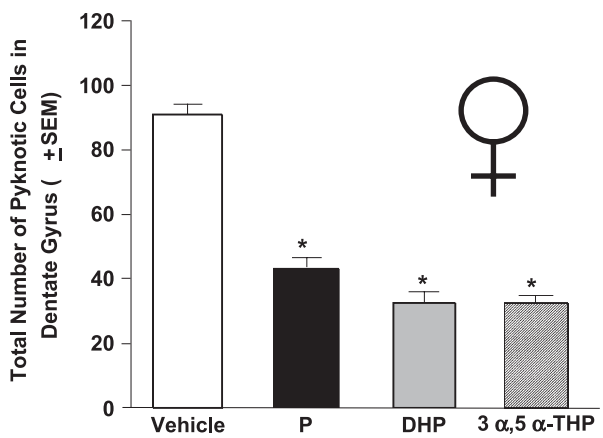


Fig. 4. Total number of pyknotic cells in the dentate gyrus of ovariectomized female rats administered with vehicle (open bar), progesterone (black bar), DHP (gray bar), or 3α,5α-THP (striped bar). * Significant difference from vehicle administration.

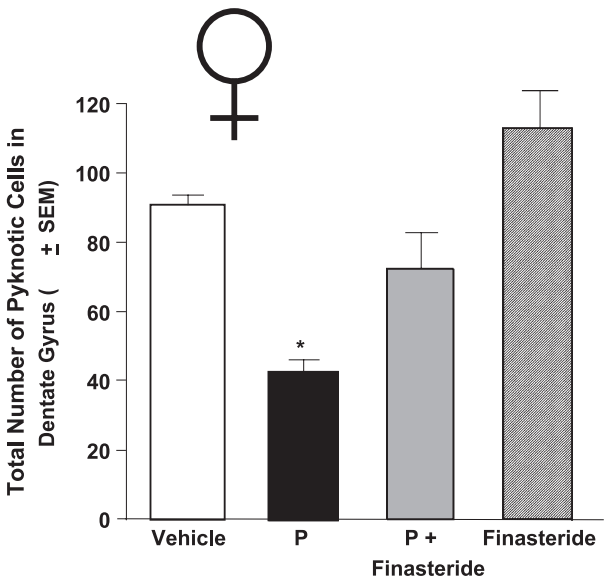


Fig. 6. Total number of pyknotic cells in the dentate gyrus of ovariectomized female rats administered with vehicle (open bar), progesterone + finasteride (gray bar), or finasteride (striped bar). * Significant difference from all other groups.

the dentate gyrus compared with vehicle administration ($P < .05$).

## 4. Discussion

Results of the present studies supported our hypothesis that 3α,5α-THP may underlie some of progesterone's effects to protect against cell death in the hippocampus following ADX. Administration of progesterone, DHP, or 3α,5α-THP had similar effects to significantly decrease the number of pyknotic cells in the dentate gyrus of ADX male and female rats compared with vehicle administration. The number of pyknotic cells in the dentate gyrus of ADX female or male rats administered progesterone, DHP, or 3α,5α-THP were analogous to numbers seen in non-ADX rats in this and previous experiments (Frye, 2001; Frye and McCormick, 2000a,b). Furthermore, blocking progesterone's metabolism to 3α,5α-THP attenuated progesterone's protective effects on cell death in the hippocampus of female rats. These data suggest that actions of 3α,5α-THP may underlie some of progesterone's neuroprotective effects in an ADX model of neurodegeneration.

Our current findings confirm previous data that progesterone can have neuroprotective effects. The ability of progesterone, DHP, and 3α,5α-THP to protect against ADX-induced cell death in the hippocampus is consistent with previous reports of progestins' protective effects in in vitro and in vivo models of neurodegeneration. Thus, these data support the idea that progestins may have an important role in protecting the brain from various types of insults.

Results from the present experiments investigating progesterone's ability to protect against ADX-induced cell death also extend previous reports of progestins' neuroprotective effects. Progesterone's antiseizure effects are due, in part, to the actions of its metabolite, 3α,5α-THP. However, to date, there have been few reports of the protective effects of progesterone metabolites in other models of neurodegeneration that do not involve seizures. The similar effects of progesterone, DHP, and 3α,5α-THP to decrease total pyknotic cells in the dentate gyrus of ADX female or male rats suggests that progesterone's ability to reduce ADX-induced cell death may be due in part to actions of its metabolite 3α,5α-THP. Second, that progesterone's protective effects were attenuated when progesterone metabolism was blocked further supports the role of 3α,5α-THP to mediate progesterone's protective effects in an ADX model of neurodegeneration. These data suggest that 3α,5α-THP may be important for protection against ADX-induced cell loss, although it should be noted that blocking progesterone's metabolism to 3α,5α-THP did not induce pyknosis to the same levels seen in ADX vehicle-administered rats.

Although the present findings suggest that 3α,5α-THP can protect against ADX-induced cell death, they do not address the mechanisms by which 3α,5α-THP may have its protective effects. 3α,5α-THP can attenuate the hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis. Administration of 3α,5α-THP to ovariectomized rats attenuates the elevation of plasma adrenocorticotropin (ACTH) and serum CORT after exposure to emotional stress (Patchev et al., 1996). Thus, this is one mechanism by which 3α,5α-THP may have its protective effects in this and other models of neurodegeneration. Furthermore, the effects of ADX are related to the absence of mineralocorticoid receptor activation in the brain. Neuron loss in the dentate gyrus is not seen in ADX animals maintained chronically on low levels of corticosterone or aldosterone, but is seen in animals maintained on the glucocorticoid receptor agonist RU28362 (Conrad and Roy, 1993; Rosenfeld et al., 1993; Sloviter et al., 1989; Woolley et al., 1991). Notably, progesterone has antagonist-like actions at mineralocorticoid receptors, which may underlie some of its effects to reduce ADX-induced cell death (Patel et al., 2003; Sitruk-Ware, 2003).

Another way in which 3α,5α-THP may protect the brain against injury is by influencing excitability in the brain. Inhibition of excitatory glutamate receptors or potentiation of inhibitory $GABA_A$ receptors can be neuroprotective. 3α,5α-THP is one of the most potent modulators of $GABA_A$ receptors and has robust effects to enhance GABA-mediated inhibition of neuronal activity (Majewska et al., 1986). 3α,5α-THP can also bind to, and alter the function of, NMDA receptors (Park-Chung et al., 1997; Smith et al., 1987a,b). Notably, both $GABA_A$ and NMDA receptors have been localized to the hippocampus (Simburger et al., 2001; Thompson et al., 2002). Thus, 3α,5α-THP may reduce ADX-induced cell death by potentiating GABA receptor activation and/or by inhibiting excitatory glutamate receptors.

The finding in Experiment 3 that the administration of finasteride alone increased the number of pyknotic cells in the dentate gyrus compared with vehicle administration also expands the current knowledge about progesterone's neuroprotective effects. Notably, although 3α,5α-THP is produced peripherally by the ovaries, it is also synthesized de novo from cholesterol by glial cells. That finasteride administration to ovariectomized rats had intrinsic effects to increase pyknotic cells in the dentate gyrus suggests that central production of 3α,5α-THP may be particularly important for protecting the brain against injury. However, it should be noted that the increase in pyknotic cells with finasteride administration alone was similar in magnitude to the decrease seen with progesterone administration. Thus, it is possible that the increase with finasteride alone offsets the decrease seen with progesterone, and this may be independent of finasteride blocking the formation of 3α,5α-THP. Indeed, finasteride may have effects other than blocking progesterone's metabolism to 3α,5α-THP that might alter cell death in the hippocampus.

Although the present data suggest that progestins can have protective effects, in part, by decreasing cell death, at this point, it is unclear if progestins' effects to protect the

brain may also include inducing neurogenesis, or a combination of these processes. First, gonadal hormones can modulate neurogenesis in the hippocampus. Estrogen and progesterone have been demonstrated to regulate synapse formation in CA1 during the estrous cycle of female rats (McEwen, 2002). Second, progestins can have trophic effects to regulate neuronal and glial differentiation and can modulate synaptic formation and synaptic plasticity in several brain areas, including the hippocampus (Bicknell, 1998; Chowen et al., 2000; McEwen, 1994; Melcangi et al., 2002; Stein, 2001). Third, 3α,5α-THP can have antidepressant effects, which may be mediated by actions in the hippocampus. The administration of 3α,5α-THP decreases depressive behavior in mice (Khisti and Chopde, 2000; Khisti et al., 2000). Fourth, antidepressant drugs (serotonin or norepinephrine selective reuptake inhibitors or monoamine oxidase inhibitors) increase neurogenesis in the dentate gyrus of adult rats (Duman et al., 2001; Malberg et al., 2000). Finally, the alleviation of depressive symptomology with antidepressant drug therapy is associated with increased levels of 3α,5α-THP (Romeo et al., 1998; Uzunova et al., 1998). Together these data suggest that, perhaps, 3α,5α-THP's effects on hippocampal cells may underlie some of the therapeutic effects of antidepressant drugs.

In summary, our data that 3α,5α-THP can similarly prevent ADX-induced cell loss in the dentate gyrus of female and male rats indicate that the ADX model may be a useful tool for further investigations of the effects and mechanisms of gonadal hormones to protect the brain from damage. Given the findings discussed above that gonadal hormones and antidepressant drugs can modulate neurogenesis, and that antidepressant drugs can increase levels of 3α,5α-THP, investigations of this nature may ultimately lead to an improved understanding of the mechanisms of depression and to the development of novel pharmacological therapies.

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