NEUROANATOMICAL AND BEHAVIORAL CORRELATES OF PROGESTERONE WITHDRAWAL

by

Ethan H. Beckley

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Ethan H. Beckley

has been approved

[redacted]

Andrey E. Ryabinin, Chair

[redacted]

Deborah A. Finn, Member

[redacted]

Cynthia D. Bethea, Member

[redacted]

Garet P. Lahvis, Member

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Abstract

Reproductive depression can be defined as symptoms or syndromes of depression in women that are presumed to occur as side effects of reproductive function. Classic examples of reproductive depression are premenstrual syndrome and postpartum depression, which can be serious medical conditions. One etiological theory of these syndromes states that natural withdrawal of progesterone circulation in the periphery during the ovulatory cycle or birthing process can sometimes manifest as a mental illness because the brain is responsive to changes in circulating levels of progesterone and its metabolites. The current work discusses a mouse model of this phenomenon, termed "progesterone withdrawal," and employs behavioral-pharmacological and neuroanatomical methods to advance this line of research.

The primary topic that is specifically addressed in the present work focuses around the question of whether it is progesterone or one of its metabolites, allopregnanolone, that underlies the connection between peripheral progesterone withdrawal and depression. Because the biosynthesis of allopregnanolone is dependent on progesterone, and because the metabolism of progesterone results in allopregnanolone, the concentrations of these steroids in the periphery and in the central nervous system are correlated. Previous attempts to study progesterone withdrawal have typically failed to delineate which changes could be due to loss of progesterone signal or to loss of allopregnanolone signal. The current work begins by establishing a general methodological framework and validating an effect of passive progesterone withdrawal on depression-like behavior in mice, then

develops a line of experimentation that uses several different methods to assess whether progesterone withdrawal or allopregnanolone withdrawal better explains increases in depression-like behavior in mice during progesterone withdrawal. Depression-like behavior is measured using the forced swim test, wherein time that a mouse spends floating immobile in water is interpreted as an index of its emotional state. An additional goal of the present research was to identify one or more candidate brain regions that might be the anatomical substrate that mediates progesterone withdrawal or allopregnanolone withdrawal and the increase in forced swim test behavior.

One initial finding was that inhibiting the biosynthesis of allopregnanolone increased forced swim test immobility, thus showing that one form of allopregnanolone withdrawal was associated with an increase in depression-like behavior. However, two additional approaches to test the effects of allopregnanolone withdrawal failed to convincingly corroborate a role for allopregnanolone withdrawal in this phenomenon. Subsequent studies with progesterone receptor antagonists demonstrated that inhibiting progesterone receptor activity reliably and robustly increased forced swim test immobility. The efforts to identify a neural substrate for this effect were inconclusive.

These studies provide new evidence that progesterone receptors are involved in the neural substrates of emotion. Inhibiting their activity with pharmacological antagonists increased depression-like behavior in mice, which suggests that loss of progesterone receptor activation during the menstrual cycle or during the birthing process may be part of the etiology of reproductive depression.

It is hoped that the current work will stimulate new interest and increased attention to progesterone within the study of reproductive depression.

Chapter 1: General Introduction

Reproductive depression

It has been recognized throughout history that some women experience depressive episodes during periods of reproductive function or change (Trede et al., 2009). This association has led some to hypothesize the existence of one or more subtypes of depression that could be called "reproductive depression" (Payne et al., 2009) that may be etiologically distinct from classic forms of depression such as a major depressive episode. The archetypical forms of reproductive depression are postpartum depression, which can occur after childbirth, and premenstrual syndrome, which can occur prior to menses. Both postpartum depression and premenstrual syndrome have related subtypes (e.g., postpartum psychosis, premenstrual dysphoric disorder). Depressed mood associated with menopause or pharmaceutical contraceptives may also be related.

It has been estimated that as many as 80% of women experience some mild degree of post-birth depression ("baby blues"), 5-20% of women experience clinically significant "postpartum depression," and less than 1% experience a severe form of "postpartum psychosis" or "puerperal psychosis" (e.g., Appolonio & Fingerhut, 2008; Grussu & Quatraro, 2009; Lolak et al., 2005; Reck et al., 2008; Spinelli, 2009; Valdimarsdóttir et al., 2009). Perimenstrual affective disorders have a graded distribution of severities, similar to postpartum depression. Less than 10% of women experience the severe perimenstrual affective disorder "premenstrual dysphoric disorder," approximately 5-30% more experience a clinically significant "premenstrual syndrome," and perhaps as many as 80% of all

women experience at least slight menstrual-related symptoms (Freeman, 2003; Halbreich et al., 2003; Potter et al., 2009; see also, Jacobi, 1877).

Susceptibility to one type of reproductive depression appears related to susceptibility to other types of reproductive depression (Bloch et al., 2006; Brockington, 2009; Frey et al., 2008; Garcia-Esteve et al., 2008; Graziottin & Serafini, 2009; Gregory et al., 2000; Payne et al., 2007; Soares & Zitek, 2008; but see Haywood et al., 2007). Several sources have proposed that susceptibility to reproductive depression and major depressive disorder may be related, although this remains controversial (Bancroft, 1993; Kessler, 2003; Phillips et al., in press; Steinberg et al., 2008). There is overwhelming evidence that sex steroids play a significant role in reproductive depressive disorders, and it is possible that sex steroids are also involved in major depression (Hyde et al., 2008; Kessler, 2003; Noble, 2005; Steiner et al., 2003).

These disorders impose material interference with life plans and activities. For example, adolescent girls with premenstrual syndrome have shown reduced academic performance (Boyle, 1997), and it has been estimated that women affected by premenstrual syndrome experience work productivity decreased by 15% and can incur medical expenses of thousands of dollars per year (including indirect expenses, Borenstein et al., 2005). Having premenstrual syndrome is estimated to result in the loss of over seven years of time living with optimal-health (Halbreich et al., 2003), and health-related quality of life when living with premenstrual dysphoric disorder is thought to be comparable to living with other chronic medical conditions such as type 2 diabetes or hypertension (Yang et al.,

2008). Individual health-care providers and entire public health agencies underestimate the burden that these disorders impose (Rapkin & Winer, 2009), and rates of reproductive depression may be even higher in less-developed countries than the prevalence rates for developed countries (Almond, 2009), such as those discussed above.

Reproductive depression is an important medical concern in its own right, but in addition to the frank symptoms associated with reproductive depression, these disorders and reproductive events are also associated with increases in suicide attempts and suicidal ideation (Saunders & Hawton, 2006), psychiatric admissions (Targum et al., 1991), and changes in drug sensitivity, craving, or use (Franklin et al., 2004; Nyberg et al., 2004; Park et al., 2009). Reproductive changes have been shown to exacerbate a great number of other disorders[1]. Thus, the lives of many women could be substantively improved by better understanding of the underlying biological mechanisms of reproductive events. Understanding reproductive depression may provide a framework for understanding other disorders that are exacerbated or precipitated by reproductive events.

Progesterone secretion and withdrawal

The underlying pathology of reproductive depression has eluded researchers, but one potential mechanism that has continued to interest investigators comes from the observation that similar hormone fluctuations precede the symptoms of both premenstrual syndrome and postpartum depression. This

[1] For example: fibromyalgia, Amital et al., in press; pulmonary hemosiderosis, Foglia & Deering, 2008; rheumatoid arthritis, Häupl et al., 2008; irritable bowel syndrome, Lea et al., 2004; human immunodeficiency virus, Patel & Grimes, 2006; asthma, Redmond et al., 2004; ATPase skin disorders, Szigeti et al., 2007.

observation has led many to hypothesize that reproductive-related depression might be a sort of hormone withdrawal syndrome (Kammerer et al., 2006; Meaden et al., 2005; Pearlstein et al., 2005; see also, Gehlert et al., 1999; Gonda et al., 2008). In particular, the steroid hormone progesterone is secreted in large amounts during menstrual cycles and pregnancies, but when its secretion is terminated (as discussed below) the normal clearance mechanisms quickly eliminate progesterone from the compartments where it circulates, resulting in a phenomenon termed progesterone withdrawal.

Women can produce over 40 mg per day (mg/d) of progesterone during the luteal phase of the menstrual cycle, and over 200 mg/d during pregnancy, compared to less than 1 mg/d in men or in women who are neither pregnant nor in the luteal phase (MacDonald et al., 1991). These rates of progesterone production result in peak plasma concentrations of approximately 40 nmol/L (about 12.5 ng/mL) during the luteal phase (Chabbert Buffet et al., 1998; Peters & McNatty, 1980), and 350-400 nmol/L (about 100-125 ng/mL) during pregnancy, although the actual values appear to vary tremendously among different women (Hill et al., in press). Plasma progesterone concentrations are approaching zero and are probably not physiologically relevant in women who are not pregnant or in the luteal phase of the menstrual cycle (Chabbert Buffet et al., 1998; Peters & McNatty, 1980). "Progesterone withdrawal" refers to the change from 40 nmol/L or 350 nmol/L progesterone to virtually-zero progesterone in as little as one or two days.

The occurrence of progesterone withdrawal is beyond any real doubt, but its link to reproductive depression remains to be more convincingly demonstrated. It

should be noted that sometimes symptoms of reproductive depression do occur prior to progesterone withdrawal. For example, sometimes the symptoms of premenstrual dysphoric disorder occur around the time of ovulation (*Diagnostic and Statistical Manual of Mental Disorders—Fourth Edition—Text Revision*, American Psychiatric Association, 2000). Cases where symptom timing does not coincide with progesterone withdrawal may be less related to the current work.

Although progesterone has several physiological roles, one of its main roles is to allow for implantation to occur after an embryo has been fertilized. While not all menstrual cycles result in a pregnancy, all pregnancies must start with an ovulatory cycle. Progesterone prepares the uterus for embryonic implantation by stimulating the endometrium to secrete glycogen and mucus, and after estrogen activity has caused sufficient endometrial proliferation (increase in cell number of the endometrium), progesterone also causes the endometrium to prepare for implantation by decidualizing the endometrium (forming several layers of tissue, the decidua, that have specific functions related to the implantation of the fetus) (Hisaw & Hisaw, 1961; see also, Ganong, 2003; Hawkins & Matzuk, 2008).

The uterus is an important target tissue of progesterone, but it is not a source of progesterone. Progesterone secretion is initially accomplished by the corpus luteum, a structure that develops from an ovarian follicle following its rupture and release of the oocyte. The most common way that progesterone withdrawal occurs is when fertilization is not achieved. In the absence of an implanted embryo, the corpus luteum undergoes first a functional regression, followed by atrophy of the tissue. This process is not entirely understood, but it is believed that the corpus

luteum becomes increasingly sensitive to prostaglandins as circulating luteinizing hormone is depleted, and that prostaglandins initiate the regression cascade of the corpus luteum[2] (luteolysis).

The second major route by which secretion of progesterone may be terminated occurs when a woman does become pregnant after ovulation. Progesterone is essential for the maintenance of pregnancy (Csapo, 1961), so secretion will eventually be terminated around the time of parturition. First, however, progesterone secretion must be prolonged past its normal duration of secretion during a menstrual cycle. The corpus luteum is "rescued" by signals from the developing embryo. After the blastocyst has successfully implanted in the endometrium, it releases chorionic gonadotropin, which, as a close homolog of luteinizing hormone, prolongs the activity of the corpus luteum.

Progesterone secretion is thus maintained for the whole of the pregnancy in many animals. A "luteo-placental shift" takes place, whereby the placenta takes over secretion of progesterone for the rest of the pregnancy (Csapo, 1969; but see Azuma et al., 1993). A key function of progesterone during pregnancy is to block myometrial activity. When progesterone is withdrawn, the myometrium becomes active and contracts to align the uterus with the vaginal canal and expel the fetus (Johnson, 2007).

A complication among species is that progesterone withdrawal immediately precedes parturition in most mammals, but seems not to occur in women until after

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[2] This paragraph and the next are based on several sources collectively: McCracken et al., 1999, Peters & McNatty, 1980, and Sugino & Okuda, 2007.

the placenta has been expelled. This presents a potential paradox, because progesterone withdrawal is generally believed to be necessary for parturition in most mammals. A number of lines of evidence indicate that progesterone is necessary to maintain pregnancy in women (e.g., Bengtsson, 1961; Corner & Long, 1961; Mazouni et al., 2006), and thus there is a growing consensus that women must experience a "functional progesterone withdrawal" that still withdraws progesterone activity in the myometrium prior to birth (R. Smith, 2007; Zakar & Hertelendy, 2007). Progesterone secretion is truly terminated in women after the placenta has been delivered in the third stage of labor.

Progesterone withdrawal and depression

A causative connection between progesterone withdrawal and reproductive depression can only be hypothesized at this point. On a broader level than will be investigated in the current work, the progesterone withdrawal hypothesis is this: *Since progesterone withdrawal occurs prior to the depressive symptoms of premenstrual syndrome and postpartum depression, it is hypothesized to play a role in the initiation of episodes of reproductive depression.* A consequence of this hypothesis is that it proposes that progesterone withdrawal is the common etiological factor in both premenstrual syndrome and postpartum depression. (This hypothesis admittedly does not explain all important aspects of these disorders.)

Frank (1931) suggested that hormones were the cause of several cases of what he called "premenstrual tension," and shortly thereafter came reports suggesting that insufficient progesterone contributed to these symptoms (Israel, 1938; see also Gray, 1940; Greenblatt, 1940; Morton, 1950). Later, R. Green and

Dalton (1953) were emphatic that progesterone insufficiency led to premenstrual syndrome, and they convincingly documented that the majority of presenting cases of premenstrual syndrome had their onset in the last week prior to menses.

Many more observations support the hypothesis that progesterone withdrawal induces premenstrual syndrome, but unfortunately most simply reiterate the temporal link. For example, it was later shown that peak progesterone secretion precedes peak severity of premenstrual syndromes symptoms by a period of approximately 3-7 days (e.g., Bäckström et al., 1983; Halbreich et al., 1986; Redie & Freeman, 1995). Experimental evidence for progesterone withdrawal in premenstrual syndrome comes from the observation that medically-induced anovulation is effective at preventing premenstrual symptoms (reviewed by Bäckström et al., 2003). Perhaps the most compelling evidence to suggest that progesterone withdrawal induces premenstrual symptoms is a study that showed premenstrual syndrome-like symptoms could be induced among women treated with the progesterone receptor antagonist mifepristone (Schmidt et al., 1991; see also below for additional discussion of this study).

Little true experimental evidence links progesterone withdrawal to postpartum depression. A similar temporal link between progesterone withdrawal and symptoms has been observed for postpartum depression, with symptom severity peaking around day 5 postpartum (reviewed in Kendall-Tackett, 2005). One important study showed that the magnitude of progesterone decline in individuals was associated with depression ratings within 10 days following parturition (Nott et al., 1976). Preventing ovulation would prevent postpartum

depression by preventing parturition, so the same research strategies used in premenstrual syndrome cannot necessarily be adapted to postpartum depression.

One good piece of experimental evidence to suggest that hormone withdrawal underlies postpartum depression comes from a study where women with one or more biological children were divided into groups according to positive or negative history for postpartum depression, and given leuprolide to suppress their menstrual cycles for five months. (Leuprolide is a gonadotropin-releasing hormone agonist that is used clinically to inhibit ovarian or testicular steroidogenesis.) The women then received estradiol and progesterone tablets to simulate pregnancy-like hormones for 8 weeks, followed by a withdrawal period. Women with histories negative for postpartum depression did not exhibit increased depression symptoms during the withdrawal phase, but women with histories positive for postpartum depression experienced increased depression symptoms during the withdrawal phase compared to their own baseline or women with negative histories (Bloch et al., 2000). This study indicates that hormone withdrawal can induce depressive symptoms consistent with postpartum depression in at least a subset of women.

From one perspective, these findings provide only limited evidence to indicate that progesterone withdrawal is a causal factor in premenstrual syndrome or postpartum depression. The limited number of studies, especially true experiments, makes it impossible to state that there is a causative relationship. Still, these studies offer an abundance of support for the hypothesis, and certainly enough to justify further research.

Effects of progesterone and its metabolites on brain function

Steroids have profound effects on neural function. A vast variety of brain functions are affected by sex steroids (e.g., Hajszan et al., 2008; Quesada & Micevych, 2008; Tokuyama et al., 2008), adrenal steroids (e.g., J.-W. Shen et al., 2009; Uchoa et al., 2009), and other steroids. Steroids are the recognized endogenous ligands for a number of receptor proteins present in the brain (e.g., androgen receptors, Claessens et al., 2008; glucocorticoid and mineralocorticoid receptors, Kawata et al., 2008; liver X receptors, S. Matsumoto et al., 2009; estrogen receptors, Kelly & Rønnekleiv, 2008; vitamin D receptors, Garcion et al., 2002). Steroids that are active in the brain are referred to as neuroactive steroids or neurosteroids.

Like the other steroids listed above, progesterone too is a recognized endogenous ligand for certain receptor proteins that are found, among other places, in the brain. These receptors include the "classic" or "nuclear" progesterone receptors, progesterone receptor A and progesterone receptor B, which are coded by a single gene which is differentially transcribed (Brinton et al., 2008). At least four other "nuclear" isoforms have been proposed, although the evidence for these is limited (Guennoun et al., 2008, Samalecos & Gellersen, 2008; A. H. Taylor et al., 2009). There are also the "membrane-bound progesterone receptors," which have seven transmembrane subunits and are coupled to G-protein mechanisms. Within this class, α, β, and γ isoforms have been characterized. Two additional receptors, each with a single transmembrane spanning unit, are called the "progesterone membrane receptor components" (PGMRCs) (Thomas, 2008; Zhu et al., 2008). New information regarding all of these receptors is rapidly emerging, and these receptors

could turn out to mediate significant physiological functions (Mani, 2008). However, very little is currently known about many of these receptors, and the purpose of introducing these receptors here is only to give some preliminary consideration to the multiple pathways by which progesterone might act.

Some steroids also modulate the activity or function of protein targets for which a separate ligand is considered to be the endogenous ligand. The best example of this class of neurosteroids is possibly the steroid allopregnanolone. Allopregnanolone is synthesized by enzymatic reduction of the A-ring of progesterone. First, the 5α-reductase enzyme attaches a hydrogen atom in the α orientation to the carbon in the fifth position of the A-ring (C5). The resulting steroid is 5α-dihydroprogesterone. Second, a reduction reaction occurs when the 3α-hydroxysteroid dehydrogenase enzyme replaces the A-ring ketone (formed by the double-bonded oxygen attached to the C3 carbon on the A-ring) with a hydroxyl group in the α orientation, resulting in allopregnanolone (see also below) (also called 5α-pregnan-3α-ol-20-one). The metabolic relationships among steroids is dealt with more extensively below (for example, see Figure 1), while the present section focuses mainly on pharmacodynamic properties of these steroids.

Allopregnanolone is a positive allosteric modulator of γ-aminobutyric acid type-A receptors ($GABA_A$ receptors), which are multimeric receptors formed by the concatenation of five subunits from a selection of at least 20 possible subunits (S. S. Smith, 2004), resulting in many possible stoichiometric combinations. Allopregnanolone is highly potent at some of the more common subunit combinations. It potentiates $GABA_A$ receptor activity at concentrations as low as

10 nM (Morrow et al., 1987), and its half-maximal effective concentration (EC_{50} value) has been reported as low as 74 nM at one receptor subtype (Olsen et al., 2004; EC_{50} for muscimol potentiation reported to be 100 nM in Morrow et al., 1987). The effect of allopregnanolone is to increase the open-time and frequency of the chloride channel through the $GABA_A$ receptor (Belelli & Lambert, 2005). The effects of allopregnanolone on that $GABA_A$ receptor are very rapid, and occur on the time scale of milliseconds, rather than hours to days as when progesterone affects gene transcription by nuclear progesterone receptors. Progesterone does not appear to significantly interact with $GABA_A$ receptors at physiological concentrations (Veleiro & Burton, 2009).

Additionally, allopregnanolone activates the intracellular pregnane X receptor, also called the NR1I2 receptor, which is expressed in human brain tissue (Lamba et al., 2004; see also Fang & Zhang, 2009). However, it is probable that the pregnane X receptor is not activated by normal physiological concentrations of allopregnanolone. In an *in vitro* study, Lamba and coworkers (2004) found that pregnane X receptor activation increased nearly 10 fold when stimulated with 10 μM allopregnanolone, the lowest concentration tested. However, the lowest-tested concentration was much higher than the highest allopregnanolone concentrations that humans should achieve. Even during pregnancy women only achieve plasma allopregnanolone concentrations of approximately 100 nM (Paul & Purdy, 1992). Additional research is needed to clarify whether pregnane X receptor is activated at physiological concentrations. Still, it has been suggested that it could mediate physiological roles in the menstrual cycle or pregnancy (Kancheva et al., 2007).

Allopregnanolone does bind to progesterone receptors (Beyer et al., 1995), but this is generally not considered to be of physiological significance.

The effect of allopregnanolone on $GABA_A$ receptor function is, perhaps, better understood than any of the receptors mentioned above that are activated by a progestin. At physiological concentrations allopregnanolone increases $GABA_A$ receptor activity by potentiating the action of GABA, and at higher concentrations it directly activates the $GABA_A$ receptor. There are interesting lines of research that implicate allopregnanolone in such disparate processes as pain (e.g., Frye et al., 2004; Mechlin et al., 2007; Peng et al., 2009), memory and cognition (e.g., Djebaili et al., 2004; Marx et al., 2009), seizure susceptibility (e.g., Biagini, 2009; Mareš et al., in press), and addictive behaviors (e.g., Anker et al., 2009; Hodge et al., 2006). Allopregnanolone is also implicated in anxiety and depression, which are discussed below.

Allopregnanolone withdrawal

It is hypothesized that progesterone withdrawal may underlie reproductive depression, but a complementary hypothesis is that allopregnanolone withdrawal may mediate this effect. Since plasma concentrations of progesterone are correlated with neural allopregnanolone levels (Corpéchot et al., 1993), changes in progesterone secretion across the menstrual cycle or pregnancy (i.e., progesterone withdrawal) should alter allopregnanolone concentrations in the brain, with corresponding changes in $GABA_A$ receptor modulation.

There is some evidence to support this hypothesis. For example, women with premenstrual syndrome have lower allopregnanolone during the luteal phase

of the menstrual cycle than women without premenstrual syndrome (F. Bernardi et al., 2004). Also, women of the general population also have decreased allopregnanolone following childbirth (Gilbert Evans et al., 2005), the period when some women experience postpartum depression. Outside the realm of reproductive depression, clinical studies in small cohorts of patients with unipolar depression (including males) have documented an inverse relationship between endogenous allopregnanolone levels and depression severity (Uzunova et al., 2006). Furthermore, *in vitro* methods have demonstrated that several antidepressant medications interact with allopregnanolone in ways that favor its production or in some other way allows allopregnanolone to increase the efficacy of the drug (Griffin & Mellon, 1999; Pinna et al., 2006; Schüle et al., 2006).

Yet, there have been conflicting reports on the role of allopregnanolone withdrawal in premenstrual syndrome in the human literature. One important study showed no difference in plasma allopregnanolone levels between women with or without premenstrual syndrome at a single time point during the luteal phase (Schmidt et al., 1994). A subsequent study found that women with premenstrual syndrome had lower plasma allopregnanolone concentrations compared to control participants at 12 days after luteinizing hormone surge but not after 5 days (Rapkin et al., 1997). Although this is only a single report, its design takes multiple time points into consideration, and supports the idea that allopregnanolone withdrawal induces premenstrual syndrome (F. Bernardi et al., 2004). Given these different lines of evidence, there is good reason to suggest that allopregnanolone withdrawal may be an important part of the pathology of reproductive depression.

Depressed mood in reproductive depression

To this point it has been described that reproductive depression exists, but the term "depression" has not been defined. *Depression* is sometimes used to describe a feeling of sadness or depressed mood, a normal emotion of life, and it is sometimes used to describe a mental illness that has depressed mood as one of its hallmark symptoms. Accordingly, reproductive depression is a mental illness, associated with reproductive events, that has depressed mood as one of its characteristic symptoms. Depressed mood is perhaps the most common symptom of premenstrual syndrome and postpartum depression. Other important symptoms of reproductive depression can include irritability, poor concentration, appetite changes, breast tenderness, swelling, changes in sleep patterns, specific food cravings anxiety, and headache (e.g., Freeman, 2003; Gotts et al., 1995; Halbreich, 2003).

Depressed mood was chosen as the symptom that would be modeled for the present work. There are several reasons for this choice. The chief and most simple reason was that depressed mood appears to be a key symptom of reproductive depression syndromes. For example, among women drawn from a general population, mood problems were reported in inverse proportion to the total number of symptoms reported (A. W. Chen & Filsinger, 1987). Factor analytic approaches have also demonstrated that depressed mood may be a key symptom (see Derman et al., 2004; Gerlert et al., 1999). Also, it was judged that there has been less animal research done to model the depressed mood symptoms of reproductive depression compared to the efforts to model anxiety.

The forced swim test model of depression

Since there are several symptoms of reproductive depression, one strategy that can be adopted is a "test battery" approach where multiple symptoms are modeled at the same time. With limited resources, the choice to study multiple domains at once can impose a tradeoff where fewer experimental manipulations get tested. Given this tradeoff, the present work makes extensive use of a single behavioral test of depression call the "forced swim test." There are many variations on this test, but the essential elements can be easily summarized. The animal (usually a mouse or rat) is placed into a tank of water and its behavior is observed. The tank size and water level physically prevent the animal from escaping the tank, but the animal spends a good deal of time navigating the tank, presumably looking for an exit. The animal is left in the tank for a fixed period of time that is normally sufficient to ensure that it will stop actively seeking escape, and will instead float in the water. Rats and mice can float without exhibiting virtually any overt behaviors, which is recorded as "immobility" behavior.

The forced swim test is perhaps the best-validated rodent model of depression, and can discriminate between compounds that do or do not act as antidepressants in humans with a high degree of accuracy (Cryan et al., 2002). That is, drugs that are antidepressants in humans tend to decrease forced swim test immobility, and drugs without antidepressant efficacy fail to do so. Immobility behavior is also decreased by non-pharmacological antidepressant therapies in humans, including electroconvulsive shock (Li et al., 2007) and other therapies (e.g., Lopez-Rodriguez et al., 2004; Schulz et al., 2008). Forced swim test immobility is

increased by a variety of treatments that have depression-inducing effects in humans (e.g., Rygula et al., 2008; Mazarati et al., 2008; T. H. Wu & Lin, 2008). For example, Stevenson and colleagues (2009) have recently reported increased forced swim test immobility in mice that were allowed to voluntarily drink alcohol, which is sometimes associated with increased depressed mood in humans.

The chief way to interpret forced swim test immobility is that increases and decreases in forced swim test immobility are indicative of increases and decreases in a rodent's level of "depression." Since mice and rats share similar neurobiology, these increases and decreases are then sometimes extrapolated to make the predication that a human's affective state would experience corresponding changes under comparable circumstances.

Additional animal models of depression and anxiety

A brief synopsis of some important animal behavioral tests is provided here prior to discussing some of the relevant background literature below. In addition to the forced swim test, one important rodent model of depression is the tail suspension test. In contrast to the forced swim test—which uses an inescapable tank of water to produce immobility—in the tail suspension test a mouse is suspended in the air by its tail. Like the forced swim test, the variable of interest is the amount of time that the animal spends immobile, and these immobility scores are generally interpreted to represent a state like depression or despair (see, Cryan et al., 2002, 2005; Petit-Demouliere et al., 2005; Porsolt et al., 1977b).

A very different model of depression is the differential reinforcement of low rate behavior model. This model puts the animal into a situation where it can earn

reinforcing stimuli by performing an arbitrary task (e.g., pushing a lever). The unusual aspect of this assay is that if the animal performs the arbitrary task at a rate that is defined as too high, then the animal receives a time-out interval rather than a reinforcer. Thus, the model differentially reinforces low-rate behavior. The animals are generally food deprived, so their internal motivation is presumably to earn as many food reinforcers as possible. The animals perform poorly when they fail to maximize their earning potential. Based on pharmacological validation methods and other evidence, it is thought that poor performance in this assay (low reinforcement rate) is a result of a depressive state in rodents (O'Donnell et al., 2005). One way to interpret this test is that it could reflect some of the cognitive aspects of depression. Specifically, the self-control model of depression is based on the observation that people who suffer from depression can be bad at delaying gratification (see Gilbert, 1992).

Lastly, the elevated plus maze is one of the most common laboratory models of anxiety for rats and mice. This task involves placing a test animal into a simple maze of two intersecting paths in the form of a plus sign. When the animal is in one pathway it is protected by walls along the edges (the "closed arms"), but when the animal is on the other pathway there is little or no protective walling (the "open arms"). The most commonly reported variables are derived from how the animal explores the open arms relative to the closed arms. Time spent in the open arms is interpreted as a non-anxious response (File, 2001).

Many other models of anxiety and depression have been proposed for mice and other model animals. The ones that have been discussed so far are especially

relevant to the current discussion because they are the models that have been most extensively used for hormone withdrawal studies, as will be discussed below. However, it should also be kept in mind that other relevant models exist, and that some of these models might have unique advantages. For example, one behavioral task relevant to depression is to measure the amount of sucrose (or saccharine) solution that a mouse will voluntarily drink. Decreases in consumption of these solutions (which are normally preferred) are commonly interpreted as an "anhedonic" response akin to a common symptom of depression when a human reports the inability to find pleasure in life (A. D. Green et al., 2009; Flaisher-Grinberg et al., 2009b). There are some clear advantages to assessing changes in sucrose consumption, compared to other tasks. Although some motor activity is required for the sucrose consumption task, it is likely that the physical activity is less strenuous than in the forced swim test, and therefore sucrose consumption may be less affected by treatments that have locomotor side effects. Another consideration is that sucrose consumption can be measured over long periods of time, which may minimize possible confounds due to changes in attention.

Some discussion is warranted to establish why the forced swim test was employed in the present work. No specific studies or reviews were found that explicitly documented the diversity of drugs that have been used to pharmacologically validate the forced swim test compare to other tests, but based on a general reading of the literature it was judged that the pharmacological validation for the forced swim test is unsurpassed by any other behavioral model of depressed mood because of the number and variety of drugs that have been used to

validate it. Also, non-pharmacological validations and the attempts to model increases in depressed mood have been well explored with the forced swim test. These efforts demonstrate that the forced swim test can detect changes in depression-like behavior in either direction, which has not been well established for other models. Since the hypotheses at hand predicted increases in depression-like behavior, it was judged to be very important that the test that was to be chosen could detect such increases.

Again, no specific studies or reviews were found that explicitly documented the diversity of non-pharmacological validation for the forced swim test compared to other models such as the tail suspension test. However, again from a general reading of the relevant literature it was judged that the forced swim test has received the greatest variety of non-pharmacological validation among the relevant depression models, such as sleep deprivation (Lopez-Rodriguez et al., 2004) and phototherapy (Schulz et al., 2008). Aside from pharmacological and non-pharmacological validation, practical considerations were also taken into account. In the process of conducting the experiments described below a pilot study was conducted where the tail suspension test was used as the behavioral test. Although many laboratories have reported using the tail suspension test with great success, the animals that were tested in this pilot study climbed their own tails no matter what suspension method was used, a behavior that is incompatible with the normal scoring scale for this test. It is unclear why this behavior emerged in the pilot study, but whatever the reason the test was abandoned for the current work.

The differential reinforcement of low rate behavior task was also judged to

be ill suited for the present purposes. Although the task has many strengths, it must be noted that mice can sometimes require a great deal of training to perform the kind of complex task that is entailed in this behavioral test. In part due to advice received, and in part due to the other experiments that were ongoing in the laboratory, it was decided that the benefits of the this task were outweighed by the large amount of work required and the limited access to the operant testing chambers that are necessary for this type of work. Therefore, the chief specific reason that the forced swim test was used was that its pharmacological and non-pharmacological validation were seen as compelling strengths of the assay compared to the other relevant models of depression.

The 5α-reductase inhibitor finasteride

The drug finasteride is often used to decrease allopregnanolone or other steroids[3]. There are three known isozymes of the enzyme 5α-reductase, also sometimes called 5α-steroid reductase. Finasteride interacts with both the type I and type II 5α-reductase isozymes, but in a species-dependent manner. It is currently unknown whether finasteride inhibits the type III isozyme (Uemura et al., 2008). In humans, finasteride binds with much greater affinity for the type II isozyme compared to the type I isozyme, but in rodents finasteride comparably inhibits both type I and type II isozymes.

These isozymes catalyze the A-ring reduction of 11-deoxycorticosterone, progesterone, and testosterone, into 5α-dihydrodeoxycorticosterone (5α-DHDOC), 5α-dihydroprogesterone (5α-DHP), and 5α-dihydrotestosterone (5α-DHT),

[3] Portions of this section are based on the reviews by Finn et al., 2006, and Mellon & Griffin, 2002.

respectively. Further reduction of these steroids results in the production of allotetrahydrodeoxycorticosterone (5α-THDOC), allopregnanolone, and androstanediol, respectively, which are positive modulators of the $GABA_A$ receptor (see Figure 1). Thus, finasteride can prevent the production of these $GABA_A$-receptor modulating neurosteroids by removing their metabolic precursors. This is the basis of the metabolic withdrawal method that will be discussed below.

Acute and withdrawal effects of neuroactive steroids on anxiety- and depression-like behaviors

A great deal of research has highlighted the roles that steroids, particularly allopregnanolone, may play in anxiety and depression. Frye and coworkers (2004) found that progesterone administration decreases forced swim test immobility in wildtype mice but not in mice missing a functional *Srd5a1* gene that encodes the type I 5α-reductase enzyme. Since the 5α-reductase enzymes are necessary to convert progesterone into allopregnanolone, this study suggests that the reason the gene-deficient mice failed to show a decrease in swim test immobility was that they were unable to produce allopregnanolone from the abundant supply of progesterone. Therefore, this study provides indirect evidence that allopregnanolone may have an antidepressant effect in the forced swim test. In a complementary study, intra-amygdala injections of the 5α-reductase inhibitor finasteride increased forced swim test immobility in rats, suggesting that acute decreases in allopregnanolone levels were associated with increased depression-like behavior (Walf et al., 2006). Another research group began by injecting rats with allopregnanolone, which resulted in decreased forced swim test immobility.

1

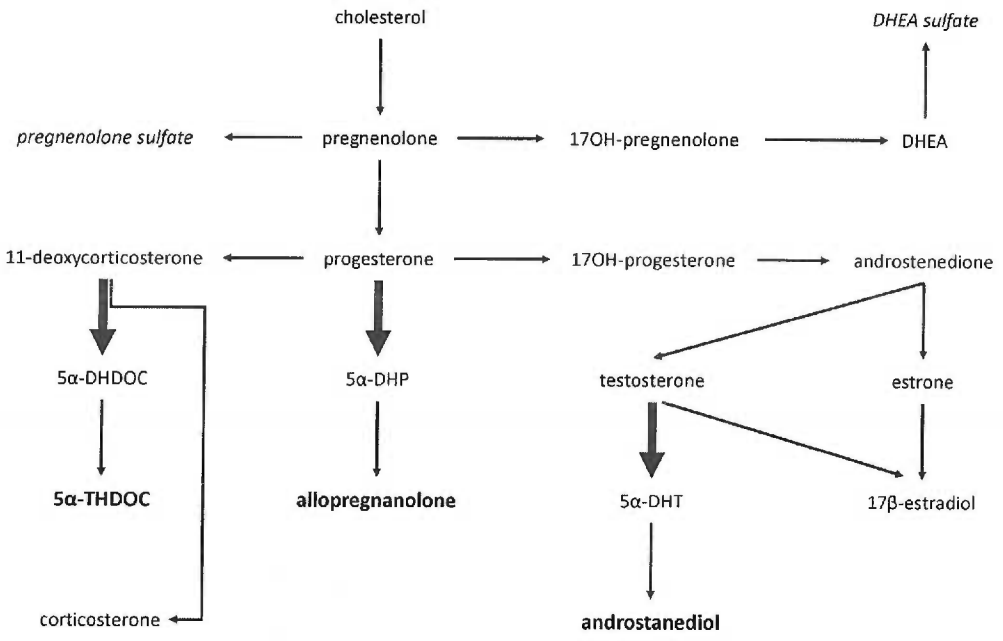


Figure 1. Biosynthetic pathway showing some key endogenous steroids. Many details are omitted for simplicity. Heavily weighted arrows show reactions catalyzed by 5α-reductase isozymes. Steroid names set in boldface are positive modulators of $GABA_A$ receptors, and names set in italics are negative modulators. Adapted from Finn et al., in press, and Mellon & Griffin, 2002.

However, when the animals were administered picrotoxin, a $GABA_A$ receptor antagonist, the antidepressant-like effect of allopregnanolone on swim test immobility was blocked (Rodríguez-Landa et al., 2007). In additional studies using the elevated plus maze, an anxiolytic effect of progesterone was observed in mice lacking the nuclear progesterone receptors A and B, suggesting that these intracellular progesterone receptors were not necessary for progesterone's anxiolytic effect (Frye et al., 2006; D. S. Reddy et al., 2005).

Fewer studies have used hormone withdrawal preparations to study emotional behaviors in rodents. Several methods have been developed to model hormone withdrawal in laboratory animals. There is no standardized nomenclature for the different methods, but several terms are proposed here to distinguish among these methods (see Table 1).

M. Bernardi and coworkers (1989) may have been the first to note that hormone withdrawal increased depression-like behavior in mice. Mice underwent surgery to remove their ovaries (ovariectomy), and following a recovery period showed increased immobility in the tail suspension test relative to mice that had undergone a sham surgery. Similar studies have used surgical and passive methods in mice and rats to induce increases in forced swim test immobility (Bekku & Yoshimura, 2005; Bekku et al., 2006; Stoffel & Craft, 2004), as well as anxiety-like behavior (Bitran & S. S. Smith, 2005), or both (Chaves et al., 2009). One example is the study by Galea and colleagues (2001) who used exogenous hormones to simulate pregnancy in rats and found an increase in forced swim test immobility during hormone withdrawal. A later study (A. D. Green et al., 2009) documented

Table 1: Methods of Inducing Hormone Withdrawal in Laboratory Animals

Withdrawal type	Method
Passive	Administer hormones for a period of time, then discontinue administration and allow hormones to decrease as a result of endogenous clearance mechanisms
Metabolic	Administer precursor hormones for a period of time, then co-administer drugs to inhibit metabolism of the precursor, thus withdrawing hormone of interest by blocking its production
Surgical	Allow the animal to produce its own hormones (often after inducing pseudopregnancy), then surgically remove the steroidogenic glands
Estrous cycle dependent	Allow the animal to produce and withdraw from its own hormones according to its estrous cycle
Precipitated	Administer hormones for a period of time, then co-administer drugs that antagonize the target receptors of interest[a]

Note. [a]The precipitated method of withdrawal is one of the primary methods that is

employed in the experiments reported here. The current use of the term "precipitated withdrawal" is generally consistent with models where a receptor antagonist is used to induce a withdrawal syndrome without actually withdrawing the substance in question (e.g., precipitated cannabis withdrawal, Budney & Hughes, 2006; precipitated opioid withdrawal, Sadée et al., 2005). However, an important point should be explained regarding the present usage of this term. As previously discussed, allopregnanolone is active at multiple receptors in the brain; but, among them, the drug picrotoxin is only an antagonist for the $GABA_A$ receptor. Therefore, although the use of the term "precipitated withdrawal" is generally consistent with other areas of research, a point of divergence is that only one mechanism of action of allopregnanolone is thought to undergo precipitated withdrawal using this method. Therefore, rather than refer to picrotoxin treatment as "precipitated allopregnanolone withdrawal," the current document uses terminology such as "precipitated $GABA_A$ receptor withdrawal" or "withdrawal of $GABA_A$ receptor activity" to specify that only this one action of allopregnanolone is blocked by this treatment. Framing withdrawal in terms of the receptor also properly acknowledges that allopregnanolone is not the only signal that is blocked by picrotoxin. By blocking the ion channel of the $GABA_A$ receptor, picrotoxin interferes with all $GABA_A$ receptor activity regardless of the ligand, including endogenous GABA. Nonetheless, it should be acknowledged that $GABA_A$ receptor activity cannot be "withdrawn" in quite the same sense that a drug or steroid can be withdrawn. "Withdrawal of $GABA_A$ receptor activity" is therefore used for lack of a better phrase.

decreased sucrose consumption in a subset of animals during hormone withdrawal, supporting the notion that this hormone-simulated pregnancy procedure increased symptoms like depression.

Similarly, post-parturition rats have been shown to exhibit increased response rates compared to pregnant rats in the differential reinforcement of low rate behavior model of depression (Molina-Hernández et al., 2000; see also de Brito Faturi et al., 2006), resulting in decreased reinforcements (a depression-like behavioral pattern). This behavior pattern could reflect a rodent form of post-partum depression. Other animal models have supported roles for progesterone withdrawal or other hormone withdrawal regimens in emotional or other behaviors of laboratory animals. Included are findings with anxiety (Löfgren et al., 2006), changes in exploratory behavior (Löfgren, 2009), changes in pain sensitivity (Devall et al., 2009) and changes in aggression or irritability (Schneider & Popik, 2006, 2007). Many methodological differences exist among these studies (such as the method of hormone withdrawal, the duration of initial hormone exposure, the length of withdrawal period), but the cited studies are all alike in that they used steroid hormone withdrawal experimental preparations, and they all report increases in behavior that are consistent with symptoms of reproductive depression.

Hypotheses

There are two principle hypotheses that were tested in the present work. Hypothesis 1: *removing or blocking progesterone signal in mice will increase depression-like behavior as assessed by the forced swim test.* This first hypothesis will

be referred to generally as the progesterone withdrawal hypothesis. Hypotheses 2: *removing allopregnanolone or blocking $GABA_A$ receptor signal will increase depression-like behavior as assessed by the forced swim test.* This hypothesis will generally be referred to as the allopregnanolone withdrawal hypothesis.

Chapters 3-5 will test the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis. The work described here also tests the following sub-hypotheses:

- Steroid withdrawal such as progesterone or allopregnanolone withdrawal increases forced swim test immobility because the activity of a specific receptor has decreased. Therefore, it is hypothesized that causing a precipitated withdrawal by using receptor antagonist drugs will result in similar changes in forced swim test immobility compared to the passive withdrawal methods (Chapter 4).
- Steroid withdrawal may increase forced swim test immobility because of loss of activity of a receptor, or as a result of loss of more than one receptor. Therefore, it is hypothesized that blocking the signal of more than one receptor (e.g., blocking both progesterone and $GABA_A$ receptors) will result in increased forced swim test immobility compared to terminating the activity of only a single receptor (Chapter 4).
- As a component of the allopregnanolone withdrawal hypothesis, a sub-hypothesis is that allopregnanolone withdrawal increases forced swim test immobility because the removal of this inhibitory neurosteroid results in a state of rebound hyperexcitation. Correspondingly, when no

exogenous progesterone is administered it is hypothesized that blocking $GABA_A$ receptor activity will not increase forced swim test immobility (Chapter 4), because no neuroadaptations will have taken place, and therefore no withdrawal could occur.

- Forced swim test immobility is a form of decreased locomotion, but an interesting question is whether increased immobility reflects a global decrease of locomotion (which could result from non-affective neurological or musculoskeletal changes), or cognitive or motivational changes in locomotion. Increased swim test immobility may be more relevant to depression when locomotor ability is normal. From this follows the hypothesis that some treatments that increase swim test immobility do so without causing generally-suppressed locomotor activity (Chapter 4).
- Estrogen induces nuclear progesterone receptors. If progesterone withdrawal is mediated by progesterone receptors then removing estrogen by ovariectomy should prevent swim test immobility from increasing during progesterone withdrawal. Thus, it is hypothesized that progesterone withdrawal will not increase forced swim test immobility in ovariectomized mice. Administering exogenous estrogen to ovariectomized mice could induce progesterone receptors, and therefore it is hypothesized that exogenous estrogen could rescue an effect of progesterone withdrawal on forced swim test immobility (Chapter 5).
- In keeping with the idea that allopregnanolone withdrawal increases

swim test immobility by a mechanism that results in a state of rebound hyperexcitability, a corollary sub-hypothesis is that allopregnanolone withdrawal and progesterone withdrawal will increase neuronal activity (Chapter 6).

Chapter 2: General Methods

Animals

Procedures were approved by the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee, and were performed according to the guidelines of "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral research" (National Research Council, 2003). DBA/2J female mice were purchased from Jackson Laboratories—West (Davis, CA) and were delivered at approximately 8 weeks old to the Veterinary Medical Unit of the Veterans Affairs Medical Center in Portland, OR. (The importance of genotype is considered in the General Discussion.) All behavioral tests and blood and tissue collections were conducted when the mice were aged approximately 11-12 weeks old, except where noted (Experiment 2).

All mice were housed in Maxi-Miser #1 cages (Thoren Caging Systems, Hazelton, PA). Some mice were housed with Bed-o'cobs bedding (Andersons Inc., Maumee, OH) (Experiments 1-7, 14, and 16), and some mice were housed with EcoFresh bedding (Absorption Corp, Bellingham, WA) (Experiments 9-13, 15, and 17). Mice in one experiment were initially housed with Bed-o'cobs bedding but were switched to EcoFresh bedding during the first phase of the experiment (Experiment 8). Mice were maintained on 12 hr/12 hr light cycles with lights on at 0600 h except where noted (Experiments 10 and 13). Animal rooms were temperature controlled at approximately 21 ± 1 °C. Mice had unrestricted access to LabDiet 5001 Rodent Diet food (PMI International) and tap water. All mice had a

small whole punched into their ear for identification prior to any injections and had over a week to acclimate to the facilities prior to onset of injections.

Treatment scheme

All but two experiments employed similar two-phase treatment procedures. (Experiments 14 and 15 also employed an additional phase where animals were ovariectomized. Details are provided in Chapter 5.) In general the first treatment phase lasted five days and consisted of exposing mice to progesterone, progesterone plus the estrogen steroid 17β-estradiol, or allopregnanolone to achieve relatively stable concentrations of these steroids that approximated a steady-state. In some experiments certain comparison groups were tested without a withdrawal phase, but in most experiments all groups proceeded to a second phase. For mice that proceeded to the second phase of the experiment, the second phase lasted 1-3 days (typically three), during which the experimental groups received a treatment that was designed to withdraw some steroid from circulation or to block its activity at its target receptors. Additional comparison groups were sometimes included that did not receive withdrawal treatments.

Drugs

Several drugs or other compounds were employed in these experiments. The main experimental compounds and general descriptions of their characteristics are shown in Table 2. Progesterone, 17β-estradiol, mifepristone, and finasteride were purchased from Steraloids (Newport, RI), except where noted (Experiments 1 and 4). Allopregnanolone was purchased from Dr. Robert Purdy (Veterans Medical Research Foundation, San Diego, CA). Picrotoxin and ketorolac were purchased

from Sigma (St. Louis, MO). Isoflurane was purchased from Butler Animal Health Supply/Abbott Laboratories (North Chicago, IL). CDB-4124 was a gift from Dr. Ronald Wiehle on behalf of Repros Therapeutics Inc. (The Woodlands, TX). All injections of progesterone were in doses of 5 mg/kg.

Within each experiment except Experiment 3 mice were given an equal number of injections regardless of treatment condition. Within each experiment (including Experiment 3) mice received the same number of injections on any given day of the experiment. Injections of vehicle were used to equalize the number of injections across groups when some groups received more drugs than others. The volume of each injection was 10 mL/kg except where noted (see Experiments 5, 11, 12, and 15). All injections were administered intraperitoneally (ip) except where noted (see Experiments 14 and 15). All injections were administered from 1000-1230 h except where noted (see Experiments 6, 14 and 15). The vehicle for all injections other than ketorolac was 20% (mass/volume) 2-hydroxypropyl-β-cyclodextrin, supplied by either Cargill (Cedar Rapids IA, Experiments 1-9, 14, 16, and 17) or by Onbio (Richmond Hill, Ontario, Canada, Experiments 10-13 and 15), prepared in 0.9% saline solution (Baxter Healthcare, Deefield, IN). The vehicle in which ketorolac was dissolved was the same 0.9% saline. Tables below show injection schedules for each experiment.

Table 2: Names and Mechanisms of Key Drugs and Compounds Administered

Common Name (Trade Name)	General Description
17β-estradiol (various)	A key physiological estrogen with intracellular and membrane receptors. Involved in primary and secondary sex characteristics. Induces nuclear progesterone receptors.
allopregnanolone	Positive modulator of $GABA_A$ receptor.
CDB-4124 (Proellex, Progenta)	Second-generation progesterone receptor antagonist. Highly selective for progesterone receptors compared to glucocorticoid receptors[a].
finasteride (Propecia, Proscar)	5α-reductase inhibitor. Clinically used for alopecia and benign prostatic hyperplasia; decreases 5α-reduced steroids such as allopregnanolone[b].
mifepristone (Mifegyne, Mifeprex)	Original, first-generation progesterone receptor antagonist. Inhibits progesterone receptors with potency comparable to CDB-4124, but has antiglucocorticoid activity[c].
picrotoxin	$GABA_A$ receptor antagonist[d].
progesterone (various)	A key physiological progestin with intracellular and membrane receptors. Involved in primary and secondary sex characteristics. Prepares for and maintains pregnancy.

Note. [a]Attardi et al., 2002. [b]Finn et al., 2006. [c]Attardi et al., 2004. [d]Yoon et al., 1993.

Forced swim test

The forced swim test was performed in most experiments (all except Experiments 3, 13 and 17). The swim test was performed using slight modifications of the methods described by Lucki and coworkers (2001). On the last day of injections each mouse was transferred to a holding cage (singly-housed) after her last injection. Testing then occurred from 1300-1800 h, during which each mouse was tested in a clear, colorless, cylindrical water tank measuring 21.5 cm × 24.5 cm (inner diameter × inner height), which was refilled with 25 ± 2 °C tap water for each mouse to a height of approximately 15 ± 1 cm.

Consistent with common laboratory practice, subjects were dropped into the tank from a height of approximately 20 cm above the upper rim of the tank, which was sufficient to submerge the mice underwater with a high degree of uniformity. A video camera placed above the tank recorded each mouse's behavior for 6 min until she was removed and transferred to a holding cage. Each experiment was scored entirely by either of two highly trained observers that together scored all of the experiments. The inter-rater reliability between these two observers was assessed using the Pearson product-moment correlation coefficient. After a training period, the inter-rater reliability was found to be $r = .94$ ($r^2 = .88$), which was judged to be suitably high. Mice were scored for forced swim test immobility from videotape in a way that the individual condition of each mouse was unknown to the scorer. For each test, only the last 4 minutes of the 6 min test session were scored (based on the methods of Lucki et al., 2001, and Porsolt et al., 1977a). Since mice tested in the forced swim test spend most of their time swimming and exhibit bouts of

immobility of variable length, total immobility was measured with a stopwatch that enabled the scorer to record cumulative time spent immobile.

Progesterone radioimmunoassay

Plasma progesterone was measured in Experiment 3 by radioimmunoassay using a commercially available kit (Coat-A-Count progesterone kit, Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions. A 100 μL aliquot of each standard (in duplicate), sample, or diluted sample was vortexed with 1.0 mL [^{125}I]progesterone and incubated in the provided tubes coated with rabbit antibodies for progesterone at room temperature for 3 hr. Following incubation, tubes were decanted and bound radioactivity was quantified with a standard gamma-counter.

Counts per minute were fit to a least-squares regression equation produced by log-logit transformation of the standards (0.1-40 ng/mL) using Prism 4 (GraphPad Software, Inc., San Diego, CA). Intra-assay variability ranged from 0.336% to 15.1% (average 5.44%), and inter-assay variability was 13.5%. Because progesterone concentrations were expected to vary widely among treatment groups, all samples were assayed at their full concentration but some samples were additionally assayed after being diluted. To improve precision, reported plasma progesterone concentrations are based on the dilution at which counts were best contained within the linear portion of the standard curve. Some additional samples were assayed after diluting plasma with the provided buffer in 1:2 or 1:5 ratios.

Allopregnanolone radioimmunoassay

Allopregnanolone was extracted according to methods published previously

(e.g., Finn et al., 2004) based on methods originally described by Janis and coworkers (1998). Brains were sonicated for one minute in 1 mL of 0.3 N NaOH. An aliquot of [^{3}H]allopregnanolone (2000 cpm/100 µL in ethanol; PerkinElmer, Boston, MA) was added to monitor the efficiency of the extraction. Samples were sonicated in the presence of a 10% (volume/volume) mixture of ethyl acetate in heptane (Fisher, Pittsburgh, PA), and centrifuged at 1000 *g* for 2 min. The supernatant was then collected, and this process was repeated for two additional sequences of sonicating the resulting pellet, centrifuging the mixture, and collecting the supernatant. The supernatants were collected and combined for further processing. The combined supernatants were eventually gravity-filtered through solid phase silica columns (Burdick & Jackson 9056, supplied by VWR, Seattle, WA). First, 5 mL isopropanol was run through the columns to activate the silica. Next, the columns were washed twice with heptane (5 mL each wash). An additional 5 mL heptane was added to the collected supernatants, then the supernatant/heptane mixture was gravity-filtered through the columns. Finally, a 25% (volume/volume) mixture of acetone in pentane was used to elute allopregnanolone from the silica columns. The elutant was dried under nitrogen and frozen at −4 °C until the radioimmunoassay was performed.

For radioimmunoassay, extracts were reconstituted with 30 µL isopropanol and 170 µL of a sodium phosphate/bovine serum albumin radioimmunoassay buffer. Extraction efficiency was calculated by liquid scintillation spectroscopy using 50 µL of the reconstituted steroid. Of the remaining reconstituted steroid, 100 µL was used for the assay.

The allopregnanolone radioimmunoassay was conducted using [^{3}H]allopregnanolone and a polyclonal antiserum that was provided by CoCensys (Irvine, CA) at a 1:240 dilution. This antiserum has been shown to cross-react only minimally to other steroids (Finn & Gee, 1994). The methods of this radioimmunoassay are described in detail elsewhere (Finn & Gee, 1994). Briefly, a standard curve was prepared in duplicate using a range of nine masses of unlabeled allopregnanolone from 156 pg to 20 ng. Total and non-specific binding were also measured. Bovine serum albumin buffer, [^{3}H]allopregnanolone, and antiserum were added, and standards were vortexed and incubated at room temperature for 1 hr. Dextran coated charcoal was used to separate bound allopregnanolone from free allopregnanolone. Tubes were vortexed again, allowed to sit on ice for 10 min, then centrifuged at 3000 rpm for 20 min. The supernatant was removed to a scintillation vial and mixed with 5 mL scintillation fluid (Safety-Solve, Research Products International, Mount Prospect, IL). Samples were counted using a standard β-counter and averaged over 2 min. Counts per minute were fit to a standard curve by least-squares regression and log-logit transformation of the standards. Mass of allopregnanolone was calculated by interpolation of the standards and corrected for extraction efficiency and brain mass. Brains were run in two separate assays. The intra-assay variabilities were 0.474% and 2.90%. The inter-assay variability was 49.9%. Based on plasma progesterone data for these mice, some steroid extractions were diluted at 1:2 or 1:5 ratios with additional isopropanol and sodium phosphate/bovine serum albumin radioimmunoassay buffer prior to radioimmunoassay.

Corticosterone radioimmunoassay

Plasma corticosterone was measured in Experiments 14 and 17 by radioimmunoassay using the ImmuChem Double Antibody Corticosterone ^{125}I kit from MP Biomedicals (Orangeburg, NY) according to the manufacturer's instructions. Plasma was diluted 1:200 using the steroid diluent. Standards were run in duplicate (25 ng/mL-1000 ng/mL). [^{125}I]corticosterone competitive antigen was added to all samples (including standards), and antibody was added to all samples except non-specific binding samples. Tubes were vortexed and incubated at room temperature for 2 hr. Precipitant solution was added and tubes were vortexed then centrifuged at 1000 *g* for 15 min. The supernatant was aspirated, and the precipitate was counted in a gamma-counter. Counts per minute were fit to a least-squares regression equation produced by log-logit transformation of the standards (25-1000 ng/mL) using Prism 4 (GraphPad Software, Inc., San Diego, CA). The intra-assay variabilities for these assays were 0.152% and 4.14%.

Locomotor Activity

Locomotor activity was measured in Experiment 13 using Accuscan activity monitors (Accuscan Activity Instruments Inc., Columbus, OH) in 30-min sessions. After their last injections on day 8 mice were transferred to holding cages. Testing took place from 1300-1500 h, during which mice were placed into one of 16 clear acrylic boxes measuring 40 cm × 40 cm × 30 cm (length × width × height), which were in turn housed in shelters that blocked light and attenuated sound. Fluorescent 8 W light bulbs provided light inside the testing chamber, and a fan provided masking for outside noise as well as ventilation. Activity monitors had an

8 × 8 array of photocell beams and detectors raised 2 cm above the floor. Beam breaks from mouse movement were automatically recorded and calculated by Accuscan software into total distance traveled (cm).

Ovariectomy

Bilateral ovariectomy was performed in Experiments 14 and 15 according to the methods described by Gililland and Finn (2007). For each of these two experiments, all mice within a given experiment had their surgeries performed within a five-day interval and were given at least a week to recover prior to proceeding to the first treatment phase of the experiment. To perform the surgery, the mouse was first placed into an induction chamber to be anesthetized by 5% isoflurane inhalation. When the mouse became unresponsive to agitations of the chamber, she was rapidly removed and placed into a face-mask ventilator where anesthesia was maintained at approximately 2% for the remainder of the surgery. Before the surgery began, anesthesia was confirmed by toe-pinch, and isoflurane concentration was adjusted occasionally throughout the surgery based on monitoring the mouse's respiration and other responses, if any.

To begin the surgery, the mouse's fur along the dorsal surface was sheared with electric animal clippers. All invasive tools were heat sterilized between animals and held in a bath of 70% ethanol (volume/volume in water) during surgery. The incision site was disinfected with betadine and a vertical incision approximately 1.0-1.5 cm in length was made alone the dorsal midline. The looseness of the skin allowed the skin, and consequently the incision, to be gently rotated around the mouse's torso to either lateral muscle wall. For each side, a

small (approximately 4-8 mm) incision was made in the lateral musculature. The ovary and associated tissue was pulled through this hole, clamped with hemostats, and excised. The hemostats were held in place long enough to bind the tissue and prevent substantial bleeding without cauterizing the tissue. The tissue was pushed back into the abdominal cavity, and the muscle incision was closed with 4-0 chromic gut absorbable suture (Davis & Geck, Danbury, CT). After both ovaries were removed, the midline dorsal incision was closed with three 9 mm EZ Clips staple-clips (Stoelting, Wood Dale, IL). Starting on the day of the surgery, ketorolac was administered for post-operative analgesia for three days (3 mg/kg sc qd).

Brain cryoprotection

For immunohistochemistry, after the brain was removed from the mouse it was placed into ice-cold 2% (mass/volume) heat-filtered paraformaldehyde (Sigma) in 10 mM phosphate-buffered saline solution over night. Phosphate-buffered saline solutions were prepared with NaOH and $NaH_2PO_4 \cdot H_2O$ (Fisher Scientific, Fair Lakes, NJ), and NaCl (Sigma), in H_2O filtered by a Milli-Q Water System (Millipore, Billerica, MA), brought to pH 7.4. A 20% stock sodium azide (NaN_3, Sigma) solution was prepared with phosphate-buffered saline and subsequently diluted 1:1000 to a final concentration of 0.1% (mass/volume) in phosphate-buffered saline. After overnight fixation in 2% paraformaldehyde solution, brains were cryoprotected by serial sucrose saturation. Brains were first bathed overnight in a 20% sucrose (EM Science, Cherry Hill, NJ) solution (in phosphate-buffered saline with 0.1% sodium azide), then bathed overnight in a 30% sucrose solution (in phosphate-buffered

saline with 0.1% sodium azide) where they remained until sectioning was performed.

Brain sectioning

To collect brain sections, brains were mounted by the brain stem or cerebellum in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, CA) and allowed to fully freeze for at least 1 hr prior to cutting. Serial 40 µm coronal sections were made at approximately −22 to −20 °C with a Leica CM1850 cryostat. After sectioning was performed, slices were stored under refrigeration in phosphate-buffered saline with 0.1% sodium azide until immunohistochemistry was performed.

Immunohistochemistry

Prior to antibody labeling, sections were chosen from among all collected sections based on neuroanatomical landmarks visible to the unaided eye. Labeling was performed in multiple cohorts, with typically 6-8 sections from each subject in each labeling cohort. Sections were typically chosen from approximately one out of every three serial 40 µm sections. Sections that were selected were transferred to Netwell 15 mm inserts with 500 µm mesh, which were nested in 12-well culture plates (Corning Incorporated, Corning, NY) filled with phosphate-buffered saline with 0.1% sodium azide until the immunohistochemistry was begun.

All immunohistochemistry was performed at room temperature, and except for minimal handling time between solutions the sections remained in well plates atop an operating orbital shaker. Sections were washed three times in phosphate-buffered saline for 5 min each, then treated with 0.3% H_2O_2 (VWR, Chester, PA) in

phosphate-buffered saline for 15 min, followed by three additional 5-min phosphate-buffered saline washes. Non-specific binding was minimized and cell membranes were simultaneously permeablized by incubating sections with normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline with Triton X (0.3% volume/volume Triton X-100 in phosphate-buffered saline, VWR) for 4 hr. Sections were then incubated overnight with primary c-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2000 in phosphate-buffered saline with Triton X and bovine serum albumin (0.1% mass/volume bovine serum albumin in phosphate-buffered saline with Triton X, Sigma).

On the second day, sections were washed three times for 5 min each in phosphate-buffered saline. Sections were then incubated for 1 hr with 1:200 biotinylated anti-rabbit secondary antibody (Vector Laboratories) in phosphate-buffered saline with Triton X. Following three additional washes with phosphate-buffered saline for 5 min each, sections were placed in a solution that was 1:200 in each of the avidin and biotinylated enzyme solutions from a Vectastain ABC kit (Vector Laboratories) for 1 hr. Sections were washed in phosphate-buffered saline three times for 5 min each, then reacted with 3-3'-diaminobenzidine in H_2O_2 buffer (Thermo Scientific, Rockford, IL) for 2 min 15 s. The reaction was terminated by washing once in H_2O. Sections were then mounted on glass microscope slides covered with a gelatin and $CrKSO_4$ coating using a mounting medium of gelatin, acetic acid, and ethanol, and allowed to air-dry overnight.

On the third day sections were prepared for coverslipping by serial dehydration. Slides were first rinsed in 70% (volume/volume in water) ethanol

(Pharmco-Aaper, Brookfield, CT), followed by 95% ethanol and 100% ethanol for 10 min each. Then slides were rinsed in mixed xylenes (Mallinckrodt, Paris, KY) for > 12 min. Slides were removed one at a time from the xylene bath. A few drops of Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI) were placed onto the slide. The coverslip was dropped onto the slide and any air bubbles were quickly removed by compressing the coverslip to push the bubbles to the edge of the slide. Slides were allowed to sit overnight for the mounting medium to harden.

c-Fos quantification

c-Fos protein expression was quantified by counting nuclei that were visibly immunoreactive. Slides were examined under a Leica DM LB2 light microscope at 100X power. All cell counting was performed by a single experimenter who was blind to the experimental condition of the individual mice. Each brain region was quantified unilaterally. By default, the side that was counted was on the right side of the slide, but since the sections were stained as floating sections it is presumed that the right and left hemispheres were randomly represented among the cell counts. If the right side structure was damaged or for some other reason difficult to quantify, the left side was counted instead. However, mere absence of cells was not sufficient reason to count the contralateral side, and if the immunohistochemistry could be observed to have been successful in other brain regions of the same slice it was accepted as valid if no cells or few cells in the region of interest were immunoreactive for c-Fos.

Prior to counting each region, a few slides were first given a preliminary examination to familiarize the rater with the topology. The slides were then compared to the mouse brain atlas of Paxinos and Franklin (2001), and visual borders for each brain region were defined. Within each brain region, a "cell" was counted if there was a visible dark spot that was judged visually to be a nucleus that had been immunohistochemically labeled. Nuclei were judged comparatively; their shape and size were required to be consistent with other nuclei, they had to be distinguishable from debris, and their labeling intensity had to be consistent with other slides (although a reasonable allowance was made for individual differences with each of these factors). Each section was rated to have been cut at a certain distance from bregma (expressed in mm) based on which micrograph it most-closely resembled from the atlas of Paxinos and Franklin. After assessing the distance from bregma the number of c-Fos-positive cells was counted and recorded for each region of interest within each section. Brain regions analyzed are compiled in Table 19. Hand-held counting devices provided an easy way to pay full attention to the cells without losing track of the count.

c-Fos count data processing

After all sections for a given brain region had been counted, data were compiled and a preliminary review took place. Because usable sections were not available for all mice for all brain regions at all distances from bregma, for some anatomical brain regions there were rostrocaudal sections defined as sub-regions. For these areas, the number of c-Fos-positive cells in the brain structure were averaged across all available sections for a single mouse. This average was then

treated as the subject's count for that brain region at that bregma range (sub-region) when the inferential statistics were calculated. At other levels, sufficient numbers of sections were available for enough mice for analysis at that specific bregma distance. The bregma ranges or specific bregma levels that were analyzed are shown in Table 21. One-way analysis of variance (ANOVA) was performed for each of these ranges of levels or specific levels. For brain regions where two rostrocaudal sub-regions were analyzed, if no omnibus significant difference was confirmed by ANOVA then a new average count for each mouse was calculated by taking the average c-Fos positive cell count from all available sections for that mouse, and these new averages were then analyzed by one-way ANOVA.

Statistical analyses

One-way or two-way ANOVA was used for all experiments except where noted. Post hoc tests were performed only if justified by an omnibus significant difference from one-way ANOVA or a significant interaction from two-way ANOVA. The α-level was set at .05 so that *p*-values less than .05 were considered significant. For purposes of discussion, estimated partial omega squared ($\tilde{\omega}_p^2$) was calculated to estimate the effect size for Experiment 10 as described by Sheskin (2007) according to equation #27.60. However, since this equation assumes a balanced design, *N* was substituted for *nqp*, which is equivalent to *N* in a balanced design but undefined in an unbalanced design. The result of substituting *N* for *nqp* is equivalent to the equation suggested by Runyon and coworkers (2000) to calculate what they called "omega square" (ω^2), but here these calculations are reported using the terminology suggested by Sheskin (see Sheskin 2007, page 1146 for details).

The original number of mice (*n* values) planned for each group of each experiment is shown in the table accompanying its respective experiment. Some mice that were originally slated for experiments were not included in the final analysis for various reasons. The final numbers of mice included in the analyses are shown in the legends of the figures that accompany each experiment. Some of the mice were removed based on automated outlier analyses performed in SYSTAT (version 11) based on Studentized residuals. Where mice were removed for reasons other than automated statistical analysis the reason for removal is also given in the relevant figure legend. For radioimmunoassays, when the counts per minute returned from the gamma-counter or β-counter were outside the range given by the standards (calibrators) then it was deemed that the sample could not to be fit to the standard curve.

Chapter 3: Passive Progesterone Withdrawal and Metabolic Allopregnanolone Withdrawal

Chapter 3 Introduction

These experiments tested the hypothesis that progesterone withdrawal and allopregnanolone withdrawal result in depression-like behavior in intact female mice, measured by forced swim test immobility. The first experiment sought to identify the time point(s) when forced swim test immobility would increase following discontinuation of progesterone administration (i.e., a passive withdrawal procedure). The next experiment tested whether blocking the conversion of progesterone to neuroactive steroids such as allopregnanolone was sufficient to induce forced swim test immobility. This experiment used the 5α-reductase inhibitor finasteride, which causes a downstream reduction in allopregnanolone levels (e.g., Frye & Walf, 2002; D. S. Reddy et al., 2001; M. E. Rhodes & Frye, 2001; VanDoren et al., 2000; for a review see Finn et al., 2006). The third experiment used the progesterone injection regimen from the first experiment to verify that this procedure is associated with decreased plasma progesterone levels and brain allopregnanolone levels during progesterone withdrawal.

Progesterone and allopregnanolone withdrawal were accomplished in these experiments by adapting procedures previously used in electrophysiological and neurochemical studies. Costa and coworkers (1995) found that treating intact female rats with daily 5 mg progesterone injections (5 days/wk for 1-3 wks) significantly increased brain allopregnanolone levels at zero days of progesterone withdrawal, but allopregnanolone levels were diminished after one, three, or five

days of withdrawal. Dazzi and coworkers (2002) found that treating male rats with 5 mg/kg progesterone for five days resulted in increased brain levels of allopregnanolone that remained elevated 25-30 hr after the fifth injection. Taken together, it was predicted that a 5 mg/kg treatment regimen in intact female mice would result in a withdrawal syndrome after three days, if not after one day.

It was predicted that forced swim test immobility would be increased during passive progesterone withdrawal (Experiment 1). The first experiment was designed both to test the progesterone withdrawal hypothesis and to validate the general procedure that is used throughout these experiments. It was also predicted that swim test immobility would be increased during metabolic allopregnanolone withdrawal (Experiment 2). If depression-like behavior increased during these treatments, these data would support the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis. It was also predicted that passive progesterone withdrawal (Experiment 3) would decrease plasma concentrations of progesterone and brain concentrations of allopregnanolone.

Chapter 3 Experiments

Experiment 1—Methods—Passive progesterone withdrawal and forced swim test

The purpose of this experiment was to test the progesterone withdrawal hypothesis by determining if and when passive progesterone withdrawal would increase depression-like behavior in the forced swim test. It was hypothesized that the passive progesterone withdrawal method would increase forced swim test immobility, which would support the progesterone withdrawal hypothesis. Mice were injected with progesterone (5 mg/kg) once daily for five days and tested in the

forced swim test on the same day as the last injection of progesterone ("0 d WD" group), or one day, two days, or three days ("1 d WD," "2 d WD," and "3 d WD" groups, respectively) after the last progesterone injection. A fifth group ("VEH" group) received vehicle injections for five days and was then tested in the forced swim test. Table 3 shows the daily schedule of progesterone or vehicle injections, and indicates the days of forced swim testing for each group. D ifferent groups were thus tested on different days of the week. Progesterone in this experiment was purchased from Sigma (St. Louis, MO).

Experiment 1—Results—Passive progesterone withdrawal and forced swim test

The omnibus test revealed that forced swim test immobility differed among treatment groups, $F_{4,60} = 3.97$, $p < .05$ (Figure 2). Tukey comparisons revealed that the 3 d WD group exhibited significantly more immobility in the forced swim test than each of the other groups. No other difference was found among these groups.

Table 3: Experiment 1 Injection and Forced Swim Test Schedule

	Days				
Group	1-4	5	6	7	8
VEH	VEH	VEH, FST			
0 d WD	PRO	PRO, FST			
1 d WD	PRO	PRO	FST		
2 d WD	PRO	PRO		FST	
3 d WD	PRO	PRO			FST

Note. Drug-injection abbreviations used: PRO, progesterone (5 mg/kg); VEH, vehicle. "FST" indicates the day of forced swim testing. Initial sample sizes were n = 12 for VEH and 0 d WD groups, and n = 14 for 1 d WD, 2 d WD, and 3 d WD groups.

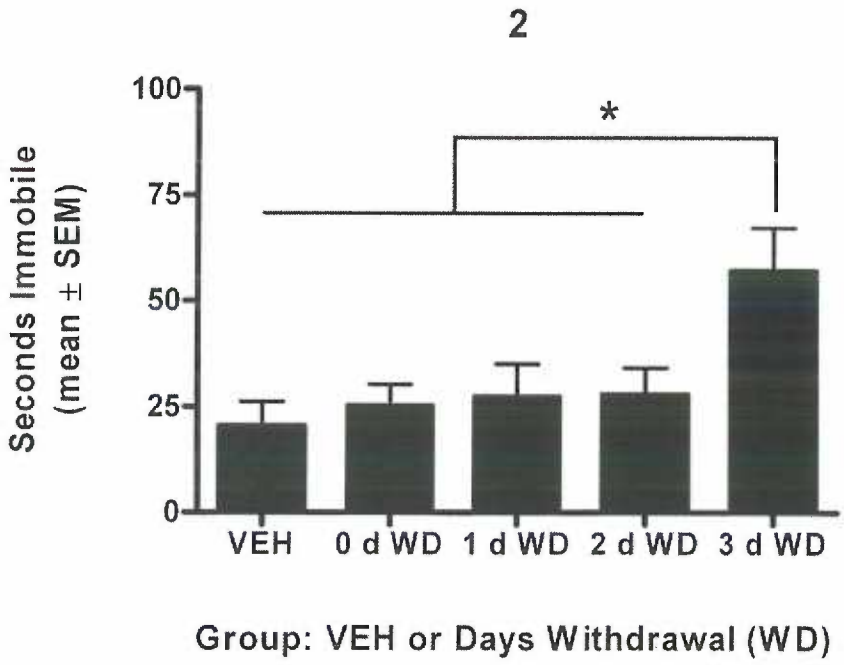


Figure 2. Mean (± SEM) seconds of immobility in the forced swim test in mice following different intervals of withdrawal (group labels) from repeated 5 mg/kg injections of progesterone, or following repeated vehicle injections. * $p < .05$ compared to all other groups by Tukey post hoc tests. Final sample sizes were n = 12 for the VEH and 0 d WD, n = 13 for the 1 d WD group, and n = 14 for the 2 d WD and 3 d WD groups.

Experiment 2—Methods—Metabolic allopregnanolone withdrawal and forced swim test

The purpose of this experiment was to test the allopregnanolone withdrawal hypothesis by determining whether using finasteride to achieve metabolic allopregnanolone withdrawal would mimic the increase in forced swim test immobility observed during progesterone withdrawal. It was hypothesized that the metabolic allopregnanolone withdrawal method would increase forced swim test immobility, which would be consistent with the allopregnanolone withdrawal hypothesis. This experiment also tested the underlying principle of this hypothesis that different methods for terminating the steroid signal should result in similar changes in forced swim test immobility. DBA/2J mice aged approximately 12-13 weeks on test day were used. On days 1-5 of the experiment, mice received daily injections of progesterone (5 mg/kg). Then on days 6-8 of the experiment, separate groups of mice received daily injections of progesterone with additional injections of vehicle ("0 mg/kg" group), 50 mg/kg finasteride ("50 mg/kg" group), or 100 mg/kg finasteride ("100 mg/kg" group). All mice were tested in the forced swim test on day 8 of the experiment, approximately 2-4 hours after the last injection. Table 4 shows the daily schedule of progesterone, vehicle, or finasteride injections, and indicates the day of testing for all groups. The doses of finasteride that were chosen have previously been shown to significantly decrease allopregnanolone concentrations in rats following a systemic progesterone injection (e.g., M. E. Rhodes & Frye, 2005) or in pseudopregnant rats (e.g., D. S. Reddy et al., 2001).

Experiment 2—Results—Metabolic allopregnanolone withdrawal and forced swim test

An overall difference was found among the finasteride dose groups, $F_{2,33} = 5.18$, $p < .05$. Post hoc Tukey tests revealed that 100 mg/kg finasteride significantly increased immobility in the forced swim test, when compared to the 50 mg/kg or 0 mg/kg doses (Figure 3). There was no difference in immobility detected between the 0 mg/kg and 50 mg/kg doses.

Table 4: Experiment 2 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
0 mg/kg	PRO	PRO, VEH	PRO, VEH, FST
50 mg/kg	PRO	PRO, FIN	PRO, FIN, FST
100 mg/kg	PRO	PRO, FIN	PRO, FIN, FST

Note. Drug-injection abbreviations used: PRO, progesterone (5 mg/kg); VEH, vehicle; FIN, finasteride (doses indicated by group names). "FST" indicates the day of forced swim testing. Initial sample sizes were n = 12 in each group.

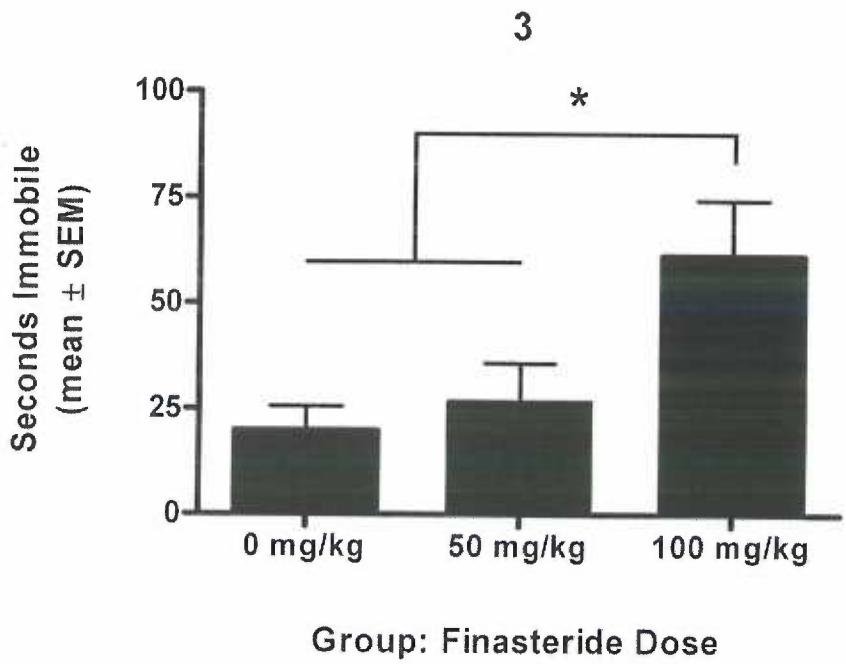


Figure 3. Mean (± SEM) seconds of immobility in the forced swim test in mice following repeated 5 mg/kg ip injections of progesterone, co-administered with different doses of finasteride (group labels). * $p < .05$ difference between 100 mg/kg group and each other group. Final sample sizes were $n = 12$ in each group.

Experiment 3— Methods—Progesterone and allopregnanolone levels

The purpose of this experiment was to characterize plasma progesterone and brain allopregnanolone concentrations that are achieved by the passive progesterone withdrawal method of Experiment 1. Plasma progesterone levels and brain allopregnanolone levels were measured in mice at three time points following a single 5 mg/kg ip injection ("0.5 hr," "2 hr," and "8 hr" groups), or at three time points following repeated daily injections of 5 mg/kg progesterone ip for 5-6 days ("0 d PWD," "1 d PWD," and "3 d PWD" groups). Blood and brains were collected from the 0 d PWD group two hours after the last progesterone injection. Mice in the 1 d PWD and 3 d PWD groups received daily vehicle injections for one or three days (respectively) following the last progesterone injection, and blood and brains were collected from these groups two hours after the last vehicle injection. A seventh group ("VEH") was used to determine average plasma progesterone levels in mice receiving daily ip injections of vehicle for six days. Blood and brains were collected from the VEH group two hours after the last vehicle injection. Table 5 shows the daily schedule of progesterone or vehicle injections, and the interval after which blood was collected following the last injection.

Mice were decapitated at the times indicated below. Trunk blood was collected into Vacutainer (BD, Franklin Lakes, NJ) 4 mL tubes containing 7.2 mg EDTA on ice, which were then centrifuged at 850 *g* and 4 °C for 20 min. Following centrifugation, the plasma fraction was aspirated and stored in separate tubes at −80 °C until assayed. At the same time when blood was collected (see Table 5)

Table 5: Experiment 3 Injection and Blood/Brain Collection Schedule

	Days						
Group	1	2-4	5	6	7	8	9
0.5 hr	PRO (0.5 hr)						
2 hr	PRO						
8 hr	PRO (8 hr)						
0 d PWD	PRO	PRO	PRO				
VEH	VEH	VEH	VEH	VEH			
1 d PWD	PRO	PRO	PRO	PRO	VEH		
3 d PWD	PRO	PRO	PRO	PRO	VEH	VEH	VEH

Note. Drug-injection abbreviations used: PRO, progesterone (5 mg/kg); VEH, vehicle. Blood and brains were collected 2 hr after the last injection shown except where noted in parentheses. One additional mouse was included in the 0 d PWD group, for brain allopregnanolone only, that was euthanized after six daily injections of progesterone rather than five daily injections. Initial sample sizes were $n = 8$ in each group.

brains were rapidly removed from the animals' skulls and frozen immediately in microcentrifuge tubes on dry ice, and then moved to storage at −80 °C until allopregnanolone extraction was performed (see General Methods).

Experiment 3—Results—Progesterone and allopregnanolone levels

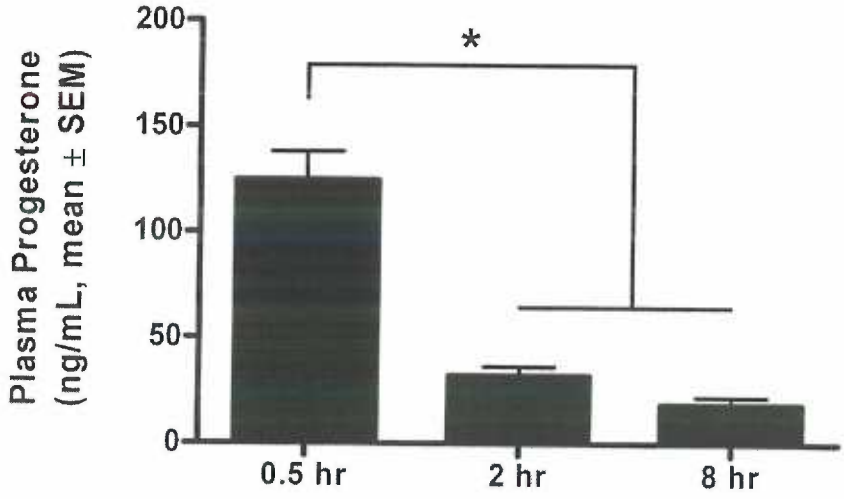
Within each physiological measure (brain allopregnanolone or plasma progesterone) separate analyses were performed for mice that received a single injection of progesterone and for mice that received repeated progesterone or vehicle injections. Although a detailed discussion is beyond the scope of the present work, essentially the separate ANOVAs were performed because the calculations in Tukey post hoc tests take into consideration the total variation present in an entire analysis, so the huge variations between groups that were sampled after only a single injection of progesterone would otherwise have prevented the ability of the Tukey test to detect the relatively-small differences among the other groups by artificially inflating the threshold for a group-mean difference to be considered significant.

An overall difference was found among plasma progesterone concentrations taken at different time points following a single injection of progesterone, $F_{2,14} = 55.0$, $p < .05$. Post hoc Tukey tests confirmed that plasma progesterone was significantly increased at 0.5 hr after injection, compared to the 2 hr and 8 hr groups. However, progesterone levels did not differ significantly between 2 hr or 8 hr after injection (Figure 4a). An omnibus significant difference was also confirmed by ANOVA of the groups that received multiple progesterone or vehicle injections, $F_{3,20} = 24.7$, $p < .05$ (Figure 4b). Progesterone levels were significantly higher in the

0 d PWD group compared to the 1 d PWD group, the 3 d PWD group, and the VEH group, but no other significant differences were detected among the multiple-injections groups.

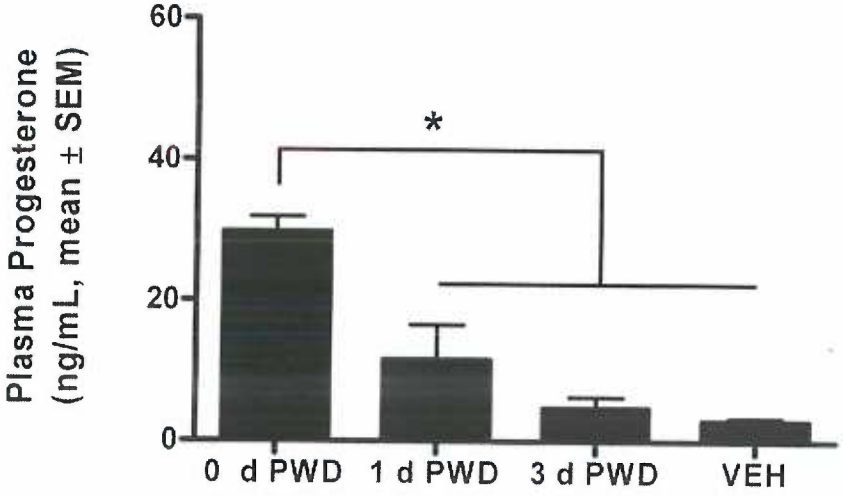
Overall differences were detected among brain allopregnanolone concentrations among mice euthanized at different time points after a single progesterone injection, $F_{2,19} = 52.3$, $p < .05$ (Figure 4c). Tukey post hoc tests showed that each group had different allopregnanolone concentrations from the other groups that received only one injection of progesterone. Differences in brain allopregnanolone also emerged among mice that received multiple injections of progesterone or vehicle, $F_{3,27} = 11.6$, $p < .05$. Post hoc Tukey tests showed that allopregnanolone concentrations were higher in the brains of mice that were allowed no withdrawal (0 d PWD group) compared to the other groups, but no other significant differences emerged (Figure 4d).

4a

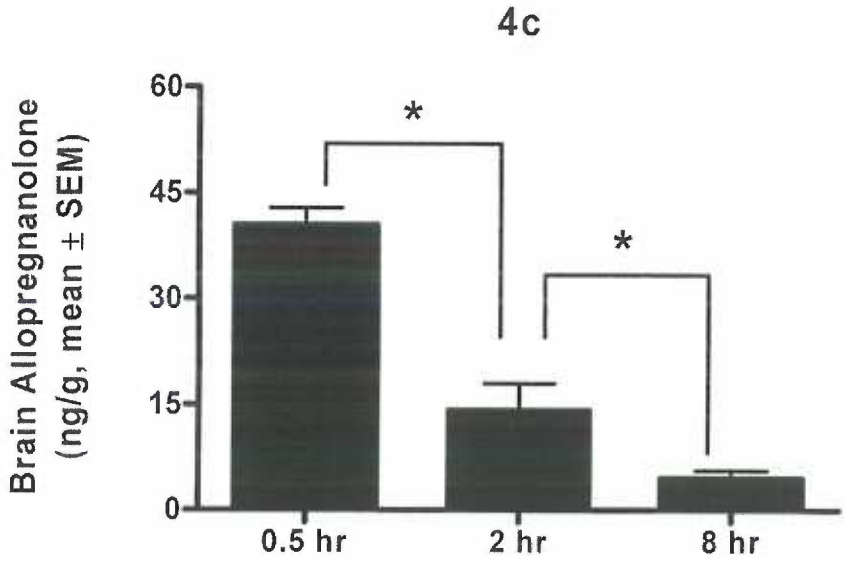


Group: Time After Single Injection

4b



Group: Days Withdrawal (PWD) or VEH


4c
Brain Allopregnanolone
(ng/g, mean ± SEM)
60
45
30
15
0
*
*
0.5 hr
2 hr
8 hr
Group: Time After Single Injection

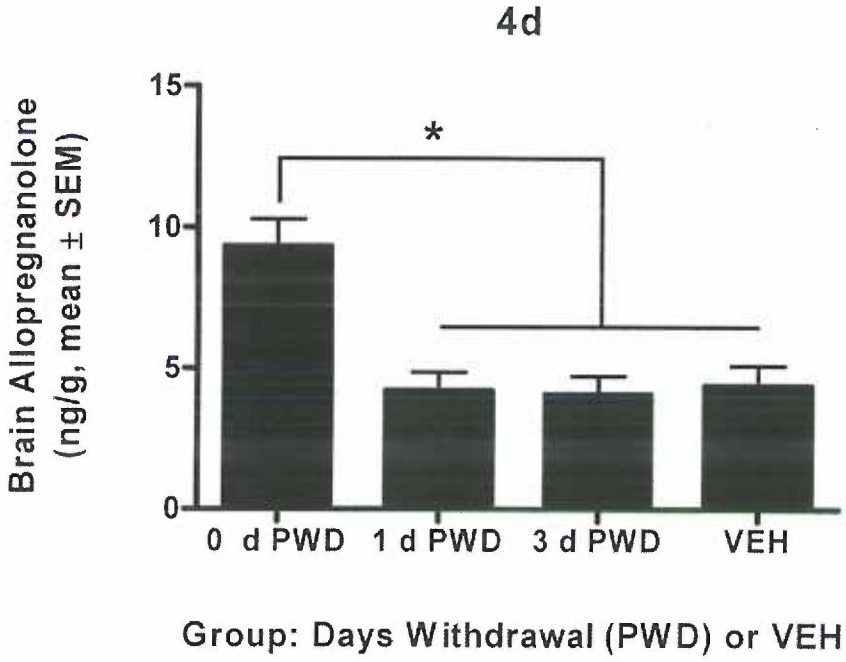

4d
Brain Allopregnanolone
(ng/g, mean ± SEM)
15
10
5
0
*
0 d PWD
1 d PWD
3 d PWD
VEH
Group: Days Withdrawal (PWD) or VEH

Figure 4. Mean (± SEM) steroid concentrations. (a) Plasma progesterone concentrations (ng progesterone per mL plasma) in mice that received a single injection of progesterone and were euthanized after various delays following injection. * $p < .05$ difference between the 0.5 hr group and both other groups. (b) Plasma progesterone concentrations among mice that received multiple injections of progesterone and were then allowed to withdraw passively for 0, 1, or 3 days, or which received multiple injections of vehicle. * $p < .05$ difference between the 0 d PWD group and remaining groups. (c) Brain allopregnanolone concentrations (ng allopregnanolone per g of whole-brain tissue) corresponding to the groups in panel 4a. * Each group had significantly different allopregnanolone concentrations from each other group, $p < .05$. (d) Brain allopregnanolone concentrations corresponding to the groups in panel 4b. * $p < .05$ difference between the 0 d PWD group and remaining groups. Final sample sizes were dependent on each group as well as which physiological measure. The sample sizes for the 0.5 hr group were $n = 5$ for plasma progesterone (three mice were not included because counts could not be fit to a standard curve) and $n = 7$ for brain allopregnanolone (one count could not be fit to a standard curve). The sample sizes for the 2 hr group were $n = 6$ for plasma progesterone (one blood sample was unusually hemolyzed and one sample could not be fit to a standard curve) and $n = 7$ for brain allopregnanolone (one count could not be fit to a standard curve). The sample sizes for the 8 hr group were $n = 6$ for plasma progesterone (two counts could not be fit to a standard curve) and $n = 8$ for brain allopregnanolone. The sample sizes for the VEH group were $n = 6$ for plasma progesterone (two counts could not be fit to a standard curve) and $n = 7$ for brain

allopregnanolone (one count could not be fit to a standard curve). The sample sizes for the 0 d PWD group were $n = 6$ (two counts could not be fit to a standard curve) and $n = 9$ for brain allopregnanolone (original $n = 8$, plus one additional mouse that was included that was intended for the 1 d PWD group but received an additional injection of progesterone on day 6 of the experiment and was euthanized 2 hr later). Sample sizes for the 1 d PWD group were $n = 5$ for plasma progesterone (one mouse was euthanized on day 6 and was included in the 0 d PWD group, and counts for two mice could not be fit to a standard curve) and $n = 7$ for brain allopregnanolone (one mouse was moved to the 0 d PWD group). Sample sizes for the 3 d PWD group were $n = 7$ for plasma progesterone (insufficient plasma was collected from one mouse) and $n = 8$ for brain allopregnanolone.

Chapter 3 Discussion

These experiments were designed to validate the passive progesterone withdrawal method and to test the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis. The passive progesterone withdrawal experiment (Experiment 1) showed that three days of progesterone withdrawal was required to detect a significant increase in forced swim test immobility. This finding is consistent with reports of delayed increases in forced swim test immobility following steroid withdrawal (e.g., Bekku et al., 2006; Stoffel & Craft, 2004), and of increased depression-like behavior in rats at three days postpartum, as assessed in the differential reinforcement of low response-rate model of depression (Molina-Hernández et al., 2000).

In the case of Stoffel and Craft (2004), rats received progesterone and estradiol injections, separately or in combination, for a period of more than three weeks. Bekku and colleagues (2006) used multiple strains of mice (ICR, C57BL/6J, DBA/2N, and CD-1) and assessed the effect of ovariectomy on forced swim test immobility. Although the results of these studies varied from the current data in some ways (most notably, the length of withdrawal time necessary to observe a depression-like response), such differences may be the result of differences in methodology. Importantly, despite dramatic differences in methods, each of these studies reported an effect of steroid withdrawal on forced swim test immobility that is consistent with the current work.

Some reports have suggested that estrogen and progesterone concentrations are normal in women with premenstrual syndrome (Halbreich, 2003). Thus, it is of

interest to study other steroids such as allopregnanolone. One potential mechanism through which progesterone withdrawal might result in depression is through a corresponding decrease in its $GABA_A$ receptor-modulating metabolite allopregnanolone. This hypothesis was tested by using a metabolic allopregnanolone withdrawal procedure. In this procedure, the 5α-reductase enzyme inhibitor finasteride was administered to produce a concomitant decrease in 5α-dihydroprogesterone and, in turn, allopregnanolone. In the metabolic allopregnanolone withdrawal experiment (Experiment 2), mice that received 100 mg/kg finasteride and progesterone exhibited a significant increase in forced swim test immobility compared to mice that received continuous progesterone or the lower dose of finasteride. Also, the magnitude of increase in immobility following 100 mg/kg finasteride administration was consistent with the magnitude of increased immobility produced by three days of progesterone withdrawal. Since progesterone levels should not be decreased in the mice receiving progesterone and finasteride injections, these results demonstrate that co-administration of the 5α-reductase inhibitor finasteride can mimic the behavioral effects of progesterone withdrawal without altering progesterone levels or inducing progesterone withdrawal. This is presumably because its administration metabolically withdraws the 5α-reduced neurosteroids such as allopregnanolone. This finding supports the hypothesis that the effect of progesterone withdrawal on forced swim test immobility is mediated through allopregnanolone withdrawal.

Experiment 3 was used to confirm the effects of the passive progesterone withdrawal procedure on plasma progesterone levels and brain allopregnanolone

levels. Plasma progesterone concentrations were significantly but transiently increased at 0.5 hr after injection compared to 2 hr and 8 hr time points. Additional analyses revealed that plasma progesterone levels had remained elevated at 2 hr after injection when compared to levels observed in mice treated with vehicle or withdrawn for three days. Progesterone levels two hours after a single progesterone injection (2 hr group) or two hours after repeated progesterone injections (0 d PWD group) did not differ in follow-up analyses, suggesting that the kinetics of progesterone were not altered during the extended injection regimen. This experiment also demonstrated a significant decrease in plasma progesterone levels at one or three days of withdrawal from repeated progesterone injections.

The brain allopregnanolone levels generally reflected the plasma progesterone levels. Some minor differences were observed (for example, compare the significant difference in allopregnanolone between the 2 hr group and the 8 hr group, a difference which is not significant for plasma progesterone). However, these discrepancies are minor differences compared to how similar the findings were between these steroids. These findings indicate that there is not a simple relationship between progesterone or allopregnanolone levels and forced swim test immobility, because plasma progesterone was already decreased after one day of withdrawal (in Experiment 3, Figure 4b), but forced swim test immobility did not increase until the third day of withdrawal (in Experiment 1, Figure 2). This is consistent with the finding in rats that an abrupt suppression of progesterone was associated with increased forced swim test immobility compared to a gradual reduction of progesterone (Saavedra et al., 2006). These data suggest that

withdrawal may initiate some physiological cascade that takes time to become apparent on the behavioral level. For example, withdrawal of allopregnanolone or progesterone may initiate some activity-dependent process, such as activity-dependent gene transcription, that takes some days to manifest as a depression-like response in the forced swim test.

Several pieces of evidence indicate that the finasteride treatments utilized in the present study would result in low allopregnanolone levels. First, data from the author's laboratory has shown an 80% decrease in brain allopregnanolone levels at 24 hr following injection of the 50 mg/kg dose of finasteride in male mice (Finn, unpublished data), which is consistent with data in rats (VanDoren et al., 2000). In other studies, a 50 mg/kg dose of finasteride significantly decreased allopregnanolone levels following a single progesterone injection (M. E. Rhodes and Frye, 2005), whereas the 100 mg/kg finasteride dose significantly reduced allopregnanolone levels by 85% in pseudopregnant female rats without altering plasma progesterone concentrations (D. S. Reddy et al., 2001). The author's laboratory has also reported a dose-dependent decrease in allopregnanolone following 100 mg/kg finasteride compared to 50 mg/kg finasteride or vehicle (M. M. Ford et al., 2008). Thus, although the precise allopregnanolone levels are unknown, it is reasonable to assume that some reduction of allopregnanolone was achieved by this finasteride/metabolic allopregnanolone withdrawal procedure.

The present work adds to a line of research indicating a role for allopregnanolone in depression-like behavior in mice. For example, Frye and coworkers (2004) previously reported that progesterone decreased forced swim

test immobility in wildtype mice but not in mice with a null mutation for the *Srd5a1* gene (which encodes the type-1 5α-reductase enzyme). Also, systemic or intra-hippocampus administration of finasteride (Frye & Walf, 2002) as well as intra-amygdala injections of finasteride (Walf et al., 2006) increased forced swim test immobility in female rats. These manipulations of allopregnanolone levels provide evidence for an inverse relationship between levels of allopregnanolone or other 5α-reduced neurosteroids and forced swim test immobility, but they do not speak specifically to the study of progesterone withdrawal. In contrast, the efforts of other researchers (e.g., Bekku et al., 2006; Molina-Hernández et al., 2000) have reported depression-like effects of progesterone withdrawal, but without specifically assessing potential mechanisms underlying the effect. Thus, the current research converges two established but separate lines of research.

Anxiety-like behaviors have been better characterized within the context of progesterone withdrawal than depression-like behaviors. Interestingly, while onset of forced swim test immobility appears to be delayed, anxiety-like responses have been demonstrated much earlier following progesterone withdrawal. For example, Bitran and S. S. Smith (2005) induced progesterone withdrawal by ovariectomizing rats after 10 days of hormonally-induced pseudopregnancy and found that one day of progesterone withdrawal increased anxiety-like behavior in the elevated plus maze compared to ovariectomized control rats. Similar effects in the elevated plus maze were detected in rats after one day of progesterone when progesterone was administered via implantation of a progesterone-filled silicone capsule, and withdrawal was achieved by removing the capsule (S. S. Smith et al. 2004; see also

Gulinello & S. S. Smith, 2003).

Research examining animal models of progesterone withdrawal has utilized several methods to manipulate progesterone levels, and the use of different species of rodents and different strains within a given species can further complicate direct comparisons among these studies. Despite variation in progesterone or other steroid hormone administration and withdrawal methods, choice of animals, and other methodological details, many groups have found consistent effects of steroid withdrawal on rodent behavior in models of anxiety or depression. Thus, the effects of progesterone withdrawal appear to be robust, despite the differences in methods.

These studies demonstrate that the present model of progesterone withdrawal is effective at increasing depression-like behavior in female mice from an inbred strain. The procedures are quick and easy to perform, requiring only eight days of experimentation, and produce behavioral differences without resorting to surgeries or manipulation of non-progestin steroids such as estrogens. The results of the metabolic allopregnanolone withdrawal experiment and the experiment characterizing brain allopregnanolone concentrations (Experiments 2 and 3) offer support for the hypothesis that allopregnanolone withdrawal may contribute to depression-like behavior associated with progesterone withdrawal. Thus, the results of these experiments form the basis for the specific studies that are reported in the remainder of the present work.

Chapter 4: Pharmacologically-Precipitated Withdrawal of $GABA_A$ Receptor Activity, Progesterone Receptor Activity, and Associated Behaviors

Chapter 4 Introduction

There are several methods that have been developed to model hormone withdrawal in laboratory animals. Since there is no agreed-upon nomenclature to describe the different methods, here some terms have been proposed that can make some basic distinctions among them (see Table 1). Most of these methods (passive, metabolic, surgical, and estrous-cycle dependent) have previously been reported at one time or another to produce depression- or anxiety-like behaviors in laboratory animals (Bekku et al., 2006; Bitran & Smith, 2005; Chaves et al., 2009; Devall et al., 2009; Gallo & S. S. Smith, 1993; Löfgren et al., 2009; Schneider & Popik, 2007; Stoffel & Craft, 2004). However, the temporal correlation between steroid withdrawal and depression symptoms in humans or rodent does not in itself identify which receptor systems are involved, since many steroids have multiple receptor targets. The current chapter utilizes steroid- and neurotransmitter-receptor antagonists as a method to pharmacologically precipitate hormone withdrawal by blocking the effects of steroids at specific receptors.

The preceding chapter discusses the development of a mouse progesterone withdrawal model as a tool for investigating behavioral changes that may model aspects of reproductive-related depression with the forced swim test. Using these procedures, it was found that passive progesterone withdrawal increased forced swim test immobility. However, changes in progesterone concentrations affect downstream steroids, which must be considered. As discussed in Chapter 1 and

Chapter 3, one notable metabolite of progesterone is allopregnanolone, which is a positive allosteric modulator of $GABA_A$ receptors. The passive progesterone withdrawal procedure results in decreased plasma progesterone (Figure 4a and 4b), and since progesterone is a precursor for allopregnanolone, it dramatically reduces brain concentrations of allopregnanolone (Figure 4c and 4d).

The tethered relationship between concentrations of allopregnanolone and progesterone led to the hypothesis that allopregnanolone withdrawal might underlie forced swim test immobility during progesterone withdrawal. The metabolic allopregnanolone withdrawal experiment (Experiment 2) showed that the 5α-reductase inhibitor finasteride increased forced swim test immobility to a level consistent with that seen during passive progesterone withdrawal. Corresponding results have led many in the field to suggest a role for allopregnanolone in depressive-like behavior in laboratory rodents (e.g., Dong et al., 2001; Molina-Hernández et al., 2005). However, as it has been noted above, results from passive or metabolic withdrawal methods cannot be used to distinguish the specific receptor systems that are affected by withdrawal of a steroid. Since metabolic withdrawal of allopregnanolone increased forced swim test immobility, the present set of experiments tested the hypothesis that the $GABA_A$ receptor antagonist picrotoxin could be used to precipitate a $GABA_A$-receptor withdrawal-induced increase in forced swim test immobility, thus mimicking the effect of passive progesterone withdrawal and metabolic withdrawal of allopregnanolone on forced swim test immobility.

At the same time, the results of the metabolic allopregnanolone withdrawal study do not rule out the involvement of progesterone receptors in increased swim test immobility during progesterone withdrawal. Thus, the effects of the nonselective progesterone receptor antagonist mifepristone (RU-38486) and the selective progesterone receptor antagonist CDB-4124 were tested on forced swim test immobility to determine whether progesterone receptor antagonism could mimic the effect of passive progesterone withdrawal in inducing swim test immobility.

Finally, the effects of CDB-4124 or finasteride were tested on locomotor activity to determine whether drug treatments that resulted in similar changes in forced swim test immobility also resulted in similar changes in general locomotor activity. Finasteride was previously observed to suppress locomotor activity immediately after injection (Gabriel et al., 2005), but it was unclear whether these effects might still be present at the time when mice would normally be tested in the forced swim test. This experiment was therefore performed to determine whether a general locomotor suppressant effect of finasteride or CDB-4124 might contribute to immobility behavior in the forced swim test.

Chapter 4 Experiments

Experiment 4— Methods—Mifepristone or picrotoxin and forced swim test

The purpose of this experiment was to test the progesterone withdrawal hypothesis and the allopregnanolone hypothesis by determining if the progesterone receptor antagonist mifepristone or the $GABA_A$ receptor antagonist picrotoxin would increase forced swim test immobility, alone or in combination. It was

hypothesized that both or either of mifepristone and picrotoxin would increase forced swim test immobility, which would support the progesterone withdrawal or allopregnanolone withdrawal hypothesis. This experiment also tested the sub-hypothesis that more than one receptor class may be involved in the increase in forced swim test immobility during progesterone withdrawal by using a combination of antagonists to test for a further increase in swim test immobility. It was therefore hypothesized that the combined treatment of both mifepristone and picrotoxin could increase swim test immobility to a level even higher than either treatment separately.

Mice received daily injections of progesterone (5 mg/kg) on days 1-8 of the experiment. On days 6-8 of the experiment, mice received injections of mifepristone (20 mg/kg; "MIF" group), picrotoxin (2 mg/kg; "PTX" group), both picrotoxin and mifepristone ("MIF/PTX" group), or vehicle ("PRO" group), all in addition to their daily progesterone injections. Mice were tested in the forced swim test on day 8 of the experiment, approximately 2-4 hours after the last injections. Table 6 shows the full schedule of injections for this experiment. The mifepristone used in this experiment was provided as a gift from Roussell-Uclaf (Romainville, France). Data for this experiment were analyzed with two-way ANOVA using progesterone antagonist treatment (mifepristone vs. vehicle) as one factor and $GABA_A$ receptor antagonist treatment (picrotoxin vs. vehicle) as another factor.

Experiment 4—Results—Mifepristone or picrotoxin and forced swim test

Neither picrotoxin nor mifepristone, alone or in combination, affected forced swim test immobility in Experiment 4 (see Figure 5). There was not a significant

main effect of mifepristone, a significant main effect of picrotoxin, nor a significant mifepristone × picrotoxin interaction ($F_{1,37}$ values all < 2.41, *ns*).

Table 6: Experiment 4 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
PRO	PRO	PRO, VEH, VEH	PRO, VEH, VEH, FST
PTX	PRO	PRO, PTX, VEH	PRO, PTX, VEH, FST
MIF	PRO	PRO, MIF, VEH	PRO, MIF, VEH, FST
MIF/PTX	PRO	PRO, MIF, PTX	PRO, MIF, PTX, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; MIF, mifepristone (20 mg/kg); PTX, picrotoxin (2 mg/kg). "FST" indicates the day of forced swim testing. Initial sample sizes were n = 11 for each group.

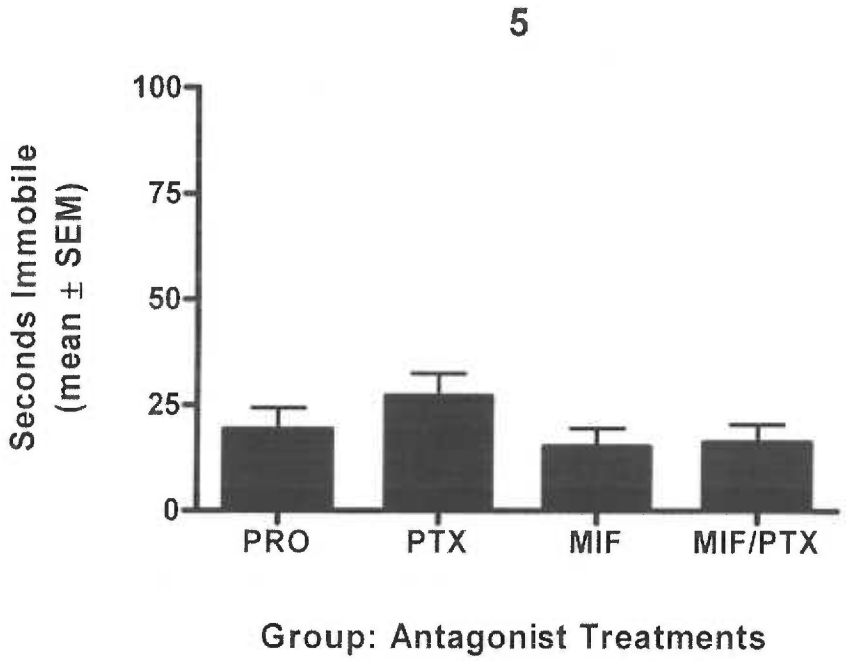


Figure 5. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered with vehicle (PRO) or with picrotoxin (PTX, 2 mg/kg) or mifepristone (MIF, 20 mg/kg), alone or in combination (group labels). Final sample sizes were $n = 11$ for the PRO and PTX groups, $n = 9$ for the MIF group, and $n = 10$ for the MIF/PTX group.

Experiment 5—Methods—Picrotoxin dose response and forced swim test

The purpose of this experiment was to test the allopregnanolone withdrawal hypothesis to determine if the $GABA_A$-receptor antagonist picrotoxin would increase forced swim test immobility. Metabolic withdrawal of allopregnanolone increased forced swim test immobility previously, so it was hypothesized that picrotoxin could induce a precipitated withdrawal of $GABA_A$ receptor activity, which would corroborate a role for allopregnanolone as a key contributor to the effects of progesterone withdrawal. Since this experiment used a different methodological approach compared to Experiment 2, this experiment also tested the sub-hypothesis that an alternate method for terminating steroid signal would increase forced swim test immobility. Since the results of the experiment with mifepristone and picrotoxin (Experiment 4) showed no increase in forced swim test immobility when mice received 2 mg/kg picrotoxin, higher doses were tested in the present experiment. Mice received daily injections of progesterone on days 1-8 of the experiment, with picrotoxin co-administered immediately following progesterone (5 mg/kg) on days 6-8 of the experiment in doses of 0 mg/kg (vehicle), 2 mg/kg, 4 mg/kg or 6 mg/kg. Mice in the 6 mg/kg group received picrotoxin in injections of 7.5 mL/kg. Table 7 shows the schedule for injections for this experiment.

Experiment 5—Results—Picrotoxin dose response and forced swim test

Picrotoxin did not significantly increase forced swim test immobility when co-administered with progesterone in Experiment 5 (see Figure 6). There was no omnibus difference in immobility by picrotoxin dose ($F_{3,29} = 1.45$, *ns*).

Table 7: Experiment 5 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
0 mg/kg	PRO	PRO, VEH	PRO, VEH, FST
2 mg/kg	PRO	PRO, PTX	PRO, PTX, FST
4 mg/kg	PRO	PRO, PTX	PRO, PTX, FST
6 mg/kg	PRO	PRO, PTX	PRO, PTX, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; PTX, picrotoxin. "FST" indicates the day of forced swim testing. Picrotoxin doses are indicated by the group names. Initial sample sizes were n = 12 for groups to receive 0 mg/kg, 2 mg/kg, 4 mg/kg or 8 mg/kg. There were initially no mice intended for a 6 mg/kg group, but a 6 mg/kg group was added during the second phase of the experiment by taking mice from other groups. No mice that received 8 mg/kg picrotoxin survived, so no data for these mice are reported.

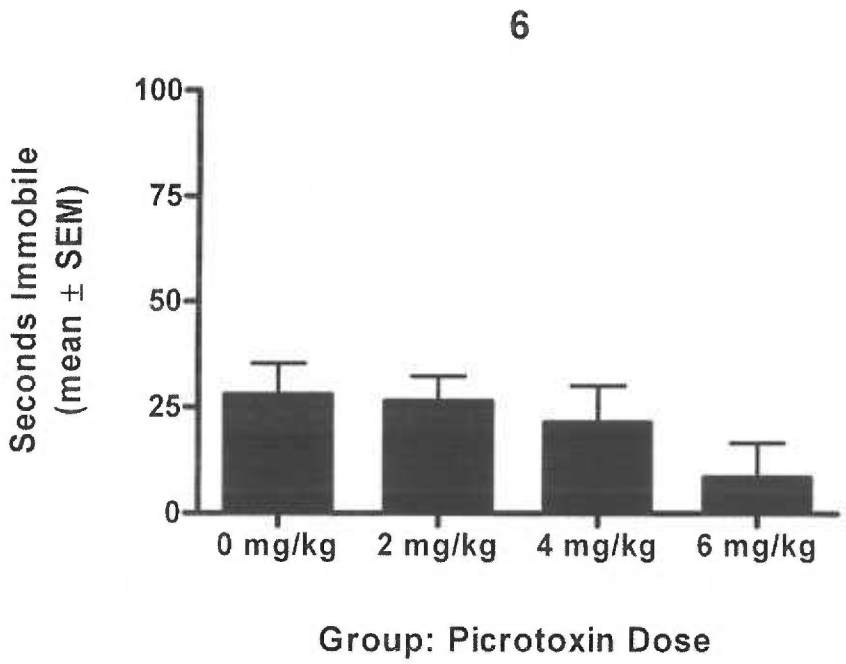


Figure 6. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered with various doses of picrotoxin (group labels). Final sample sizes were adjusted because of fatalities and group reassignments. The sample size for the 0 mg/kg group was $n = 11$ (one mouse was moved to the 6 mg/kg group). The sample size for the 2 mg/kg group was $n = 10$ (one mouse was found dead in its cage before the picrotoxin phase was commenced, and one mouse was moved to the 6 mg/kg group). The sample size for the 4 mg/kg group was $n = 10$ (two mice died following picrotoxin administration). The sample size for the 8 mg/kg group was $n = 0$ (six mice died after picrotoxin injections and six mice were moved to the 6 mg/kg group). The sample size for the 6 mg/kg group was $n = 3$ (eight mice were moved from other groups into this group, but five of these mice died following picrotoxin injections).

Experiment 6—Methods—Picrotoxin following various delays and forced swim test

Since progesterone must be metabolized into allopregnanolone, it was reasoned that a delay might be necessary between injection of progesterone and picrotoxin in order for picrotoxin to antagonize the effect of allopregnanolone on $GABA_A$ receptors, and as a result increase forced swim test immobility. Again, it was hypothesized that picrotoxin could be used as a method of precipitated withdrawal of $GABA_A$ receptor activity, and it was predicted that picrotoxin would increase forced swim test immobility consistent with the allopregnanolone withdrawal hypothesis. This experiment also tested the principle that alternate methodological techniques could be used to increase forced swim test immobility by decreasing a steroid signal. Mice received daily injections of progesterone (5 mg/kg) on days 1-8 of the experiment, with vehicle immediately co-administered on days 6-8 (PRO group), or with 2 mg/kg picrotoxin co-administered on days 6-8 with a 0.5 hr, 1.0 hr, or 1.5 hr delay following progesterone injection ("0.5 hr," "1.0 hr," and "1.5 hr" groups, respectively). A complete schedule of injections is shown in Table 8.

Experiment 6—Results—Picrotoxin following various delays and forced swim test

In Experiment 6, immobility in the forced swim test was influenced by the time delay between administration of progesterone and picrotoxin ($F_{3,38} = 3.81$, $p < .05$). Tukey post hoc tests confirmed a significant increase ($p < .05$) in swim test immobility in the 1.0 hr delay group compared to the PRO group (see Figure 7). Data also suggested that picrotoxin increased swim test immobility in the 1.0 hr group compared to the 0.5 hr and 1.5 hr groups (both $.05 < p < .1$).

Table 8: Experiment 6 Injection Schedule

	Injections			
Group	Days 1-8	Days 6-8, Immediate	Days 6-8, Delay	Delay
PRO	PRO	VEH		
0.5 hr	PRO		PTX	0.5 hr
1.0 hr	PRO		PTX	1.0 hr
1.5 hr	PRO		PTX	1.5 hr

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; PTX, picrotoxin. The forced swim test took place on day 8 of the experiment. Injections of picrotoxin (2 mg/kg) were administered after a delay relative to the injection of progesterone, indicated in the "Delay" column. Initial sample sizes for this experiment were $n = 12$ for each group.

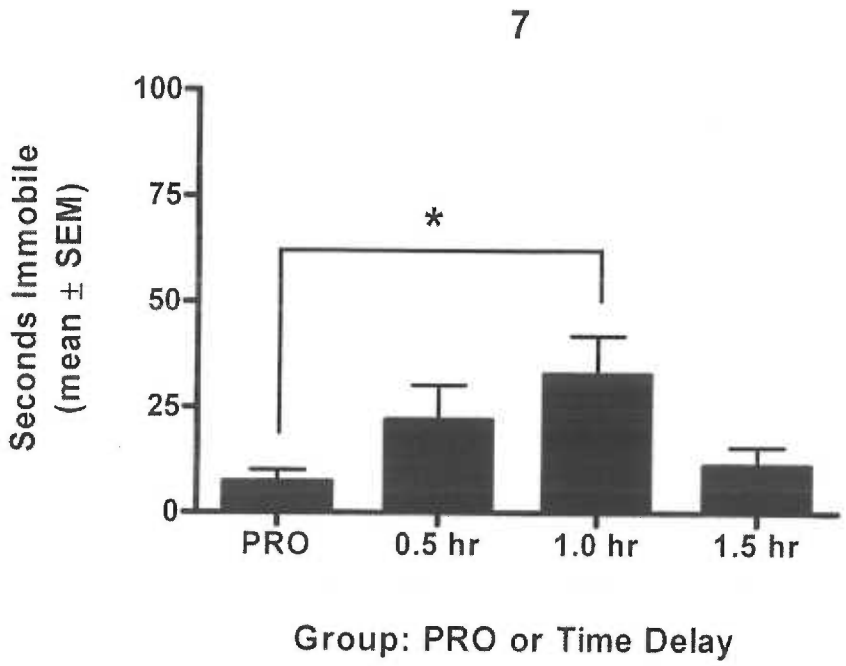


Figure 7. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered with vehicle (PRO), or with picrotoxin administered (2 mg/kg) after a delay (group labels). * $p < .05$ difference of 1.0 hr group compared to PRO group. Final sample sizes were $n = 10$ for the PRO and 0.5 hr groups, and $n = 11$ for the 1.0 hr and 1.5 hr groups.

Experiment 7—Methods—Picrotoxin with or without progesterone and forced swim test

One of the sub-hypotheses under investigation proposes that it is actually withdrawal of $GABA_A$ receptor activity—as opposed to $GABA_A$ receptor activity that is merely low—which increases forced swim test immobility. Therefore, the purpose of this study was to examine whether the effects of picrotoxin in increasing forced swim test immobility require progesterone. (In other words, the null hypothesis was that picrotoxin would not require progesterone to increase forced swim test immobility.) It was predicted that forced swim test immobility would only be increased in mice that had received progesterone. Another purpose of this experiment was to replicate (in a broad sense) the finding that 2 mg/kg picrotoxin administered 1 hr after progesterone increased forced swim test immobility. However, a different comparison condition was used in this experiment (vehicle plus picrotoxin) compared to the previous experiment.

Two groups of mice received daily injections of vehicle on days 1-8, and two groups received daily injections of progesterone (5 mg/kg) on days 1-8. All mice received daily injections of 2 mg/kg picrotoxin on days 6-8. One vehicle group received picrotoxin immediately after vehicle on these days (V. I. group) and one received picrotoxin after a 1.0 hr delay (V. D. group). Similarly, one progesterone group received picrotoxin immediately following progesterone (P. I.) and one group after a 1.0 hr delay (P. D.). Mice were tested in the forced swim test approximately 2-4 hours after their last injection of vehicle or progesterone. A schedule of injections is shown in Table 9. The data from this experiment were analyzed with

two-way ANOVA with steroid condition (progesterone vs. vehicle) as one factor, and picrotoxin condition (picrotoxin vs. vehicle) as another factor.

Experiment 7—Results—Picrotoxin with or without progesterone and forced swim test

Forced swim test immobility was dependent on initial steroid condition in Experiment 7 (see Figure 8). A main effect for steroid condition confirmed that swim test immobility was increased in mice that had received progesterone on days 1-8 of the experiment, compared to mice that had received vehicle ($F_{1,41} = 5.29$, $p < .05$). No main effect of timing (immediate co-administration compared to co-administering picrotoxin after a 1-hr delay) was detected. Similarly, no steroid × timing interaction was detected. Since a significant interaction was not detected, post hoc tests were not considered justified and were not performed. Additional mice were added from a replicate experiment for the purpose of increasing statistical power, but the pattern of results was not altered (data not shown).

Table 9: Experiment 7 Injection Schedule

	Injections			
Group	Days 1-8	Days 6-8, Immediate	Days 6-8, Delay	Delay
V. I.	VEH	PTX		
V. D.	VEH		PTX	1.0 hr
P. I.	PRO	PTX		
P. D.	PRO		PTX	1.0 hr

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; PTX, picrotoxin. The forced swim test took place on day 8 of the experiment. Injections of picrotoxin (2 mg/kg) for two groups were administered immediately following injection of progesterone or vehicle, and for two groups they were administered after a 1.0 hr delay relative to the injection of progesterone or vehicle. Initial sample sizes were $n = 12$ for each group.

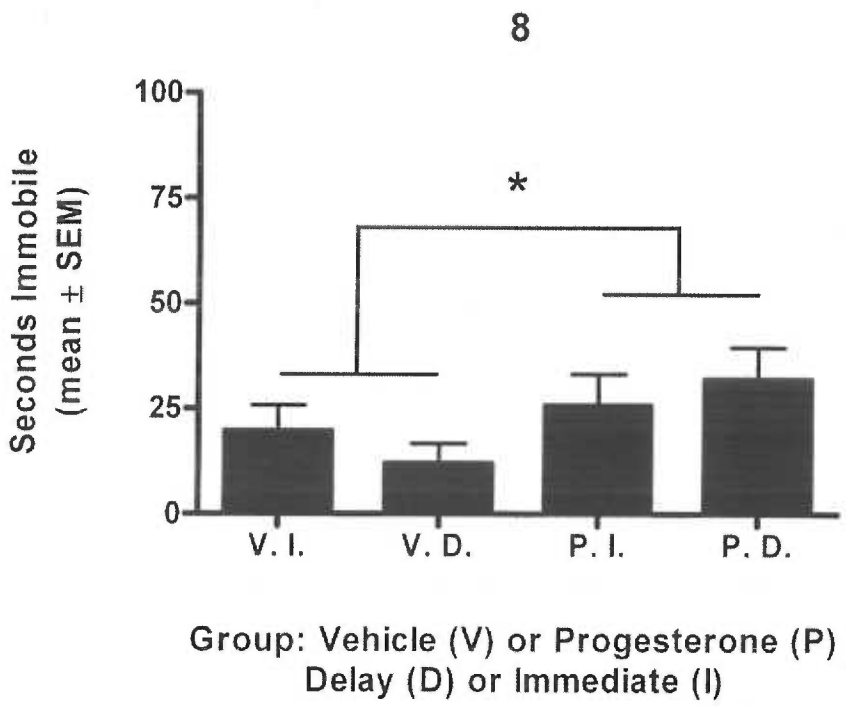


Figure 8. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone or vehicle, co-administered with picrotoxin (2 mg/kg) that was injected immediately, or following a delay. * $p < .05$ main effect of mice that received progesterone, compared to mice that received vehicle. Final sample sizes were $n = 11$ for the V. I. and P. I. group. The sample size for the V. D. group was $n = 12$. The sample size for the P. D. group was $n = 11$ (one mouse died after receiving a picrotoxin injection on day 8 of the experiment).

Experiment 8—Methods—Picrotoxin dose response at 1.0 hr delay and forced swim test

The purpose of this experiment was to find an optimal dose of picrotoxin, administered 1 hr after progesterone, for precipitating $GABA_A$ receptor withdrawal, based on the variable results seen with the 2 mg/kg dose in previous experiments. This experiment tested the allopregnanolone withdrawal hypothesis, but the main goal was to determine the optimal dose of picrotoxin for increasing swim test immobility. It was predicted that some dose or doses of picrotoxin would increase forced swim test immobility more strongly than other doses. All mice received progesterone (5 mg/kg) injections on days 1-8 of the experiment, and an injection of vehicle or picrotoxin on days 6-8 of the experiment following a 1.0 hr delay from the progesterone injection. One group received vehicle on these days (0 mg/kg group), while the other groups received 1 mg/kg, 2 mg/kg, or 4 mg/kg picrotoxin on these days. Forced swim testing occurred approximately 2-4 hours after the last progesterone injections, and a complete schedule of injections is shown in Table 10.

Experiment 8—Results—Picrotoxin dose response at 1.0 hr delay and forced swim test

No significant differences in forced swim test immobility were detected in Experiment 8 (see Figure 9). There was no omnibus significant difference in swim test immobility across the different doses tested at 1.0 hr ($F_{3,42} = 0.972$, *ns*).

Table 10: Experiment 8 Injection Schedule

	Injections			
Group	Days 1-8	Days 6-8, Immediate	Days 6-8, Delay	Delay
0 mg/kg	PRO		VEH	1.0 hr
1 mg/kg	PRO		PTX	1.0 hr
2 mg/kg	PRO		PTX	1.0 hr
4 mg/kg	PRO		PTX	1.0 hr

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; PTX, picrotoxin. The forced swim test took place on day 8 of the experiment. Doses of picrotoxin injections are indicated by the group names. Injections of picrotoxin or vehicle for all groups were administered after a 1.0 hr delay relative to the injection of progesterone (the "Immediate" column is retained only to emphasize the similarities and differences between this design and those of other experiments). Initial sample sizes were $n = 12$ for each group.

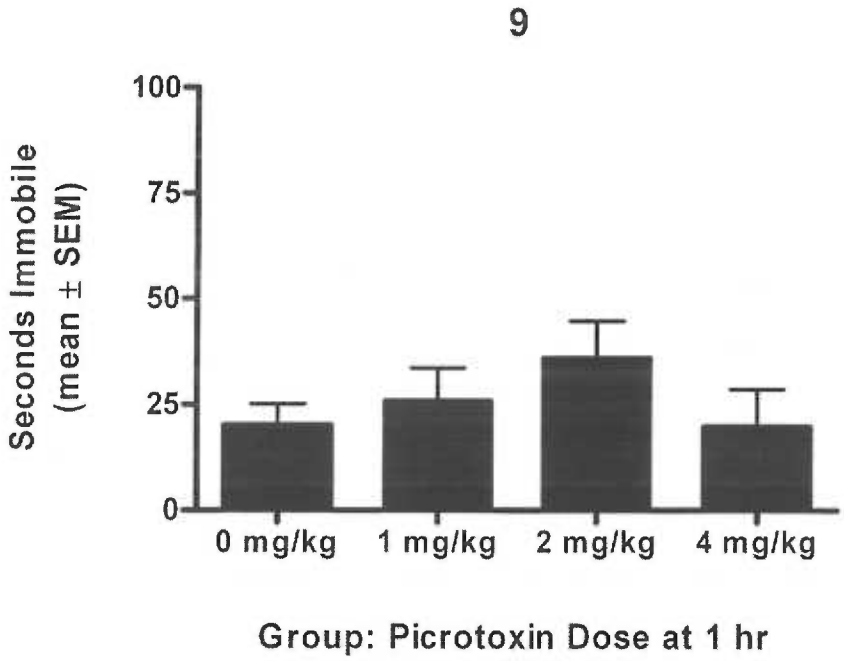


Figure 9. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered with various doses of picrotoxin after a 1-hr delay following progesterone. Final sample sizes were $n = 12$ for the 0 mg/kg group, 1 mg/kg group, and 2 mg/kg group, and $n = 10$ for the 4 mg/kg group (two mice exhibited visible seizures and were euthanized).

Experiment 9—Methods—CDB-4124 dose response and forced swim test

Metabolic allopregnanolone withdrawal was previously observed to mimic the effect of passive progesterone withdrawal on forced swim test immobility, suggesting that $GABA_A$ receptors might play an important role in forced immobility during progesterone withdrawal. The purpose of this experiment was to determine if progesterone withdrawal could increase forced swim test immobility without concurrent withdrawal of other steroids, including the $GABA_A$ receptor-modulating neurosteroids such as allopregnanolone. Therefore, this experiment tested the progesterone withdrawal hypothesis, but the approach was to use the specific progesterone receptor antagonist CDB-4124 to pharmacologically precipitate progesterone receptor withdrawal, thus avoiding concomitant withdrawal of other steroids. As such, compared to the passive progesterone withdrawal experiment (Experiment 1), this experiment also tested the principle that blocking a steroid signal at the receptor level would result in equivalent increases in forced swim test immobility. It was predicted that CDB-4124 would increase forced swim test immobility in a dose-dependent fashion.

All mice were injected daily with progesterone (5 mg/kg) on days 1-8 of the experiment. One group received additional injections of vehicle on days 6-8 of the experiment (0 mg/kg group), while other groups received additional injections of CDB-4124 on these days in doses of 20 mg/kg, 40 mg/kg, or 60 mg/kg (the respective group names). All mice were tested in the forced swim test approximately 2-4 hours after their last injection. A schedule of progesterone,

vehicle, and CDB-4124 injections, as well as forced swim testing, is displayed in Table 11.

Experiment 9—Results—CDB-4124 dose response and forced swim test

Experiment 9 revealed that CDB-4124 increased forced swim test immobility (see Figure 10). A significant omnibus difference in forced swim test immobility was detected by ANOVA ($F_{3,44} = 3.73$, $p < .05$). Post hoc comparisons revealed that swim test immobility was significantly increased ($p < .05$) in the 60 mg/kg group compared to the 0 mg/kg group.

Table 11: Experiment 9 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
0 mg/kg	PRO	PRO, VEH	PRO, VEH, FST
20 mg/kg	PRO	PRO, CDB	PRO, CDB, FST
40 mg/kg	PRO	PRO, CDB	PRO, CDB, FST
60 mg/kg	PRO	PRO, CDB	PRO, CDB, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; CDB, CDB-4124. "FST" indicates the day of forced swim testing. Doses of CDB-4124 administered are indicated by the group names. Initial sample sizes were $n = 12$ for each group.

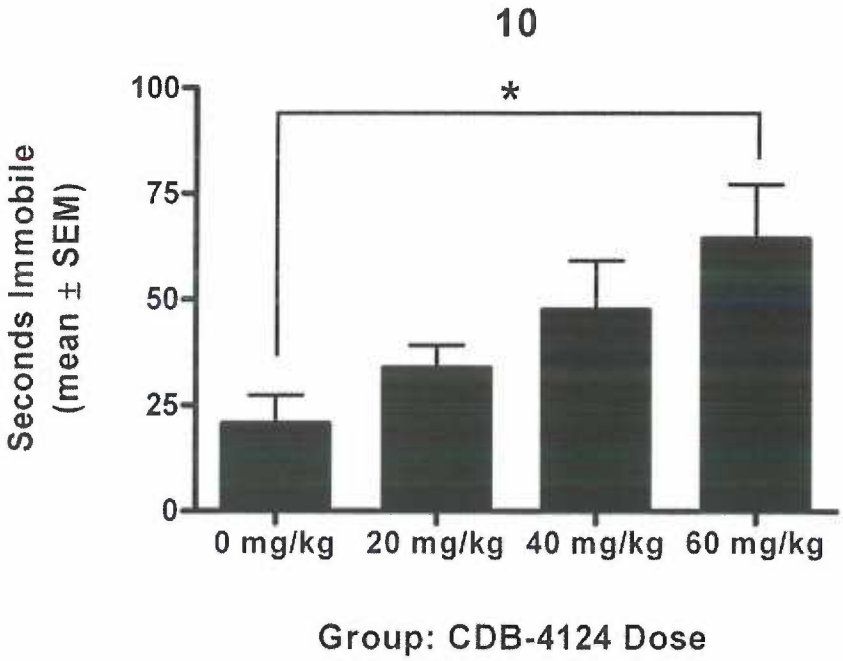


Figure 10. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered various doses of CDB-4124 (group labels). * $p < .05$ difference between 60 mg/kg group and 0 mg/kg group (vehicle). Final sample sizes were $n = 12$ for each group.

Experiment 10—Methods—CDB-4124 or picrotoxin and forced swim test

Little research has previously addressed whether progesterone receptors and $GABA_A$ receptors might work in concert to increase forced swim test immobility. The present experiment tested the hypothesis that picrotoxin and CDB-4124 could have a greater immobility-inducing effect when administered in combination than alone, similar to the experiment that tested mifepristone and picrotoxin in combination (Experiment 4), but with dosage regimens chosen based on the results of the other picrotoxin experiments and one CDB-4124 experiment (Experiments 6, 7, and 9). Thus, the current experiment tested the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis, as well as the sub-hypothesis that more than one steroid may be involved in forced swim test immobility during steroid withdrawal and the sub-hypothesis that multiple strategies for inhibiting steroid signal should result in similar changes in forced swim test immobility. It was predicted that both picrotoxin and CDB-4124 would increase forced swim test immobility, and that these drugs would more strongly increase immobility behavior combined than individually.

Mice received daily injections of progesterone (5 mg/kg) on days 1-8 of the experiment. On days 6-8 of the experiment, mice received additional injections of vehicle both immediately and after a 1.0 hr delay (PRO group), additional injections of vehicle immediately and 2 mg/kg picrotoxin after a 1 hr delay (PTX group), additional injections of 40 mg/kg CDB-4124 immediately and vehicle after a 1.0 hr delay (CDB group), or additional injections of 40 mg/kg CDB-4124 immediately and 2 mg/kg picrotoxin after a 1.0 hr delay (PTX/CDB group). All mice were tested in

the forced swim test approximately 2-4 hours after their last injection of progesterone. Table 12 shows details about injections. This experiment was analyzed with two-way ANOVA, with CDB-4124 condition (CDB-4125 vs. vehicle) as one factor, and picrotoxin condition (picrotoxin vs. vehicle) as another factor. Mice were maintained on 12 hr/12 hr light cycles with lights on at 0600 h, but the light cycle was phased forward 1 hr over the course of two weeks following the change from Pacific Standard Time to Pacific Daylight Time.

Experiment 10—Results—CDB-4124 or picrotoxin and forced swim test

In Experiment 10, forced swim test immobility was significantly increased by the progesterone receptor antagonist CDB-4124. There was a significant main effect of progesterone receptor antagonist condition (CDB-4124 compared to vehicle) on swim test immobility ($F_{1,38} = 9.86$, $p < .05$). Estimated partial omega squared ($\tilde{\omega}_p^2$) for the main effect of CDB-4124 on immobility was 0.17 (see Figure 11). There was no significant main effect of $GABA_A$ receptor antagonist condition (picrotoxin compared to vehicle). Similarly, there was no significant progesterone receptor antagonist × $GABA_A$ receptor antagonist interaction (consequently, post hoc comparisons were not performed). Since there was not a significant picrotoxin × CDB-4124 interaction these data provide no evidence that picrotoxin could enhance the effect of CDB-4124 on forced swim test immobility, nor was CDB-4124 found to enhance the effect of picrotoxin.

Table 12: Experiment 10 Injection Schedule

	Injections			
Group	Days 1-8	Days 6-8, Immediate	Days 6-8, Delay	Delay
PRO	PRO	VEH	VEH	1.0 hr
PTX	PRO	VEH	PTX	1.0 hr
CDB	PRO	CDB	VEH	1.0 hr
PTX/CDB	PRO	CDB	PTX	1.0 hr

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; PTX, picrotoxin; CDB, CDB-4124. The forced swim test took place on day 8 of the experiment. Each group received injections, either picrotoxin (2 mg/kg) or vehicle, that were administered after a 1.0 hr delay relative to the injection of progesterone and vehicle or CDB-4124 (40 mg/kg). Initial sample sizes were $n = 12$ for each group.

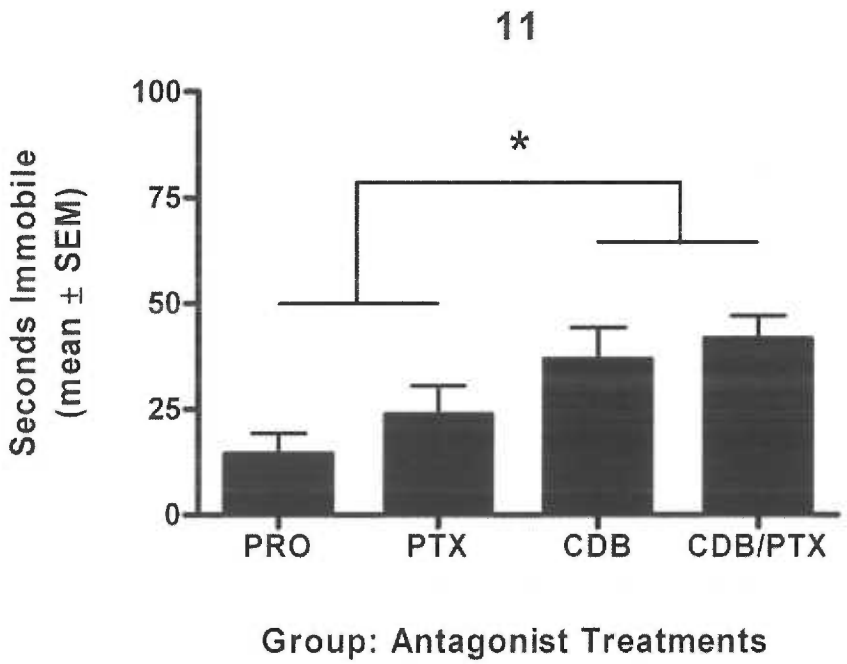


Figure 11. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered with CDB-4124 (CDB, 40 mg/kg) immediately following progesterone, and/or picrotoxin (PTX, 2 mg/kg) that was injected after a delay following progesterone. * $p < .05$ main effect difference between mice that received CDB-4124 (plus vehicle or picrotoxin), compared to mice that did not receive CDB-4124 (but did receive vehicle or picrotoxin). Final sample sizes were adjusted based on laboratory accidents. The sample size for the PRO group was $n = 10$ (one mouse was euthanized based on its unkempt physical appearance after its cage was flooded, which was taken to indicate that the mouse had possibly been adversely affected by the experience, and one mouse was moved to the PTX group). The sample size for the PTX group was $n = 11$ (one mouse was euthanized based on its physical appearance after its cage was flooded). The sample size for the CDB group was $n = 11$ (one mouse was removed based on its physical

appearance after its cage was flooded). The sample size for the PTX/CDB group was $n = 10$ (one mouse was removed based on its physical appearance after its cage was flooded, and one mouse was moved to the CDB group).

Experiment 11—Methods—CDB-4124 and forced swim test replication

The purpose of this experiment was to confirm an immobility-inducing effect of 60 mg/kg CDB-4124 and to test for a similar effect at a higher dose. Mice were administered daily injections of progesterone (5 mg/kg) on days 1-8, and on days 6-8 received additional daily injections of vehicle (0 mg/kg group), 60 mg/kg CDB-4124 (60 mg/kg group), or 80 mg/kg CDB-4124 (80 mg/kg group). It was predicted that the two drug groups would have increased forced swim test immobility compared to the vehicle group. Vehicle and CDB-4124 were administered in volumes of 20 mL/kg in this experiment. Mice were tested in the forced swim test approximately 2-4 hours after their last injections. Injection and swim tests schedules are shown in Table 13.

Experiment 11—Results—CDB-4124 and forced swim test replication

Forced swim test immobility differed among groups in Experiment 11 ($F_{2,28}$ = 15.6, $p < .05$). CDB-4124 significantly increased forced swim test immobility in both the 60 mg/kg and 80 mg/kg groups compared to 0 mg/kg (both $p < .05$), although immobility did not differ between the two drug groups (see Figure 12).

Table 13: Experiment 11 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
0 mg/kg	PRO	PRO, VEH	PRO, VEH, FST
60 mg/kg	PRO	PRO, CDB	PRO, CDB, FST
80 mg/kg	PRO	PRO, CDB	PRO, CDB, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; CDB, CDB-4124. CDB-4124 doses are indicated by the group name. "FST" indicates the day of forced swim testing. Initial sample sizes were n = 12 for each group.

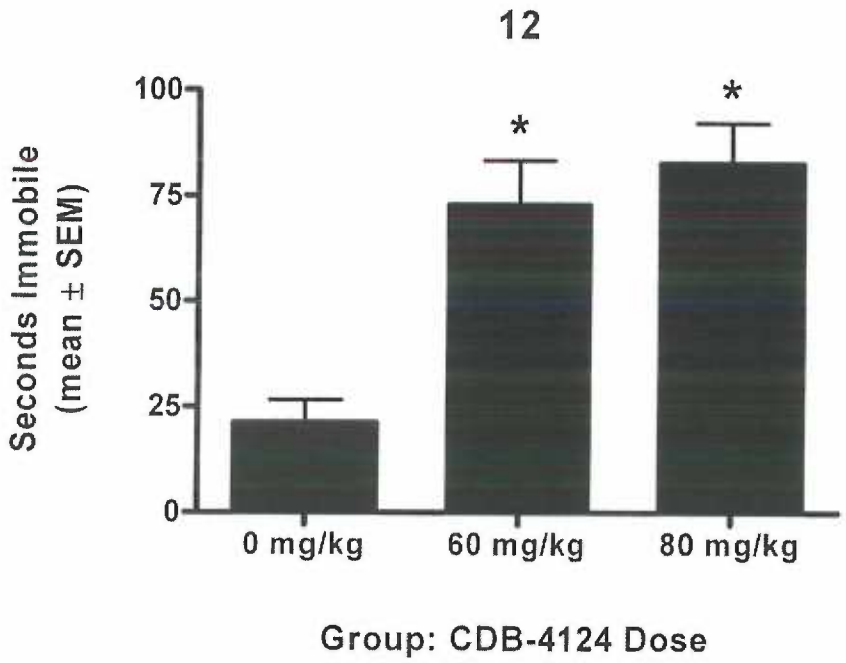


Figure 12. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered various doses of CDB-4124 (group labels). * $p < .05$ compared to mice that received 0 mg/kg (vehicle). Final sample sizes were $n = 12$ for the 0 mg/kg and 80 mg/kg groups, and $n = 10$ for the 60 mg/kg group (one mouse was removed based on unusual swim posture and labored breathing while swimming).

Experiment 12—Methods—Mifepristone and forced swim test

The purpose of this experiment was to use higher doses of the non-selective progesterone receptor antagonist mifepristone (compared to Experiment 4) to confirm the finding that the progesterone receptor antagonist CDB-4124 increased forced swim test immobility. All mice received daily injections of progesterone (5 mg/kg) on days 1-8. One group received additional injections of vehicle on days 6-8 of the experiment (0 mg/kg group), while additional groups received daily injections of 60 mg/kg mifepristone or 80 mg/kg mifepristone (60 mg/kg and 80 mg/kg groups, respectively). It was predicted that immobility behavior would be increased in the two drug groups compared to the vehicle group. Vehicle and mifepristone injections were administered in volumes of 20 mL/kg in this experiment. All mice were tested in the forced swim test approximately 2-4 hours after the last injections. Forced swim testing and injections are shown in schedule form in Table 14.

Experiment 12—Results—Mifepristone and forced swim test

In Experiment 12, forced swim test immobility was different among mifepristone dose groups ($F_{2,33} = 4.44$, $p < .05$). Post hoc Tukey tests revealed that immobility was significantly increased among mice receiving 80 mg/kg compared to 0 mg/kg ($p < .05$; see Figure 13).

Table 14: Experiment 12 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
0 mg/kg	PRO	PRO, VEH	PRO, VEH, FST
60 mg/kg	PRO	PRO, MIF	PRO, MIF, FST
80 mg/kg	PRO	PRO, MIF	PRO, MIF, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; MIF, mifepristone. Mifepristone doses are indicated by the group name. "FST" indicates the day of forced swim testing. Initial sample sizes were $n = 12$ for each group.

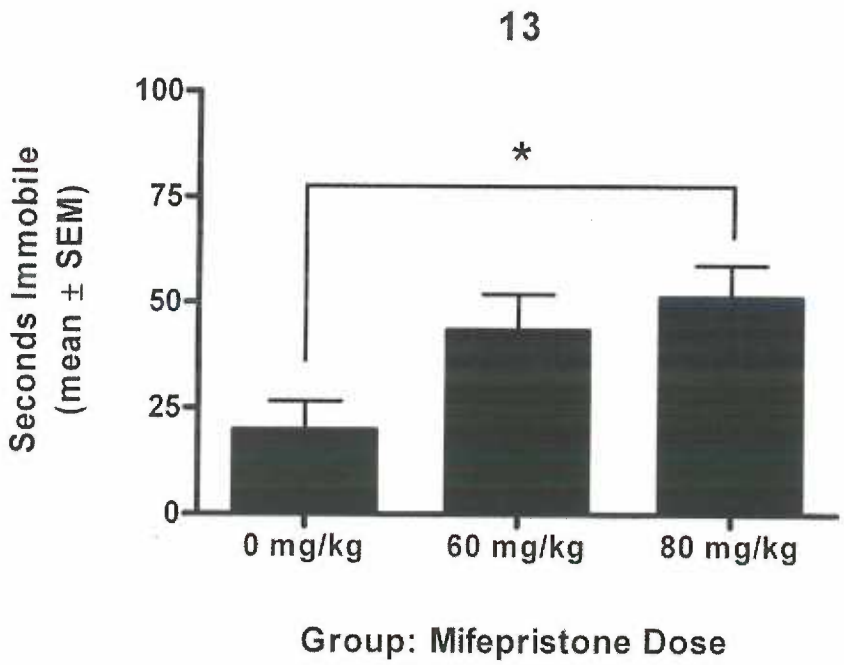


Figure 13. Mean (± SEM) seconds of immobility in the forced swim test in mice following injections of progesterone, co-administered various doses of mifepristone (group labels). * $p < .05$ difference between 80 mg/kg group and 0 mg/kg (vehicle) group. Final sample sizes were $n = 12$ for each group.

Experiment 13—Methods—Effect of CDB-4124 or finasteride on locomotion

CDB-4124 and finasteride have been seen to increase forced swim test immobility, but it is not clear from forced swim test studies whether immobility results from gross motor changes or from higher-order changes, such as changes in motivation. The purpose of this experiment was to determine whether CDB-4124 or finasteride would suppress spontaneous locomotor activity, which could explain increases in forced swim test immobility. Therefore, this experiment tested the sub-hypothesis that only some drug treatments that increase forced swim test immobility might do so without affecting voluntary locomotor activity. Since metabolic allopregnanolone withdrawal (with finasteride) robustly increased forced swim test immobility, but precipitated $GABA_A$ receptor withdrawal (with picrotoxin) did not, it was hypothesized that voluntary locomotor activity would be abnormal in the group that had received finasteride.

Mice received daily injections of vehicle on days 1-8 (VEH group), or daily injections of progesterone (5 mg/kg) on days 1-8 of the experiment. Of the mice that received progesterone, on days 6-8 of the experiment one group also received daily vehicle injections (PRO group), one received daily 100 mg/kg finasteride injections (metabolic allopregnanolone withdrawal group, MAW), and one group received daily 60 mg/kg CDB-4124 injections (CDB group). Mice were tested in locomotor activity chambers as described in the General methods approximately 2-4 hours after their final injections on day 8. A full schedule of drug injections and locomotor testing is shown in Table 15. Mice were maintained on 12 hr/12 hr light cycles with lights on at 0600 h, but the light cycle was phased forward 1 hr over the

course of two weeks following the change from Pacific Standard Time to Pacific Daylight Time. ANOVA with repeated measures was used to analyze differences in locomotor activity during 6-min time bins. Sphericity was assessed using Mauchly's test of sphericity and by inspecting the Greenhouse-Geisser and Huynh-Feldt estimates of ε. Significant ANOVA with repeated measures were followed with one-way ANOVA of the individual time bins.

Experiment 13—Results—Effect of CDB-4124 or finasteride on locomotion

Locomotor activity in Experiment 13 was divided into five 6-minute bins and analyzed with ANOVA with repeated measures. Based on Mauchly's test and estimates of sphericity violation (ε statistic) it was concluded that the sphericity assumption was met and that no adjustment of the degrees of freedom was necessary. There was a significant time (bin) by drug interaction, ($F_{12,156} = 2.34$, $p < .05$). Analyzing total distance travelled (activity across time bins), Tukey post hoc tests did not reveal a difference in locomotor activity between mice in the VEH and PRO groups (*ns*). Tukey post hoc tests revealed that total locomotor activity was significantly decreased in the MAW group mice compared to all other groups (all $p < .05$). CDB group mice exhibited significantly decreased overall locomotor activity compared to progesterone and vehicle group mice ($p < .05$), but increased locomotor activity compared to the MAW group mice (see Figure 14a). An overall view of locomotor activity for each group, across the 30-min session, is provided in Figure 14b.

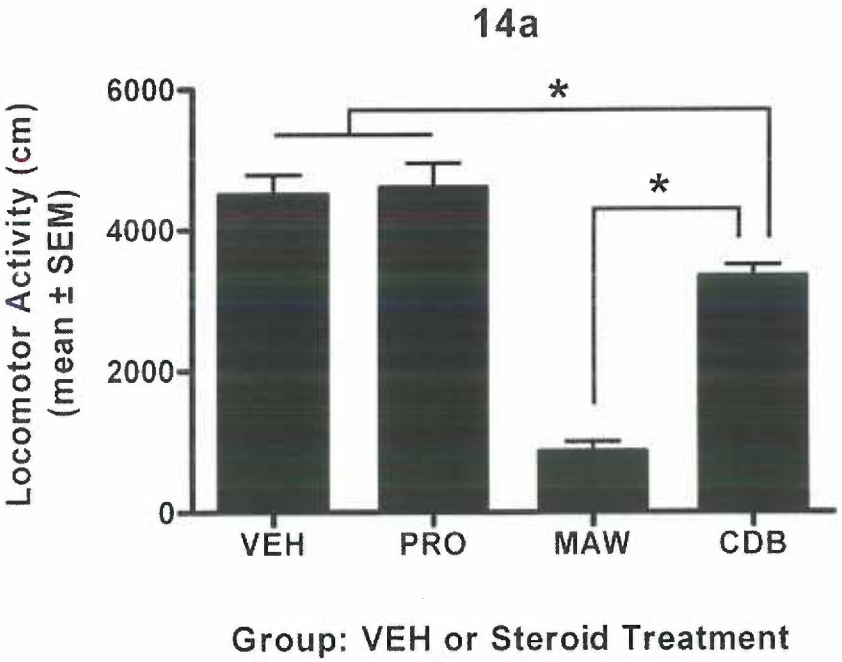
Given the significant interaction, these findings led to the examination of locomotor activity in smaller time bins using ANOVA. Group differences in

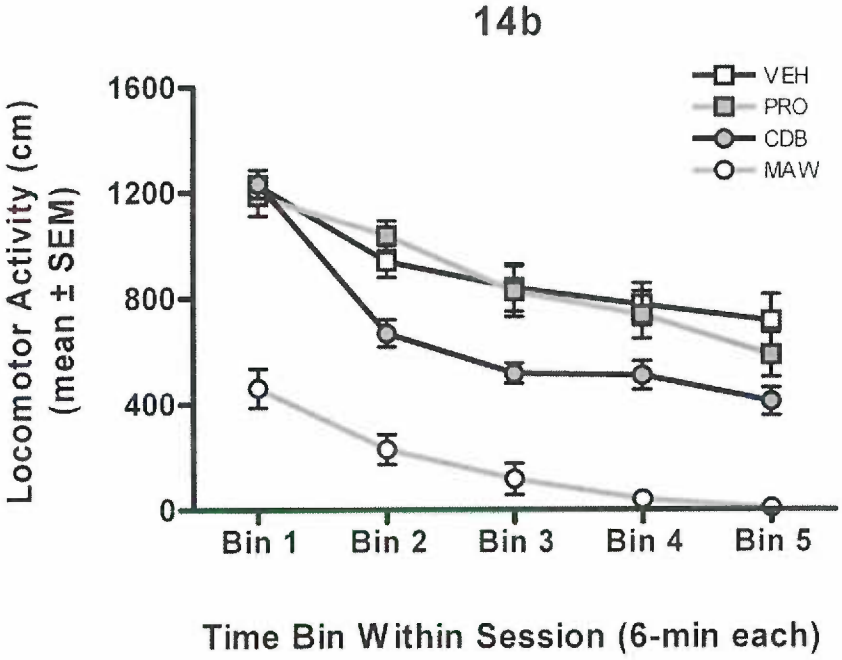
locomotor activity were evident in each of five 6-min time bins ($F_{3,39}$ value range: 17.9-38.7, all $p < .05$). Tukey post hoc tests revealed the following pairwise differences within each time bin ($p < .05$ if stated to be significant): PRO and VEH were not significantly different from one-another in any time bin. CDB group mice did not differ significantly from PRO or VEH in time bin 1 (Figure 14c). However, CDB mice had significantly lower locomotor activity compared to PRO group mice in bins 2 and 3, and compared to VEH group mice in bins 2-5. Locomotor activity was significantly lower in the MAW group compared to all other groups in all time bins.

Table 15: Experiment 13 Injection and Locomotor Activity Test Schedule

	Days		
Group	1-5	6-7	8
VEH	VEH	VEH, VEH	VEH, VEH, LOC
PRO	PRO	PRO, VEH	PRO, VEH, LOC
MAW	PRO	PRO, FIN	PRO, FIN, LOC
CDB	PRO	PRO, CDB	PRO, CDB, LOC

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; FIN, finasteride; CDB, CDB-4124. CDB-4124 was administered in doses of 60 mg/kg, and finasteride was administered in doses of 100 mg/kg. "LOC" indicates the day of locomotor activity testing. Initial sample sizes were n = 12 for each group.


14a
Locomotor Activity (cm)
(mean ± SEM)
6000
4000
2000
0
*
*
VEH
PRO
MAW
CDB
Group: VEH or Steroid Treatment


14b
Locomotor Activity (cm)
(mean ± SEM)
1600
1200
800
400
0
VEH
PRO
CDB
MAW
Bin 1
Bin 2
Bin 3
Bin 4
Bin 5
Time Bin Within Session (6-min each)

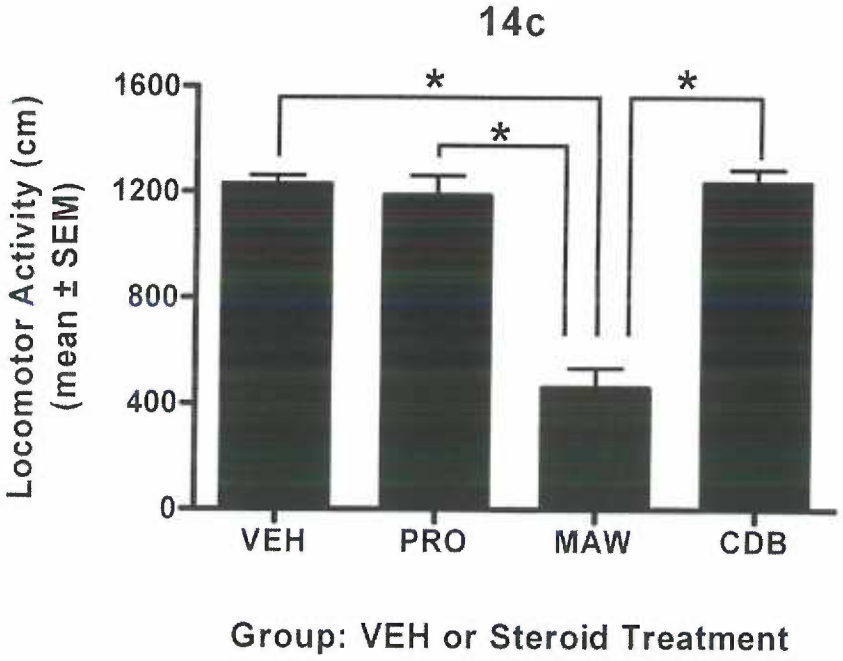


Figure 14. Mean (± SEM) centimeters distance traveled in the locomotor activity test in mice following injections of vehicle (VEH) or progesterone (PRO), or in mice following injections of progesterone, co-administered with finasteride (MAW) or CDB-4124 (CDB). (a) Total locomotor activity in a 30-min session. * $p < .05$ difference between the CDB group and the PRO or VEH groups, and between the MAW group and all other groups. (b) Activity across the 30-min session, divided into 6-min bins. Significant differences not shown due to graphical complexity; see text for narrative description of differences. (c) Activity in the first 6-min bin. * $p < .05$ difference between the MAW group and all other groups. One mouse was removed from each of the VEH, PRO and MAW groups based on their unkempt physical appearances after their cage had been flooded. The final sample sizes for one-way ANOVAs and pairwise comparisons were $n = 11$ for the VEH, $n = 10$ for the PRO and MAW groups, and $n = 12$ for the CDB group.

Chapter 4 Discussion

The present series of experiments made extensive use of the forced swim test to determine potential depression-inducing effects of precipitated withdrawal of $GABA_A$ receptor activity and progesterone receptor activity in female mice with high levels of progesterone. The main way to interpret changes in forced swim test immobility is that they indicate changes in a rodent's level of "depression." A prediction that follows from this interpretation is that corresponding changes in depression will be observed in humans under comparable circumstances. In this manner the forced swim test acts as a screening tool for making predictions about humans based on the behavior of laboratory animals (Porsolt et al., 1978, 2001). Obviously, these predictions are only hypotheses until they are actually tested.

Picrotoxin Studies

The effect of picrotoxin on forced swim test immobility was tested in several experiments (Experiments 4-8 and 10) to test the hypothesis that a $GABA_A$ receptor antagonist could increase forced swim test immobility. It was hypothesized that $GABA_A$ receptor blockade would increase forced swim test immobility because the metabolic allopregnanolone withdrawal experiment (Experiment 2) had showed that inhibiting allopregnanolone production with the 5α-reductase inhibitor finasteride mimicked the effect of passive progesterone withdrawal on forced swim test immobility (in Experiment 1). Combined with the finding that passive progesterone withdrawal also results in collateral decreases in allopregnanolone levels (Experiment 3; see also Costa et al., 1995), it was hypothesized that

allopregnanolone withdrawal increased immobility by decreasing $GABA_A$ receptor activity. These experiments demonstrated only limited support for this hypothesis.

In the mifepristone and picrotoxin combination study (Experiment 4) only a single dose each of picrotoxin (2 mg/kg) and mifepristone (20 mg/kg) was tested, alone or in combination. The picrotoxin dose was chosen based on reports that it had certain, presumably-unrelated, behavioral effects (Chester & Cunningham, 1999), but that it was a subconvulsant dose (K. Matsumoto et al., 2003). The dose of mifepristone was chosen based on reports that it was a reproductively-relevant dose in mice (Clark et al., 2005; Georén et al., 2005). When this initial study failed to reveal any significant effects on swim test immobility, it was hypothesized that higher doses might be necessary to see depressogenic effects. A full range of picrotoxin doses was tested without observing any significant change in swim test immobility (a 8 mg/kg dose was lethal in this design, so the range of doses reported essentially covers the range of non-lethal or not-always-lethal doses). Based on the results that brain allopregnanolone concentrations were maximal sometime approximately 0.5-1.0 hr following progesterone injection (Experiment 3), it was hypothesized that a delay interval between the administration of progesterone and picrotoxin might be necessary in order to observe an increase in forced swim test immobility. This hypothesis was tested in the picrotoxin delay experiment (Experiment 6), where it was found that 2 mg/kg picrotoxin significantly increased forced swim test immobility when administered 1.0 hr after progesterone injection. It should be noted that even though the difference was statistically significant, this increase in swim test immobility was not as large as the increase during passive

progesterone withdrawal or metabolic allopregnanolone withdrawal (Experiments 1 and 2).

Since the results of the picrotoxin delay experiment (Experiment 6) suggested that picrotoxin could increase forced swim test immobility, an additional experiment was conducted to determine whether picrotoxin would increase swim test immobility in the absence of progesterone (Experiment 7). The significant main effect showed that progesterone was required for picrotoxin to increase forced swim test immobility, although again this statistically significant effect was modest. Since the results of Experiments 6 and 7 were less robust than with passive progesterone withdrawal or metabolic allopregnanolone withdrawal, an additional experiment was undertaken to test whether a different picrotoxin dose might increase immobility on a scale more consistent with passive progesterone withdrawal (Experiment 8). In this experiment, however, there was no statistically significant change in forced swim test behavior among any of the dose groups. Also, the experiment that tested CDB-4124 and picrotoxin, alone or in combination, failed to reveal a significant effect of picrotoxin (Experiment 10).

Ultimately, these experiments with picrotoxin neither clearly support nor refute the hypothesis that picrotoxin increases forced swim test immobility. At most, the immobility-inducing effects of picrotoxin would have to be characterized as small and unreliable. On the other hand, there are numerous reports of picrotoxin increasing anxiety and depression-like behaviors in laboratory animals, or even blocking the antidepressant and anxiolytic effects of allopregnanolone (e.g., Bitran et al., 1999; Molina-Hernández et al., 2005; Rodríguez-Landa et al., 2007).

Collectively, these findings might be reconciled by the proposition that $GABA_A$ receptors are likely to be involved in emotional behaviors in mice, but perhaps the contribution of $GABA_A$ receptors to forced swim test immobility during progesterone withdrawal is limited. The anxiolytic effect in humans of benzodiazepines, acting on $GABA_A$ receptors, certainly speaks to the role of these receptors at least in relieving emotional distress.

Progesterone antagonist studies with forced swim test

Several experiments tested the effects of progesterone receptor antagonists on forced swim test immobility (Experiments 4 and 9-12). After the initial finding that a low dose of mifepristone (20 mg/kg) had no effect on immobility (Experiment 4), the experimental strategy was switched to using higher doses and a more specific antagonist. CDB-4124 is a potent progesterone receptor antagonist with greatly reduced potency as a glucocorticoid receptor antagonist (Attardi et al., 2002), and is currently being investigated for clinical applications. In these studies, a significant increase in forced swim test immobility was detected when CDB-4124 was administered in doses of 60 mg/kg (Experiments 9 and 11) or 80 mg/kg (Experiment 11), but not 40 mg/kg (Experiment 9). However, a significant increase in forced swim test immobility was also detected when the 40 mg/kg dose was administered with larger sample sizes (Experiment 10). (The sample sizes were effectively larger because of the 2 × 2 design, which for the main effect analysis pooled subjects that had received CDB-4124 regardless of whether they had also received picrotoxin.)

For the reasons discussed above, these findings can be interpreted as indications that CDB-4124 induced an emotional state in the mice that was similar, in some sense, to a state of depression (a "depression-like" state). These findings also lead to the prediction that humans could experience depression as a side effect of clinical use of CDB-4124 if they were to take the drug in very large doses. This prediction is based on the fact that humans and mice share many similar characteristics, that the mice respond to CDB-4124 by exhibiting a depression-like behavior, and that humans might therefore be similarly affected (see also discussion below of Schmidt et al., 1991).

It is important to note that the lowest dose of CDB-4124 to induce a statistically-significant depression-like state (as indicated by the forced swim test) was the 40 mg/kg dose (in Experiment 10), but that this dose did not significantly increase immobility in Experiment 9. Even in Experiment 10 when the sample size was higher, CDB-4124 only explained approximately 17% of the observed variability ($\tilde{\omega}_p^2 = 0.17$), indicating that a large proportion of the variance was unaccounted for by this model. Dose comparisons between species must be made with extreme caution, but with that caution in mind, it is interesting to consider the doses of CDB-4124 used in a recent clinical trial, where women received daily CDB-4124 doses of 12.5 mg, 25 mg, or 50 mg (Ioffe et al., 2009). For illustrative purposes, if the highest dose used in this clinical trial were administered to a woman weighing 50 kg (about 110 lbs) the dose would be 1 mg drug per 1 kg body mass (1 mg/kg), a dose that is a great deal lower than the doses that induced depression-like behavior in mice in the studies reported here (40 mg/kg or higher).

While the present results do lead to the prediction that CDB-4124 could induce depression in women, the dose required to do so would probably be much higher than the doses currently being tested for clinical use.

Perhaps more importantly, the current experimental preparation used CDB-4124 in large doses to precipitate progesterone withdrawal in mice that already had high levels of circulating progesterone. It is hypothesized that these high levels of progesterone are probably necessary for the depressogenic effect of CDB-4124, which suggests that it would be unlikely for any dose of CDB-4124 to affect mood states in women unless the initial administration coincided with a phase of high progesterone secretion. In summary, these data indicate that CDB-4124 could potentially induce depression in women if administered in a way that rapidly induced a progesterone withdrawal state. This is likely to have little relevance for the chronic, low dose clinical uses of the drug, and much greater theoretical or experimental relevance to perimenstrual and postpartum affective disorders, which are temporally associated with abrupt withdrawal from high levels of progesterone.

Locomotor activity

The effects of picrotoxin, mifepristone, and CDB-4124 were tested alone or in combination on forced swim test immobility. Some previous research has found that picrotoxin alters forced swim test immobility behavior, but the current experiments yielded only mixed evidence of the ability of picrotoxin to increase swim test immobility in the present experimental preparation. In contrast, CDB-4124 produced robust increases in forced swim test immobility. The metabolic allopregnanolone withdrawal experiment (Experiment 2) showed that finasteride

increased forced swim test immobility in a way that mimicked progesterone withdrawal, but the finding that picrotoxin did not reliably increase immobility led to the question of whether finasteride had increased forced swim test immobility by a mechanism that affected all locomotor activity in general, as opposed to the emotionally-motivated immobility that the forced swim test is supposed to reflect. It was previously noted that finasteride suppressed locomotor activity immediately after injection (Gabriel et al., 2005), but it was not known whether finasteride would decrease activity at later time points after injections (i.e., 2-4 hr later).

When finasteride was tested for effects on locomotor activity using the same treatment regimen as that which had increased forced swim test immobility, it was clear that finasteride had a strong locomotor depressant effect (Experiment 13). It is not clear whether finasteride decreases locomotor activity through a mechanism related to allopregnanolone, or if this locomotor depressing effect may be due to an unknown action of the drug. Furthermore, it is not clear whether this decrease in locomotor activity is a side effect that is unrelated to emotion, or if it perhaps is a symptom of "depression" in the mouse. Further research is necessary to clarify these issues, including the potential contribution of finasteride-induced changes in activity to forced swim test immobility.

CDB-4124 decreased locomotor activity over the course of the 30-min testing session, but analyzing these data in smaller time intervals revealed that locomotor activity was unaffected across the initial 6 min of testing. These smaller intervals were chosen for analysis specifically so that locomotor activity in the activity chambers could be compared to behavior in the 6-min forced swim test session.

Also, the treatment regimen for the CDB group in this experiment was identical to the treatments that increased swim test immobility, although the physical demands of locomotion in water or dry land could be different. Since locomotor activity was unchanged by CDB-4124 in the first 6 min of locomotor activity testing, these data suggest that CDB-4124 did not alter forced swim test immobility by causing outright motor deficits. The swim tank for the forced swim test and the activity monitors for locomotor activity were both novel environments, so it is not likely that the dissimilarity in activity between locomotor testing and forced swim test immobility is due to differences in novelty-induced activity. Therefore, the finding that CDB-4124 increased forced swim test immobility is likely to reflect some other behavioral domain, and clearly the hypotheses of the present work predict that this domain would be along the lines of emotion or motivation.

CDB-4124 did decrease overall activity compared to the groups that received only progesterone or vehicle. Examination of Figure 14b shows that all four groups experienced habituation to the environment. The CDB group was different from the VEH group in every time bin after the first time bin, but it could be argued that the PRO group is a more appropriate comparison group, since both received progesterone all eight days, and only differ in whether they received the progesterone receptor antagonist. In addition to the first time bin, locomotor activity was not significantly different between the CDB and PRO groups in the last two time bins. Thus, it is possible that the CDB treatment decreased overall activity by making the mice adapt more quickly to the environment, but without net any difference in adaptation at the end of the 30 minute session. This could indicate that

CDB-4124 treatment caused animals to "give up" exploring the locomotor chamber earlier than the PRO group mice. Since CDB-4124 also increased forced swim test immobility, its effects on locomotor activity across the whole session would be consistent with the hypothesis that forced swim test immobility indicates that an animal has "given up hope" (Porsolt et al., 2001, p. 1).

Additional tests might help to clarify what domains of locomotor activity are affected (or unaffected) by finasteride or CDB-4124 treatment. Anecdotally, mice that received either drug seemed to have the capacity for normal movement. For example, on in some experiments with either drug the mice were able to jump out of their cages in an apparent attempt at escape whenever the cage lids were opened. Based on these casual observations, it could be hypothesized that the drugs may have affected expression of voluntary locomotion without simultaneously affecting the animals' ability to walk or perform other locomotor behaviors.

The acts of walking or running require motivation to initiate these behaviors, but other factors must also be considered. Locomotor behaviors involve the complex integration of multiple systems in the body, and alterations in the function of any one system might influence the overall expression of locomotor behavior. Among these systems are the neural systems of proprioception, which allows the animal to sense the position and tension of its body parts relative to each other, equilibrioception, which allows the animal to sense its dynamic and static positional relationships relative to the environment, and neuromuscular system, which transmits neural signals into muscle activity. Additionally, the non-neural biological systems such as the muscular system must be competent to perform work.

Since both CDB-4124 and finasteride treatments were associated with decreased locomotor activity in the current work, more specific tests could be employed to determine whether these decreases were related to one of the specific systems mentioned above. For example, the H-reflex (Hoffmann reflex, an electrical way to elicit the muscle stretch reflex) test has previously been used in mice as a test of proprioception (e.g., X.-J. Chen et al., 2007), and could be used in conjunction to determine whether proprioceptive differences contribute to locomotor deficits in drug-treated mice. The stationary dowel test and the rotarod test have both been proposed to measure balance (Boehm et al., 2000), and could therefore be used to rule out altered equilibrioception as a consequence of finasteride or CDB-4124 administration. It may be more difficult to specifically test for muscle incoordination, since any behavior that appears to be poorly coordinated could actually reflect differences in proprioception, equilibrioception, or muscle competency, rather than the specific coordination of these systems. However, one test with some face validity for neuromuscular coordination could be the grid test (e.g., E. H. Shen et al., 1996), which requires mice to avoid stepping through holes in a gridded floor.

Finally, in terms of basic biological competence to perform work, a test such as the horizontal screen test could be used to further understand the effects of finasteride and CDB-4124 on locomotor behavior. The horizontal screen test (Coughenour et al., 1977) and related test measure the muscular strength of an animal without requiring the animal to move (isometric work). Therefore, if finasteride or CDB-4124 did not alter animals' ability to perform isometric work,

then the decreases in locomotor activity associated with these drug treatments could reasonably be proposed to be unrelated to an outright loss of muscle strength. If mice treated with either finasteride or CDB-4124 demonstrated good muscle strength, proprioception, equilibrioception, and neuromuscular coordination, then one of the most parsimonious explanations for their decreased locomotor activity could be a lack of motivation to move, perhaps related to a depression-like state.

Mifepristone

The prototypic progesterone receptor antagonist mifepristone was also tested for immobility-inducing effects in the forced swim test. The finding that mifepristone increased FST immobility (at the highest dose tested) offers additional support for the hypothesis that progesterone withdrawal increases forced swim test immobility by withdrawing activation of progesterone receptors. This finding is surprising in light of other lines of research. Mifepristone is a very potent progesterone receptor antagonist (Edwards et al., 2000), but its structure also allows it to bind with high affinity to glucocorticoid receptors, resulting in its dual action as an antagonist to both progesterone receptors and glucocorticoid receptors (Attardi et al., 2004; Benagiano et al., 2008a). (Mifepristone is also reported to have progesterone-enhancing or -agonist effects in certain instances; see Chien, 2009; R. N. Taylor et al., 1998). Given the hypothesized role of the hypothalamic-pituitary-adrenal axis in depression, there has been considerable interest in using antiglucocorticoid treatments, including mifepristone, for the treatment of various forms of depression (e.g., Benagiano et al., 2008b; Flores et al., 2006). Furthermore, in a single report, mifepristone (25 mg/kg) was demonstrated to decrease forced

swim test immobility in rats (an antidepressant-like effect), an effect which the authors attributed to the antiglucocorticoid actions of mifepristone (Korte et al., 1996).

The current results present a paradox in light of the more extensive evidence that shows potential for using mifepristone as an antidepessant. It may be possible that mifepristone can have depressogenic effects as an antiprogestin, but that these effects might be mitigated by its antiglucocorticoid characteristics. One way to reconcile the discrepant findings regarding mifepristone is to consider the unique features of the experimental preparations in the current experiments. These data do not dispute reports that show that mifepristone could have an antidepressant effect when administered chronically (presumably because of its antiglucocorticoid effects), but the current data lead to the hypothesis that high levels of progesterone could potentially unmask an ability of mifepristone to produce the opposite effects by a second mechanism of action (that is, it is possible that the antiprogestin effects became unmasked at higher doses of mifepristone combined with exogenously-administered progesterone).

It is not precisely known how mifepristone affected glucocorticoid receptors, progesterone receptors, or other targets when administered in the manner currently reported. Assuming that mifepristone and progesterone might have comparable pharmacokinetic properties, then even a dose of mifepristone lower than the 5 mg/kg dose of progesterone should be sufficient to inhibit the progesterone that was administered, because mifepristone is a substoichiometric inhibitor of progesterone receptors (Leonhardt et al., 2003). Since mifepristone

completely inhibits progesterone activity when concentrations of the two are equal, but the dose of mifepristone required to increase swim test immobility was many times higher than the exogenous progesterone dose, this might suggest that mifepristone increased swim test immobility by some mechanism of action other than progesterone receptors.

Little work has been done previously to examine specifically the role of progesterone receptors in the affective behaviors of rodents. Auger and Forbes-Lorman (2008) found that mifepristone blocked the anxiolytic effects of progesterone in male rats as assessed in the elevated plus maze. In spite of the important differences in sex, species, and affective modality, this study is essentially consistent with the present data showing that mifepristone increased depression-like behavior in female mice. The only other comparable study that has been found is the recent report by Galeeva and coworkers (2007) who tested elevated plus maze behavior in female mice. Although they reported an anxiolytic effect of mifepristone (in contrast to Auger & Forbes-Lorman), the level of methodological detail provided was insufficient to adequately compare the studies. Importantly, the dose of mifepristone used by Galeeva and colleages was only 10 μg/kg, or approximately 1,000 times lower than the dose used by Auger and Forbes-Lorman, and 8,000 times lower than the dose of mifepristone that increased forced swim test immobility in the current report (Experiment 12). It is possible, therefore, that very low doses of mifepristone may have anxiolytic effects while the higher doses have anxiogenic or depressogenic effects.

No other studies have been found that have assessed the effects of progesterone receptor antagonists on emotion-related behavior in rodents. However, Schmidt and colleagues (1991) administered mifepristone to women with premenstrual syndrome and assessed its effects on premenstrual symptoms. They found that mifepristone increased ratings of all premenstrual symptoms. However, because mifepristone is a progesterone receptor antagonist they interpreted this finding as an indication that endocrine functions during the menstrual cycle were *not* involved in premenstrual syndrome (i.e., since blocking the receptor did not block the symptoms, it was concluded that progesterone must not be involved). In light of the present data, it might be suggested that the study by Schmidt and colleagues could instead be used to support the opposite conclusion: that removal of progesterone may contribute to premenstrual syndrome, and that administration of a progesterone blocker increased these symptoms because administering the antagonist mimicked removal of progesterone. This is also the interpretation of the study by Schmidt and coworkers (1991) that was made by MacDonald and coworkers (1991).

Conclusions

In conclusion, these experiments provide only limited evidence to suggest that $GABA_A$ receptors contribute to progesterone withdrawal, measured by forced swim test immobility. In contrast, the progesterone receptor antagonists were found to robustly and reliably increase forced swim test immobility in a manner that mimicked (or even exceeded) the effect of passive progesterone withdrawal. The finding that CDB-4124 increased forced swim test immobility at a dose that did not

alter initial locomotor behavior in a novel environment suggests that this increase in immobility may not have been due to an alteration of the animals' locomotor ability. These findings are contrary to the allopregnanolone withdrawal hypothesis, which states that GABAergic steroids such as allopregnanolone might underlie the affective effects of progesterone, a hypothesis that has been explored by many laboratories (see above). However, the findings that progesterone receptor antagonists increase forced swim test immobility is consistent with the clinical study by Schmidt and coworkers (1991). The main theoretical alternative to the progesterone withdrawal hypothesis is the allopregnanolone withdrawal hypothesis. The locomotor activity data could explain why metabolic allopregnanolone withdrawal appeared to increase depression-like behavior, and therefore the strongest evidence in favor of the allopregnanolone evidence is possibly confounded by a general locomotor suppressant effect. However, the progesterone withdrawal hypothesis does not appear to be confounded by such an effect.

Chapter 5: Alternative-Approach Studies to Progesterone and Allopregnanolone Withdrawal

Chapter 5 Introduction

The preceding chapters have discussed several methods that have been used to study the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis. A passive progesterone withdrawal method was associated with increased forced swim test immobility, as was a metabolic allopregnanolone withdrawal method. Later, there was a set of experiments which showed that pharmacologically-precipitated progesterone receptor withdrawal increased forced swim test immobility, but precipitated $GABA_A$ receptor withdrawal had no reliable effect on immobility. The apparent discrepancy between the metabolic allopregnanolone withdrawal study (which presumably decreased activity at $GABA_A$ receptors) and the precipitated $GABA_A$ receptor withdrawal studies warranted further investigation.

Although the major thrust of the present work has been to study the effects of manipulating progesterone on the depression-like behavior of mice, there is also a considerable literature that addresses the effects of estrogens on depression (for review see Solomon & Herman, 2009). Specifically, a great number of studies have reported antidepressant effects of estradiol on forced swim test immobility in mice and rats (e.g., Dhir & Kulkarni, 2008; Tasset et al., 2008). As discussed in Chapter 3, in the initial passive progesterone method it was not deemed necessary to account for estrogen actions, because the doses of progesterone would be presumed to inhibit the animals' endogenous ovarian activity. Furthermore, because every effort

was taken to counterbalance all group treatments (e.g., controlling for cage effects), it was also presumed that if ovarian cyclicity was not prevented, that it would at least be a randomized factor and that it would introduce no systematic bias to the data. As it was shown, passive progesterone withdrawal was sufficient to increase forced swim test immobility without any additional control or manipulation of estrogens. However, this is not to conclude that estrogens are not involved in emotion regulation, nor should this be taken to suggest that the interactions between the estrogen and progesterone systems are unimportant. For example, it was recently shown that finasteride blocked the antidepessant effect of 17β-estradiol on forced swim test immobility (Molina-Hernández et al., 2009).

One consideration regarding estrogens is that there is a similar withdrawal of estrogen that occurs at the end of pregnancy in parallel to the progesterone withdrawal. This has led some to study estrogen withdrawal as a component of their animal models of reproductive depression (e.g., Doornbos et al., 2009b; Flaisher-Grinberg et al., 2009a; A. D. Green et al., 2009; Suda et al., 2008). In fact, given that progesterone is a precursor for both 17β-estradiol and allopregnanolone, it cannot be ruled whether down stream estrogen withdrawal contributed to the increase in forced swim test immobility during progesterone withdrawal in the passive progesterone withdrawal experiment (Experiment 1).

It is also important to recognize that progesterone receptor regulation is one of the most widely acknowledged functions of estrogen receptor activity (a relationship that is important, for example, in breast cancer; see Cui et al., 2005). For this reason, estrogen induction of progesterone receptors might be necessary

for peripheral progesterone withdrawal to elicit a neural/behavioral response. For this reason, experiments were designed to assess whether removal of endogenous estrogen or removal of endogenous estrogen followed by replacement with exogenous 17β-estradiol would increase or otherwise modulate the effects of progesterone withdrawal. Thus, the first two experiments reported in this chapter provide further tests of the progesterone withdrawal hypothesis.

The current chapter also reports a third experiment that offers an additional test of the allopregnanolone withdrawal hypothesis. In the metabolic allopregnanolone withdrawal experiment (Experiment 2) metabolic allopregnanolone withdrawal increased forced swim test immobility. Just as this single approach was not judged to be sufficient to accept the underlying hypothesis, the mixed evidence from the picrotoxin studies was not judged to be sufficient to refute this hypothesis. Therefore, an additional approach was tested where administration of exogenous allopregnanolone and its passive withdrawal preceded a test for forced swim test immobility, analogous to the passive progesterone method used in Experiment 1.

Chapter 5 Experiments

Methods—Experiment 14—Progesterone withdrawal, allopregnanolone withdrawal, forced swim test, and corticosterone in ovariectomized mice

The purpose of this experiment was to test the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis by determining whether passive progesterone withdrawal or metabolic allopregnanolone withdrawal would have increased immobility-producing effects in the forced swim

test in mice that had undergone ovariectomy to inhibit the majority of endogenous sex steroid production. It was hypothesized that progesterone withdrawal would not increase forced swim test immobility, because without endogenous estrogen there would be no progesterone receptors to mediate a progesterone withdrawal response. After the requisite period of recovery following ovariectomy, mice received either daily injections of vehicle or progesterone (5 mg/kg) on days 1-5 of the experiment. The group that received vehicle on days 1-5 continued to receive vehicle on days 6-8 of the experiment ("VEH" group). Of the mice that received progesterone on days 1-5, one group continued to receive daily injections of progesterone on days 6-8 of the experiment ("PRO" group), one group received daily injections both of progesterone and finasteride (100 mg/kg) on days 6-8 of the experiment (metabolic allopregnanolone withdrawal "MAW" group), and one group received only vehicle injections on days 6-8 of the experiment (progesterone withdrawal or "PWD" group). Mice were tested in the forced swim test on day 8 of the experiment approximately 2-4 hours after the last injection. Following forced swim testing mice were decapitated and trunk blood was collected into Vacutainer 4 mL tubes containing 7.2 mg EDTA on ice, which were then centrifuged at 850 g and 4 °C for 20 min. Following centrifugation, the plasma fraction was aspirated and stored in separate tubes at −80 °C until assayed for corticosterone concentrations as described under General Methods. Table 16 shows a complete schedule of treatment injections and when forced swim testing occurred.

Experiment 14—Results—Progesterone withdrawal, allopregnanolone withdrawal, forced swim test, and corticosterone in ovariectomized mice

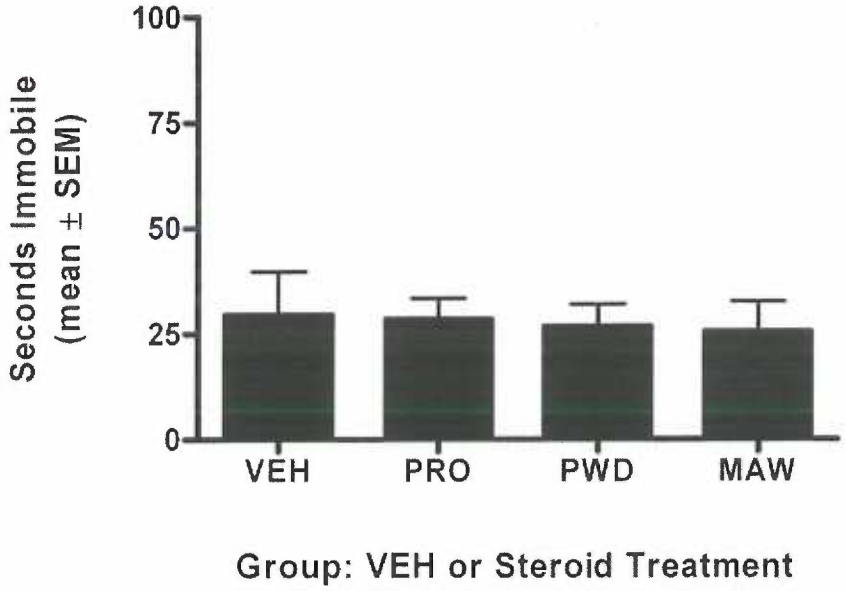
Forced swim test immobility was not altered as a result of passive progesterone withdrawal or metabolic allopregnanolone withdrawal in ovariectomized mice (see Figure 15a). There was no significant overall change in forced swim test immobility ($F_{3,50}$ = 0.064, *ns)*. There was also no difference in plasma corticosterone concentrations among these groups ($F_{3,49}$ = 0.359, *ns*), as shown in Figure 15b.

Table 16: Experiment 14 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6-7	8
VEH	VEH	VEH, VEH	VEH, VEH, FST
PRO	PRO	PRO, VEH	PRO, VEH, FST
PWD	PRO	VEH, VEH	VEH, VEH, FST
MAW	PRO	PRO, FIN	PRO, FIN, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; FIN, finasteride. Finasteride was administered in doses of 100 mg/kg. "MAW" indicates a metabolic allopregnanolone withdrawal group; "PWD" indicates a (passive) progesterone withdrawal group. "FST" indicates the day of forced swim testing. Initial sample sizes were n = 14 for each group.

15a


Seconds Immobile
(mean ± SEM)
100
75
50
25
0
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

15b

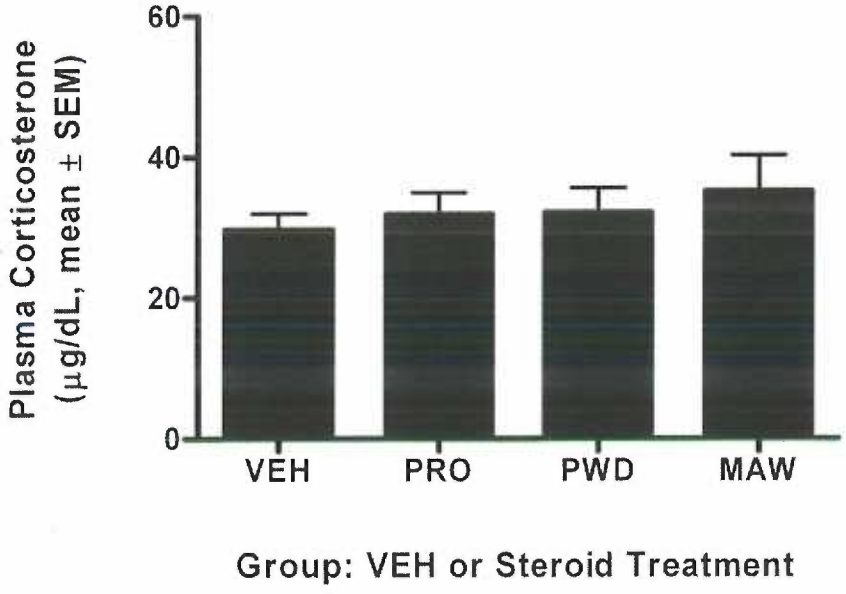

Plasma Corticosterone
(µg/dL, mean ± SEM)
60
40
20
0
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 15. Effects of passive progesterone withdrawal and metabolic allopregnanolone withdrawal on (a) forced swim test immobility and (b) plasma corticosterone concentration (μg/dL) in ovariectomized mice. (a) Mean (± SEM) seconds immobile in the forced swim test was unaffected by progesterone or allopregnanolone withdrawal. (b) Mean (± SEM) plasma corticosterone was unaffected by progesterone or allopregnanolone withdrawal. Final sample sizes for forced swim test immobility were $n = 12$ for the VEH group (two mice were removed after they received the incorrect injections) and $n = 14$ for the PRO, PWD, and MAW groups. Final sample sizes for plasma corticosterone levels were $n = 12$ for the VEH group (see above), $n = 13$ for the PRO group (one blood sample was not available), and $n = 14$ for the PWD and MAW groups.

Experiment 15—Methods—Progesterone withdrawal, estrogen withdrawal, and forced swim test in ovariectomized mice

The purpose of this experiment was to determine the effects of estrogen replacement on forced swim test immobility in ovariectomized mice. The first goal was to find whether estrogen replacement would increase the immobility-inducing effect of progesterone withdrawal ("PWD" group below) compared to mice that received continuous estrogen and progesterone injections ("CEP" group below). Another purpose was to compare the effect of estrogen withdrawal in mice that received continuous progesterone injections ("EWD" group below) and the effect of combined estrogen and progesterone withdrawal ("DWD" group below) with the PWD and CEP groups. It was hypothesized that progesterone withdrawal, and possibly estrogen or dual withdrawal, would increase forced swim test immobility because the exogenous estrogen would induce progesterone receptors to mediate a progesterone withdrawal condition.

All estrogen injections were 100 ng 17β-estradiol in 0.1 mL of vehicle. After the one-week recovery period had passed, all mice received a priming injection of estrogen on day 0. As with all other experiments, progesterone injections (5 mg/kg) were administered intraperitoneally, but it was originally planned that all estrogen injections would be administered subcutaneously (sc). On days 0 and 1 estrogen injections were administered sc. By day 2, however, the mice had begun to resist scruffing so much that it was judged to be more hazardous than scientifically justified to continue performing the sc injections. It was thought that sc injections might pose a greater risk for injuring the mouse if it is not sufficiently still because it

was thought that the muscles and organs along the dorsal thoracic section could be more easily damaged by a needle than the organs in the peritoneum. It is not clear why the mice increased their resistance to physical manipulation, although one might hypothesize that the resistance reflects irritability or pain sensitization. Therefore, approximately one-third of the mice received estrogen by sc injection on day 2, and the rest received estrogen injection by intraperitoneal injection. On day 3 and beyond, all injections were administered intraperitoneally.

Such were the routes of administration by which all mice received daily injections of progesterone on days 1-5 and estrogen on days 0-5 of the experiment. On days 6-8 mice continued to receive progesterone, estrogen, both, or only vehicle, depending on which steroid was to be withdrawn. The estrogen withdrawal group ("EWD") received progesterone but not estrogen on these days. The progesterone withdrawal group ("PWD") received estrogen on these days but not progesterone. The dual withdrawal group ("DWD") receive only vehicle on these days, while the continuous estrogen and progesterone group ("CEP") received both estrogen and progesterone on these days. All mice were tested in the forced swim test on day 8 of the experiment, approximately 2-4 hours after their last injections. Table 17 shows a schedule of injections and forced swim testing. Data for this experiment were analyzed with two-way ANOVA using progesterone treatment during days 6-8 (progesterone vs. vehicle) as one factor, and estrogen treatment during the same days (estrogen vs. vehicle) as a second factor.

Experiment 15—Results—Progesterone withdrawal, estrogen withdrawal, and forced swim test in ovariectomized mice

Neither estrogen withdrawal nor progesterone withdrawal alone increased forced swim test immobility in ovariectomized mice, as evidenced by the lack of a significant main effect of estrogen withdrawal (vs. continuous estrogen) or a significant main effect of progesterone withdrawal (vs. continuous progesterone). Also, progesterone withdrawal and estrogen withdrawal combined did not increase forced swim test immobility in the dual withdrawal group, indicated by the lack of a significant estrogen × progesterone interaction (see Figure 16). (All were $F_{1,41} < 2.80$, *ns.*)

Table 17: Experiment 15 Injection and Forced Swim Test Schedule

	Days			
Group	0	1-5	6-7	8
CEP	EST	EST, PRO	EST, PRO	EST, PRO, FST
EWD	EST	EST, PRO	VEH, PRO	VEH, PRO, FST
PWD	EST	EST, PRO	EST, VEH	EST, VEH, FST
DWD	EST	EST, PRO	VEH, VEH	VEH, VEH, FST

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; EST, estrogen. "FST" indicates the day of forced swim testing. Initial sample sizes were $n = 12$ for each group. "CEP" indicates the "continuous estrogen and progesterone" group. "EWD" indicates the "estrogen withdrawal" group. "PWD" indicates the "progesterone withdrawal" group. "DWD" indicates the "dual withdrawal" group.

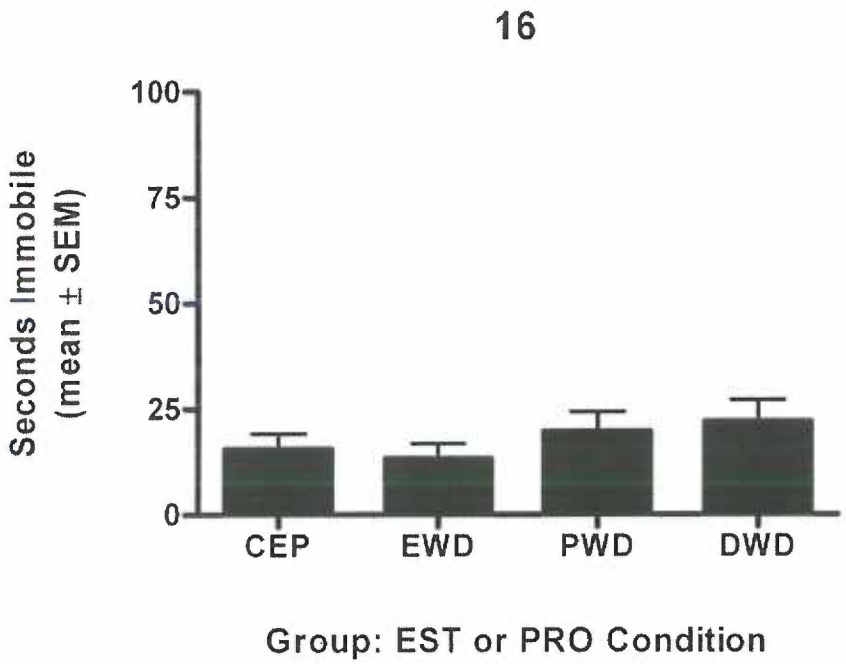


Figure 16. Mean (± SEM) seconds of immobility in the forced swim test in mice following continuous estrogen and progesterone (CEP), estrogen withdrawal (EWD), progesterone withdrawal (PWD), or dual withdrawal (DWD) in ovariectomized mice. Final sample sizes were $n = 12$ for the CEP group and $n = 11$ for each of the EWD, PWD, and DWD groups.

Experiment 16—Methods—Passive allopregnanolone withdrawal and forced swim test

The purpose of this experiment was to test the allopregnanolone withdrawal hypothesis by testing whether passive withdrawal from administration of exogenous allopregnanolone would increase forced swim test immobility in a manner consistent with passive progesterone withdrawal and metabolic allopregnanolone withdrawal (Experiments 1 and 2). It was hypothesized that passive allopregnanolone withdrawal would increase forced swim test immobility, consistent with the allopregnanolone withdrawal hypothesis and with the metabolic allopregnanolone treatment of Experiment 2. All mice received daily injections of vehicle ("VEH") or allopregnanolone (17 mg/kg) on days 1-5 of the experiment. VEH group mice were tested in the forced swim test approximately 2-4 hours after their last injections (on day 5). Of the mice that received daily injections of allopregnanolone, one group ("0 d AWD") was tested in the forced swim test approximately 2-4 hours after their last injections (also on day 5). Remaining groups that received allopregnanolone on days 1-5 were tested in the forced swim test approximately 2-4 hours after their last injections plus one day ("1 d AWD"—tested on day 6) or plus three days ("3 d AWD"—tested on day 8). A schedule of injections and swim testing is shown in Table 18.

Experiment 16—Results—Passive allopregnanolone withdrawal and forced swim test

There was no significant overall difference in forced swim test immobility among the groups that received continuous vehicle injections, continuous

allopregnanolone injections, or passive allopregnanolone withdrawal, $F_{3,39} = 1.33$, *ns* (see Figure 17).

Table 18: Experiment 16 Injection and Forced Swim Test Schedule

	Days		
Group	1-5	6	8
VEH	VEH, FST		
0 d AWD	ALLO, FST		
1 d AWD	ALLO	FST	
3 d AWD	ALLO		FST

Note. Drug-injection abbreviations used: ALLO, allopregnanolone; VEH, vehicle. "FST" indicates the day of forced swim testing. Allopregnanolone was administered in doses of 17 mg/kg. On day 7, the 3 d AWD group remained in their cages undisturbed. Initial sample sizes were n = 12 for each group.

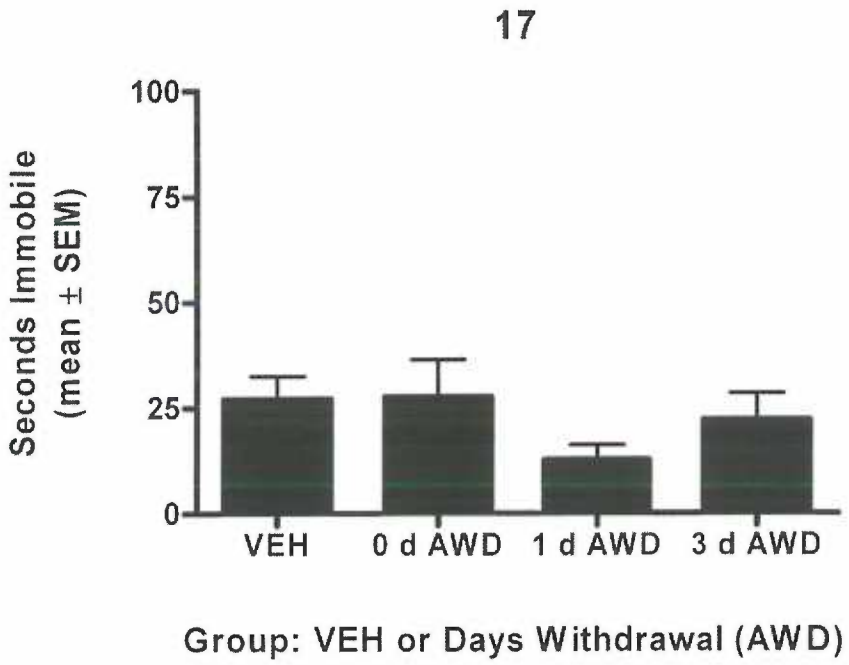


Figure 17. Mean (± SEM) seconds of immobility in the forced swim test in mice following continuous vehicle administration (VEH), continuous allopregnanolone administration (0 d AWD), or one (1 d AWD) or three (3 d AWD) days of passive withdrawal from allopregnanolone administration. Final sample sizes were $n = 11$ for the VEH group (one mouse received an incorrect injection), $n = 9$ for the 0 d AWD group (one mouse was found dead in its cage and two mice received incorrect injections), $n = 11$ for the 1 d AWD group (one mouse received an incorrect injection), and $n = 12$ for the 3 d AWD group.

Chapter 5 Discussion

Experiment 14 was tested whether removal of the ovaries—and thereby definitively controlling endogenous ovarian activity—would alter the effects of passive progesterone withdrawal or metabolic allopregnanolone withdrawal. No changes in forced swim test immobility emerged. Two possible explanations will be considered. One way to explain this finding comes from the observations of Bekku and coworkers (2006) who made an extensive study of the effects of ovariectomy in mice on forced swim test immobility. Although many important methodological details differed between their studies and the present experiments, the clear implication of their experiment is that ovariectomy itself increases forced swim test immobility. Thus, although the present work controlled the effects of ovulatory cycles on forced swim test immobility by ovariectomy, it could be possible that the ovariectomy itself introduces a profound physiological change that confounded the design. It may be that the ovariectomies that were performed resulted in a transient hormone withdrawal before the experimental phases were even commenced, and that depression-like behavior would have been detected if it had been tested during the recovery period.

An alternate interpretation of the results of Experiment 14 is that progesterone receptors are necessary for the effect of passive progesterone withdrawal, and that removing the ovaries made it so that no effective progesterone withdrawal could be achieved. In other words, regardless of what changes may have occurred in the plasma as progesterone was added and then removed, if the relative lack of estrogen resulted in no induction of progesterone receptors in the

brain, then peripheral progesterone fluctuations may be without any neurobiological consequence. This explanation is favorable when considered in the context of the previous chapter, which highlighted the effect of increased forced swim test immobility when progesterone receptor antagonists were administered. Thus, taking the results of Experiment 14 into consideration along with the CDB-4124 and mifepristone experiments, it is possible that passive progesterone withdrawal could not increase forced swim test immobility in ovariectomized mice because there was no progesterone receptor activity in the first phase of the treatment that was left to withdraw in the second phase regardless of how much progesterone was present to activate the receptors during the first phase.

Since estrogen depletion by ovariectomy may have prevented the effect of progesterone withdrawal on forced swim test immobility during Experiment 14, Experiment 15 tested whether estrogen replacement would restore the effect of progesterone withdrawal on forced swim test immobility. This was not the case. In Experiment 15 no changes in forced swim test immobility were observed even though estrogen was administered to induce progesterone receptors. One important consideration is that it may not be possible for exogenous estrogen to completely restore progesterone receptor expression. Additional exploratory statistical analyses of the data were also performed, which provided some support for a weak effect of progesterone withdrawal on forced swim test immobility (not shown), because when no outliers were removed from the analysis there was a trend towards significance for a main effect of progesterone withdrawal to increase forced swim test immobility. However, judging by criteria that are reported, the

same criteria that were used throughout the remainder of the present work, there were no differences.

Again, it must be stated that ovariectomy is not an innocuous procedure, so these data must be taken as only a preliminary examination of the effects of estrogen on progesterone withdrawal within the context of the present model. Another possibility that should be considered is that the route of estrogen administration might have confounded the experiment. The choice to inject estrogen might have prevented discovering an effect of progesterone withdrawal on swim test immobility either because of pharmacokinetics or simply the added stress of additional injections. Finally, it is possible that progesterone receptors were not sufficiently induced by the estrogen treatment regimen that was used.

Since pharmacologically precipitated withdrawal of $GABA_A$ receptor activity failed to reliably increase forced swim test immobility, the passive allopregnanolone experiment (Experiment 16) tested the effect of allopregnanolone withdrawal on forced swim test behavior using alternative methodology. Given that allopregnanolone is rapidly metabolized and cleared from the brain (for example, see Figure 4c) it is possible that exogenous administration of allopregnanolone never achieved the level of $GABA_A$ receptor activation that would be necessary in order for rebound disinhibition to result in increased forced swim test immobility. However, two pieces of information from the preceding chapter must be taken into account. One piece is that picrotoxin failed to reliably increase swim test immobility. The second piece is that finasteride was observed to result in a marked suppression of locomotor activity, which suggests that finasteride may only have

increased forced swim test immobility (in Experiment 2) because of a general motor deficit. Taking these two pieces of information into consideration, it is likely that Experiment 16 is an additional piece of evidence that allopregnanolone withdrawal does not increase forced swim test immobility as had been hypothesized.

These experiments represent only preliminary evaluations of the role of estrogen in progesterone withdrawal and the effects of passive allopregnanolone withdrawal. Although only a single experiment with a preliminary design, the chief finding of the allopregnanolone withdrawal experiment was that passive allopregnanolone withdrawal failed to increase forced swim test immobility, a finding that contradicts the allopregnanolone withdrawal hypothesis.

Chapter 6: Neural Activation During Progesterone Withdrawal and Allopregnanolone Withdrawal

Chapter 6 Introduction

Chapters 3 through 5 focused almost entirely on immobility behavior in the forced swim test. The experiments in these chapters tested the progesterone withdrawal hypothesis and the allopregnanolone withdrawal hypothesis. The general hypotheses and specific sub-hypotheses tested in the preceding chapters were oriented towards identifying which steroids and receptor systems in the brain might underlie the phenomenon of increased swim test immobility during progesterone or allopregnanolone withdrawal. In contrast, the current chapter addresses the localization of brain regions that respond to some of the key pharmacological treatments that were previously characterized in behavioral tests. Thus, the current experiment was an attempt to identify the brain regions that are affected by these procedures. Previous work has established that site-specific, acute manipulations of allopregnanolone have behavioral consequences in a few brain regions; for example, intra-hippocampal and intra-amygdalar injections of finasteride have been reported to increase forced swim test immobility (Frye & Walf, 2002; Walf et al., 2006). However, it is not known whether the same brain areas may be involved during progesterone withdrawal, or whether those brain areas that are involved during progesterone withdrawal are the same as for allopregnanolone withdrawal.

The current chapter discusses a brain "mapping" project using immunohistochemistry for c-Fos protein. A technical and highly-detailed review of

inducible and constitutive transcription factors and their molecular biology, with special reference to the nervous system, is provided by Herdegen and Leah (1998). A review of the strategy for using c-Fos protein to "map" brain activity is provided by Kovács (1998), and some important considerations for interpreting result from this sort of strategy are discussed by Ryabinin (2000). Although this approach has been used in the area of steroids and the nervous system, no attempt has previously been made to map activity across brain regions during hormone withdrawal.

In one relevant study, Doornbos and coworkers (2009b) measured c-Fos protein expression in the periventricular nucleus of the hypothalamus. One treatment group was made to experience an abrupt progesterone and 17β-estradiol withdrawal by the implantation and subsequent removal of hormone-filled silastic capsules. A second group received estrogen and progesterone injections that tapered in dose during the later phase of the experiment in a manner designed to gradually withdraw these steroids. A third "no-decline" group received similar injections with no taper at the end. Finally, a control group received only vehicle injections during the treatment phase of the study. Although c-Fos expression was increased in the periventricular nucleus in the control group compared to the remaining groups, there were no other differences among the groups. Since there were no differences in periventricular nucleus c-Fos expression among the abrupt withdrawal, gradual withdrawal, and no withdrawal conditions, these results suggest that the relative increase in c-Fos expression in the vehicle-treated control group was due to lack of exogenous hormone administration, but that the increase is not relevant to steroid hormone withdrawal specifically. The lack of difference

among groups that received steroid hormones and different withdrawal treatments is consistent with other studies showing no differences in paraventricular nucleus c-*fos* mRNA between prepartum and postpartum females (reviewed in Lightman et al., 2001; see also Luckman, 1995).

However, in this study by Doornbos and coworkers (2009b) there were important behavioral differences in the rats that are unaccounted for by c-Fos protein expression, as there was no expression difference in the periventricular nucleus among groups with differences in behavior. The rapid withdrawal group exhibited increased anxiety-related behaviors as measured by startle-response magnitude and time spent in the interior zone of an open field (Doornbos et al., 2009b). Since these differences must emerge from differences in the brain, one possibility is that the authors simply did not study the brain areas that respond to the withdrawal treatments, or that underlie the anxiety behaviors that were assessed.

Currently, one of the most active areas in progesterone research focuses on the potential neuroprotective effects of progesterone, for example during traumatic brain injury (for reviews see, Hu et al., 2009; Schumacher et al., 2008). From this research topic came a single report that showed that c-Fos protein expression, measured by Western blot, was increased in whole-lobe brain homogenates during abrupt progesterone withdrawal in rats that had received a medial frontal cortex contusion (Cutler et al., 2005). Although further details of the study become complicated to describe with brevity, it is important to note that the effect of abrupt

progesterone withdrawal and lesion on c-Fos expression did not appear to be simply a function of the lesion.

As a preliminary investigation into what brain regions might be activated during progesterone withdrawal and allopregnanolone withdrawal, the experiment described in this chapter measured c-Fos expression in various regions of the brain that are thought to be important for mood and motivation (see Table 19). This experiment addresses the sub-hypothesis that predicts that allopregnanolone withdrawal would increase neural activity. Since metabolic allopregnanolone withdrawal and passive progesterone withdrawal induced similar changes in forced swim test immobility, both treatments were studied for changes in neural activity as measured by c-Fos immunohistochemistry. It was hypothesized that some brain regions would demonstrate increased expression of c-Fos protein in the metabolic allopregnanolone withdrawal and passive progesterone withdrawal groups.

Table 19: Experiment 17 Brain Regions and Abbreviations Used

Brain Region	Abbreviation
Caudate putamen	CPu
Nucleus accumbens shell	AcbSh
Lateral nucleus accumbens shell	LAcbSh
Nucleus accumbens core	AcbC
Dorsal lateral septum	LSD
Ventral lateral septum	LSV
Bed nucleus of the stria terminalis	BST
Granule layer of the dentate gyrus	GrDG
Central nucleus of the amygdala	CeA
Basolateral amygdala	BLA
Periaqueductal grey	PAG
Ventral tegmental area	VTA
Dorsal raphe nucleus	DR
Median raphe nucleus	MnR

Note. Abbreviations are generally based on those proposed by Paxinos and Franklin (2001). The BST, PAG, and DR cell counts include all sub-regions shown in the atlas. The CeA cell counts are based on the lateral portion. The BLA cell counts can include the anterior and posterior aspects, depending on the distance from bregma.

Chapter 6 Experiment—Experiment 17

Methods—c-Fos expression

The purpose of this experiment was to determine specific brain regions with increased c-Fos immunoreactivity during passive progesterone withdrawal or metabolic allopregnanolone withdrawal. All groups in this experiment began with eight subjects (*n*), but not all subjects had usable tissue sections from each of the regions discussed. Details on group sizes are provided in the table and figure legends. Two groups of mice received daily injections of vehicle on days 1-5 of the experiment. Of these two groups, one group continued to receive daily injections of vehicle on days 6-8 of the experiment (the vehicle group, "VEH"), while the other received daily injections of finasteride (100 mg/kg) on days 6-8 of the experiment (the finasteride-only group, "FOG"). Three groups received daily injections of progesterone (5 mg/kg) on days 1-5 of the experiment. The first of these groups continued to receive daily injections of progesterone on days 6-8 of the experiment (the continuous progesterone group, "PRO"). The second of these groups received only vehicle injections on days 6-8 of the experiment (the passive progesterone withdrawal group, "PWD"). The last group received daily injections of both progesterone and finasteride (100 mg/kg) on days 6-8 of the experiment (metabolic allopregnanolone withdrawal group, "MAW"). On day 8 of the experiment, each mouse was euthanized. A schedule of injections and euthanasia is shown in Table 20.

Mice were decapitated 120 ± 1 min after their last injection and trunk blood was collected into Vacutainer 4 mL tubes containing 7.2 mg EDTA on ice. Blood

collection tubes were then centrifuged at 850 *g* and 4 °C for 20 min. Following centrifugation, the plasma fraction was aspirated and stored in separate tubes at −80 °C until assayed for corticosterone concentrations as described under General Methods. At the same time that trunk blood was being collected, the brain was being rapidly removed from the skull and cryoprotected as described under General Methods. Later, the brains were sectioned at 40 μm as described under the General Methods. Of the brain regions chosen study (see above, Table 19) sections from these brain regions were chosen based on landmarks visible to the unaided eye. These sections were taken from the rostro-caudal distances from bregma indicted in Table 21, and were treated with the immunohistochemical procedures described under the section on General Methods. Statistics were performed as described under the General Methods. Where more than one span of bregma distances were analyzed separately, if neither span yielded significant differences then both spans were combined and reanalyzed in the same manner.

Table 20: Experiment 17 Injection and Euthanasia Schedule

	Days		
Group	1-5	6-7	8
FOG	VEH	FIN, VEH	FIN, VEH, EUT
VEH	VEH	VEH, VEH	VEH, VEH, EUT
PRO	PRO	PRO, VEH	PRO, VEH, EUT
PWD	PRO	VEH, VEH	VEH, VEH, EUT
MAW	PRO	PRO, FIN	PRO, FIN, EUT

Note. Drug-injection abbreviations used: PRO, progesterone; VEH, vehicle; FIN, finasteride (100 mg/kg). "EUT" indicates the day of euthanasia. Initial sample sizes were n = 8 for each group.

Table 21: Experiment 17 Brain Regions Counted at Various Distances from Bregma

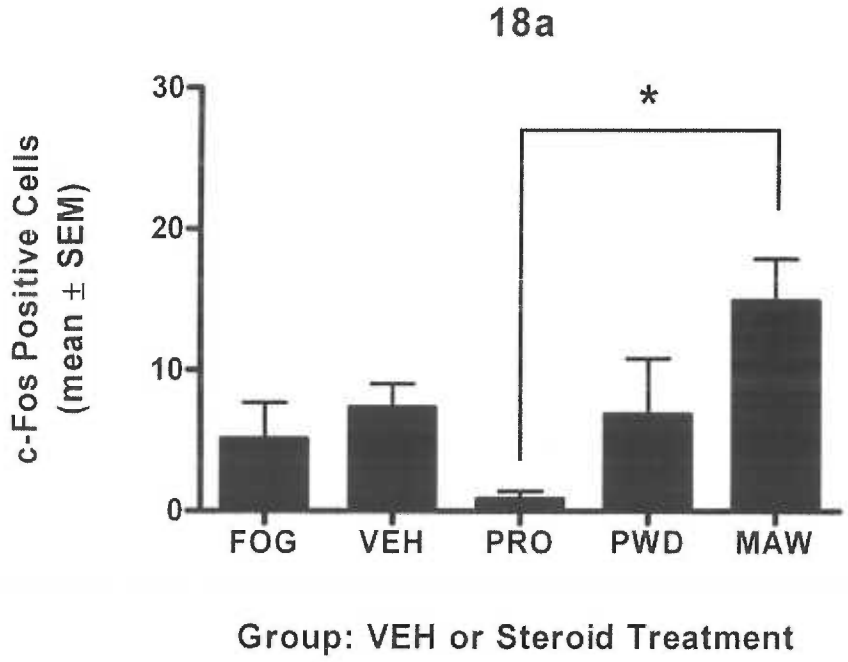
Brain Region	Bregma Distances Counted
Caudate putamen	1.34-1.10, 0.98-0.74
Nucleus accumbens shell	1.34-1.10, 0.98-0.74
Lateral nucleus accumbens shell	1.34-1.10, 0.98-0.74
Nucleus accumbens core	0.98-0.74
Dorsal lateral septum	1.18-1.10, 0.98-0.74
Ventral lateral septum	1.18-1.10, 0.98-0.74
Bed nucleus of the stria terminalis	0.26, 0.14
Granule layer of the dentate gyrus	−1.46--1.94, −2.06--2.54
Central nucleus of the amygdala	−1.82--1.94, −2.06--2.18
Basolateral amygdala	−1.82--1.94, −2.06--2.18
Periaqueductal grey	−2.54--2.80, −2.92--3.52
Ventral tegmental area	−2.54--2.80
Dorsal raphe nucleus	−4.36--4.84
Median raphe nucleus	−4.36--4.84

Results—Caudate putamen

The numbers of c-Fos positive cells were significantly different across treatment groups in both of the regions of the CPu that were tested. There was an overall difference in the number of c-Fos positive cells in the range of 1.34-1.10 mm from bregma in the CPu, $F_{4,17} = 3.30$, $p < .05$. Post hoc tests revealed that there were significantly fewer c-Fos positive cells in the PRO group compared to the MAW group (Figure 18a). There was also an overall difference in the number of c-Fos positive cells in the CPu in the range from 0.98-0.74 mm from bregma, $F_{4,18} = 14.2$, $p < .05$. There were significantly more c-Fos positive cells in the MAW group compared to all other groups, and there were also significantly more c-Fos labeled cells in the VEH group compared to the PRO group (Figure 18b).

Results—Nucleus accumbens shell

There were no significant differences in the number of c-Fos positive cells found among the different treatment groups in either of the regions of the AcbSh that were counted. There was no significant difference in the number of labeled cells in the range of 1.34-1.10 mm from bregma, $F_{4,15} = 0.775$, *ns*. There was also no significant difference in the number of labeled cells in the 0.98-0.74 range, $F_{4,18} = 1.07$, *ns*. When the two levels were combined together no difference in the number of labeled cells was detected across the whole range counted, $F_{4,19} = 1.13$, *ns* (Figure 19).


18a
c-Fos Positive Cells
(mean ± SEM)
30
20
10
0
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

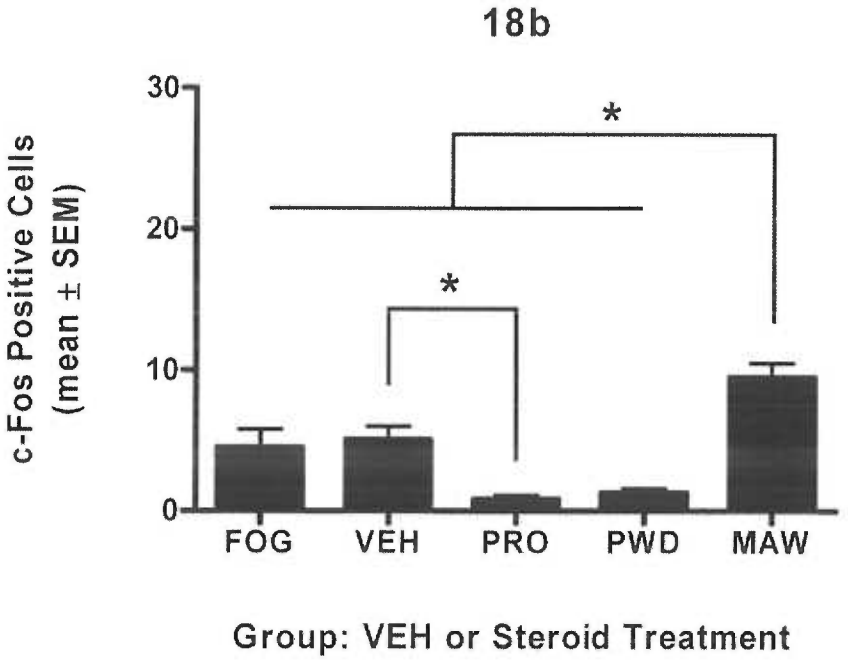

18b
c-Fos Positive Cells
(mean ± SEM)
30
20
10
0
*
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 18. Mean (± SEM) number of c-Fos positive cells the in caudate putamen at (a) 1.34-1.10 mm from bregma, and (b) 0.98-0.74 mm from bregma. (a) * $p < .05$ difference in immunoreactive cells between the MAW group and the PRO group. (b) * $p < .05$ difference in immunoreactive cells between the VEH group and the PRO, and between the MAW group and each other group. Final sample sizes in the 1.34-1.10 mm range were $n = 4$ for the FOG group (no usable sections were collected from four mice), $n = 5$ for the MAW group (no usable sections were collected from three mice), $n = 3$ for the PRO group (no usable sections were collected from five mice), $n = 4$ for the PWD group (no usable sections were collected from four mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice). Final sample sizes in the 0.98-0.74 mm range were $n = 4$ for the FOG group (no usable sections were collected from three mice), $n = 5$ for the MAW group (no usable sections were collected from three mice), $n = 5$ for the PRO group (no usable sections were collected from three mice), $n = 3$ for the PWD group (no usable sections were collected from three mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

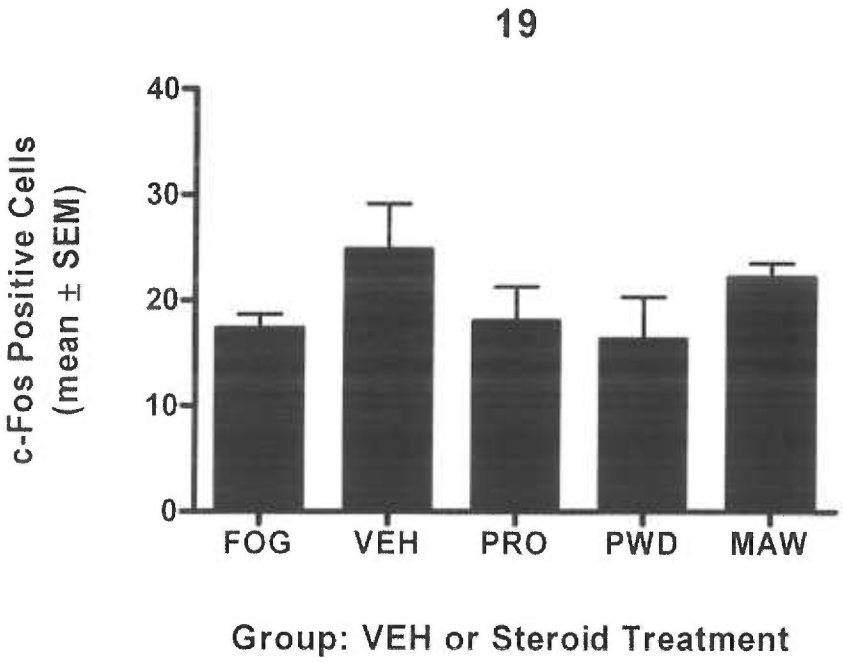


Figure 19. Mean (± SEM) number of c-Fos positive cells in the nucleus accumbens shell at 1.34-0.74 mm from bregma. Final sample sizes were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 3$ for the MAW group (no usable sections were collected from five mice), $n = 5$ for the PRO group (no usable sections were collected from three mice), $n = 5$ for the PWD group (no usable sections were collected from three mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

Results—Lateral nucleus accumbens shell

Only one difference in c-Fos positive cells was found between the two regions of the LAcbSh that were assessed. In the region of 1.34-1.10 mm from bregma there was a significant difference in c-Fos positive cells among the different groups, $F_{4,15} = 4.25$, $p < .05$ (Figure 20a). Post hoc tests revealed that there were significantly more c-Fos positive cells in the PRO group compared to all other groups. There were no significant differences in c-Fos in the range of 0.98-0.74 mm from bregma, $F_{4,16} = 0.518$, *ns* (Figure 20b).

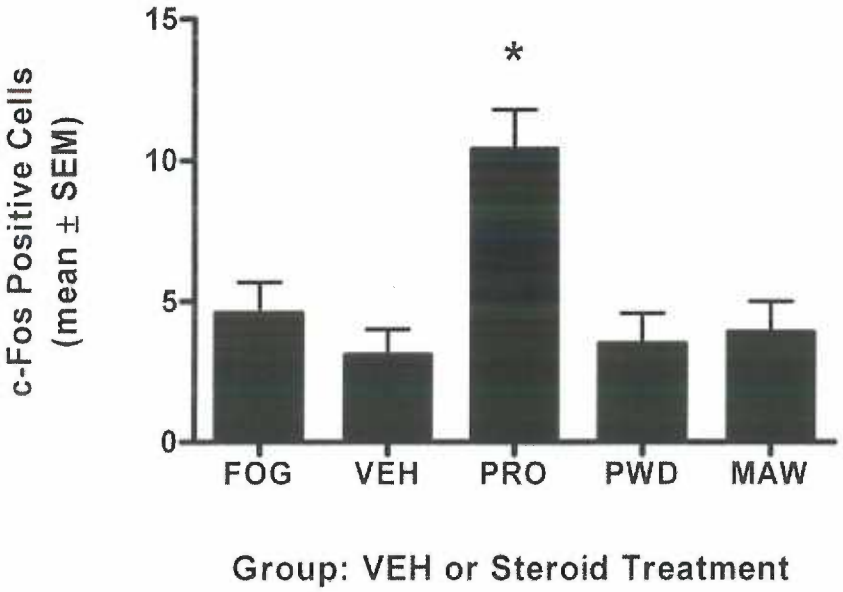
Results—Nucleus accumbens core

There were no significant differences in c-Fos immunoreactive cells in the AcbC. ANOVA revealed no differences among the different treatment groups in the 0.98-0.74 mm range from bregma, $F_{4,16} = 1.28$, *ns* (Figure 21).

Results—Dorsal lateral septum

There were no significant differences in c-Fos cells in either of the areas of the LSD that were measured. There were no significant differences in c-Fos positive cells in the range of 1.18-1.10 mm from bregma, $F_{4,13} = 1.28$, *ns*. There were also no significant differences in c-Fos positive cells in the 0.98-0.74 mm distance range from bregma, $F_{4,19} = 1.87$, *ns*. When both ranges were combined there remained no significant differences among groups, $F_{4,19} = 0.818$, *ns* (Figure 22).

20a


c-Fos Positive Cells
(mean ± SEM)
15
10
5
0
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

20b

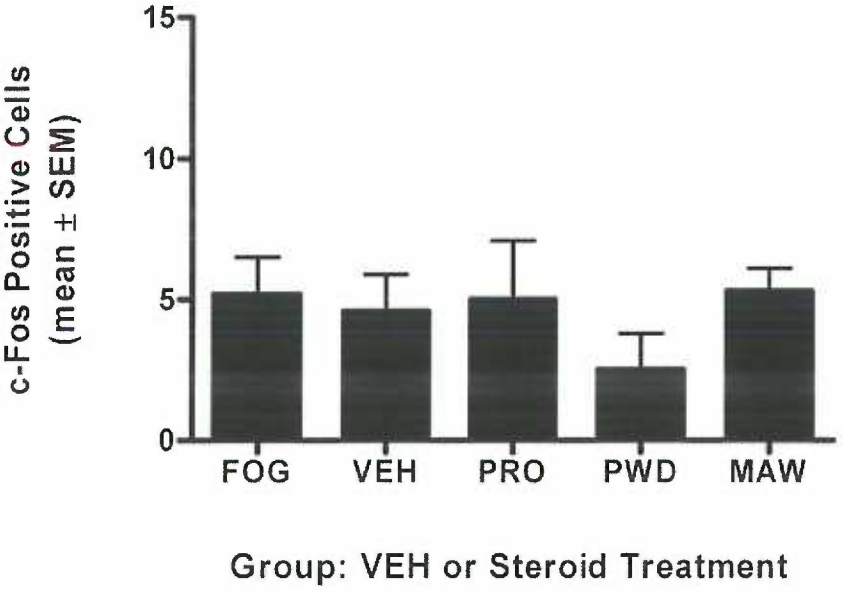

c-Fos Positive Cells
(mean ± SEM)
15
10
5
0
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 20. Mean (± SEM) number of c-Fos positive cells in the lateral nucleus accumbens shell at (a) 1.34-1.10 mm from bregma, and (b) 0.98-0.74 mm from bregma. (a) * $p < .05$ difference in immunoreactive cells between the PRO group and each other group. Final sample sizes in the 1.34-1.10 mm range were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 3$ for the MAW group (no usable sections were collected from five mice), $n = 2$ for the PRO group (no usable sections were collected from six mice), $n = 5$ for the PWD group (no usable sections were collected from three mice), and $n = 5$ for the VEH group (no usable sections were collected from two mice). Final sample sizes in the 0.98-0.74 mm range were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 2$ for the MAW group (no usable sections were collected from six mice), $n = 4$ for the PRO group (no usable sections were collected from four mice), $n = 4$ for the PWD group (no usable sections were collected from four mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

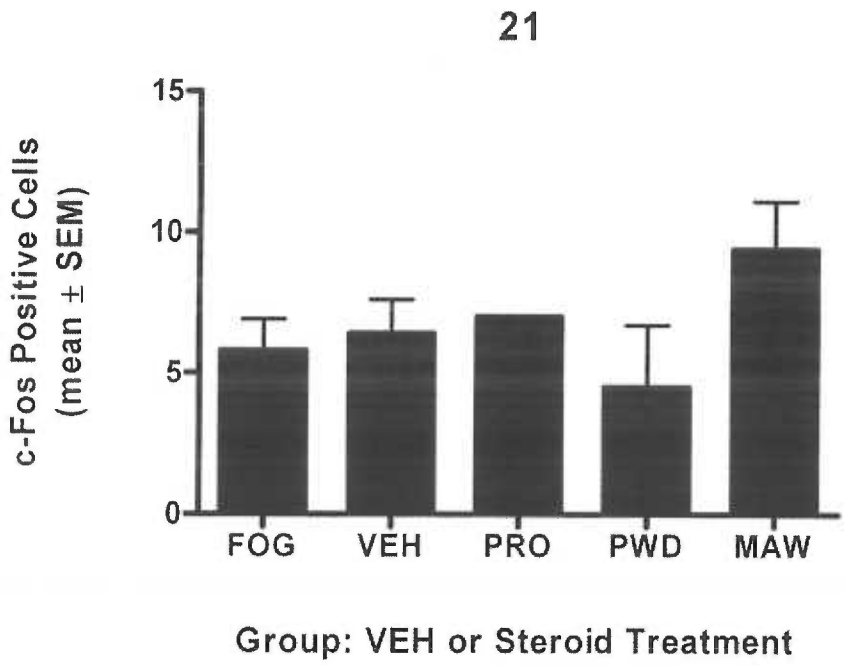


Figure 21. Mean (± SEM) number of c-Fos positive cells in the nucleus accumbens core at 0.98-0.74 mm from bregma. Final sample sizes were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 4$ for the MAW group (no usable sections were collected from four mice), $n = 2$ for the PRO group (no usable sections were collected from five mice), $n = 4$ for the PWD group (no usable sections were collected from four mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

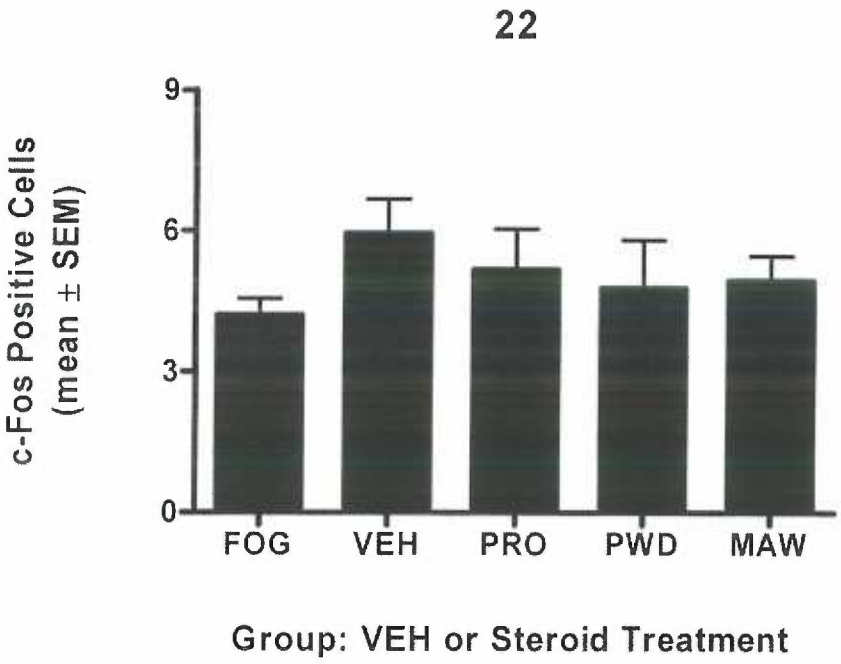


Figure 22. Mean (± SEM) number of c-Fos positive cells in the dorsal lateral septum at 1.18-0.74 mm from bregma. Final sample sizes were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 4$ for the MAW group (no usable sections were collected from three mice), $n = 4$ for the PRO group (no usable sections were collected from three mice), $n = 5$ for the PWD group (no usable sections were collected from three mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

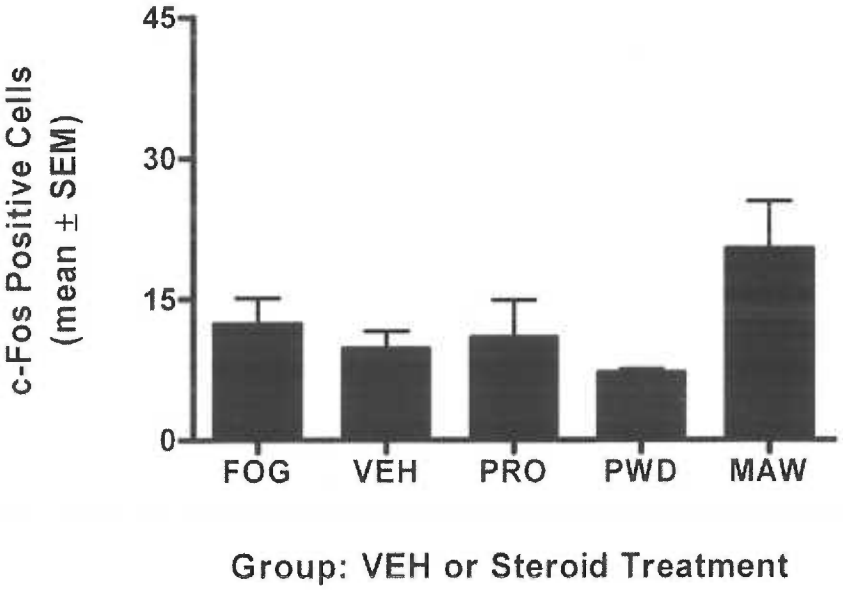
Results—Ventral lateral septum

Only one difference in the numbers of c-Fos positive cells was observed between the two regions of the LSV that were counted. There were no overall differences in c-Fos positive cells in the range of 1.18-1.10 mm from bregma, $F_{4,15}$ = 2.59, *ns* (Figure 23a). However, ANOVA did reveal an omnibus difference in the number of c-Fos immunoreactive cells in the range of 0.98-0.74 mm from bregma in the LSV, $F_{4,20}$ = 2.87, $p < .05$. Post hoc analyses revealed that the only pairwise difference among these groups was that there was a significantly lower count of c-Fos positive cells in the PRO group compared to in the MAW group (Figure 23b).

Results—Bed nucleus of the stria terminalis

There was only one significant difference among the different treatment groups between the two regions of the BST that were counted. There was a significant overall difference in c-Fos labeled cells at 0.26 mm from bregma, $F_{4,21}$ = 3.55, $p < .05$. Post hoc analyses showed that there were fewer labeled cells in the PRO group compared to the MAW group. No other pairwise comparisons were significant (Figure 24a). There was no significant overall difference among treatment groups at 0.14 mm from bregma, $F_{4,20}$ = 0.217, *ns* (Figure 24b).

23a


c-Fos Positive Cells
(mean ± SEM)
45
30
15
0
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

23b

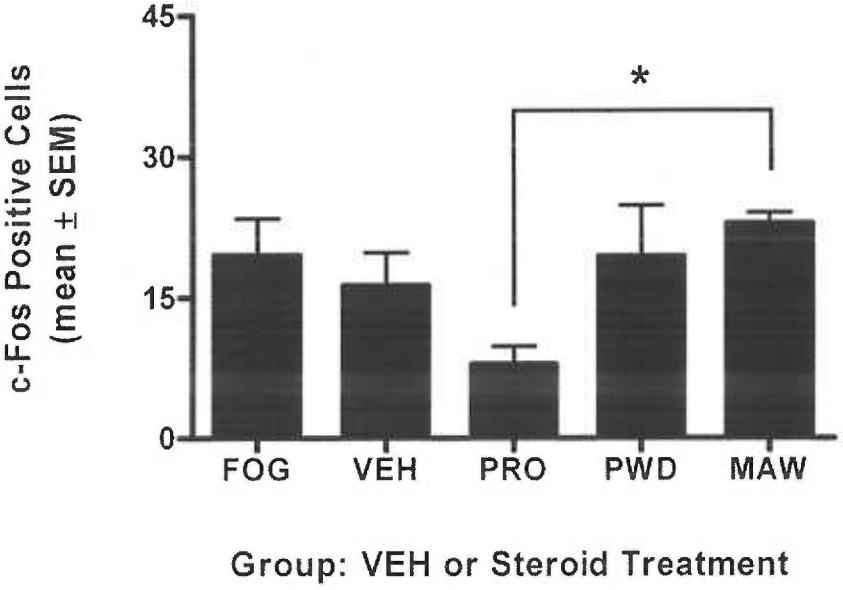
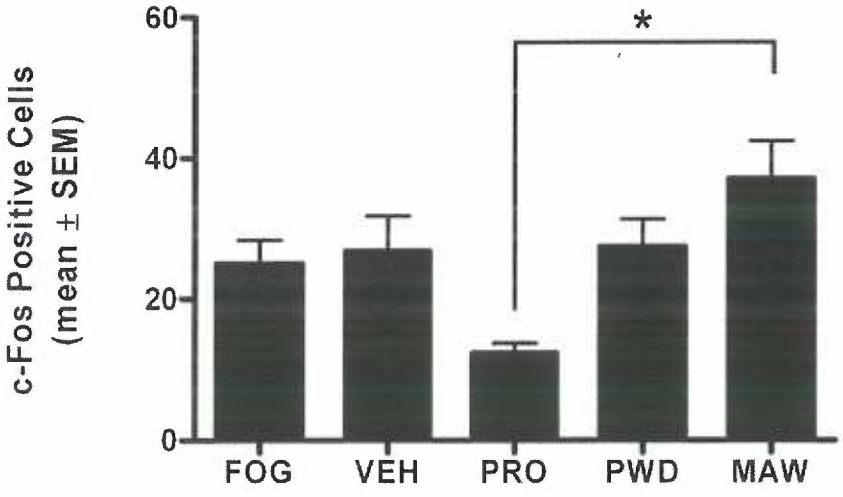

c-Fos Positive Cells
(mean ± SEM)
45
30
15
0
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 23. Mean (± SEM) number of c-Fos positive cells in the ventral lateral septum at (a) 1.18-1.10 mm from bregma, and (b) 0.98-0.74 mm from bregma. (b) * $p < .05$ difference in immunoreactive cells between the PRO group and the MAW group. Final sample sizes in the 1.18-1.10 mm range were $n = 4$ for the FOG group (no usable sections were collected from four mice), $n = 4$ for the MAW group (no usable sections were collected from four mice), $n = 3$ for the PRO group (no usable sections were collected from five mice), $n = 4$ for the PWD group (no usable sections were collected from three mice), and $n = 5$ for the VEH group (no usable sections were collected from two mice). Final sample sizes in the 0.98-0.74 mm range were $n = 5$ for the FOG group (no usable sections were collected from three mice), $n = 5$ for the MAW group (no usable sections were collected from three mice), $n = 5$ for the PRO group (no usable sections were collected from three mice), $n = 4$ for the PWD group (no usable sections were collected from four mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

24a


60
40
20
0
*
c-Fos Positive Cells
(mean ± SEM)
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

24b

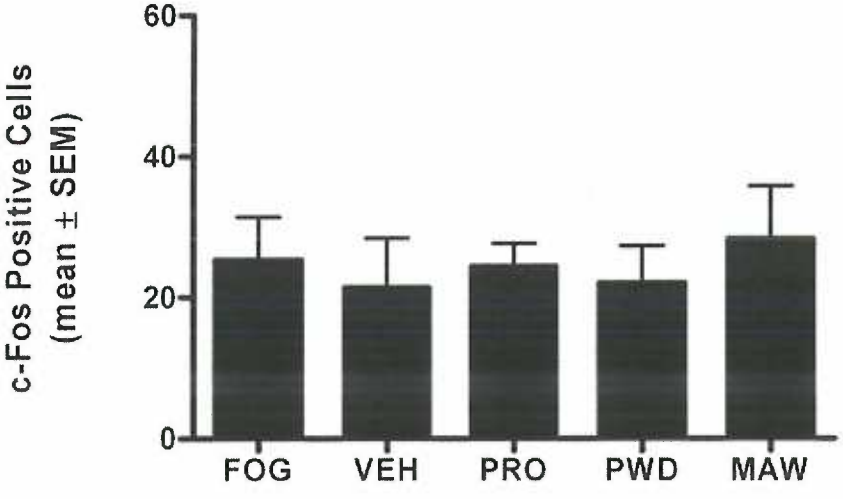

60
40
20
0
c-Fos Positive Cells
(mean ± SEM)
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 24. Mean (± SEM) number of c-Fos positive cells in the bed nucleus of the stria terminalis at (a) 0.26 mm from bregma, and (b) 0.14 mm from bregma. (a) * $p < .05$ difference in immunoreactive cells between the PRO group and the MAW group. Final sample sizes at 0.26 mm were $n = 5$ for the FOG group (no usable sections were collected from two mice), $n = 6$ for the MAW group (no usable sections were collected from two mice), $n = 4$ for the PRO group (no usable sections were collected from four mice), $n = 5$ for the PWD group (no usable sections were collected from three mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice). Final sample sizes at 0.14 mm were $n = 4$ for the FOG group (no usable sections were collected from three mice), $n = 6$ for the MAW group (no usable sections were collected from two mice), $n = 5$ for the PRO group (no usable sections were collected from three mice), $n = 5$ for the PWD group (no usable sections were collected from three mice), and $n = 5$ for the VEH group (no usable sections were collected from three mice).

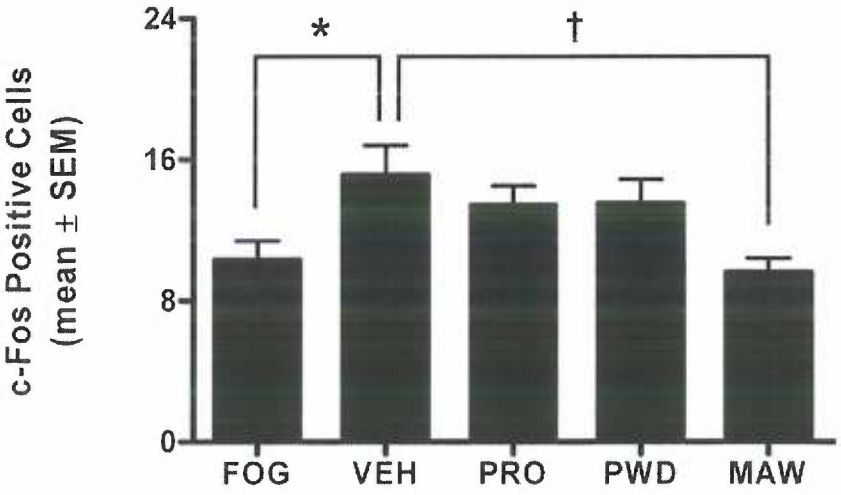
Results—Granule layer of the dentate gyrus

Significant differences in c-Fos positive cells were found in both of the areas of the GrDG that were assessed. There was an overall difference in c-Fos positive cells in the −1.46--1.94 mm range from bregma, $F_{4,27} = 3.56$, $p < .05$. Post hoc tests revealed that there were significantly fewer c-Fos labeled cells in the FOG group than in the VEH group at this level, see Figure 25a. There was a similar significant omnibus difference in c-Fos positive cells in the −2.06--2.54 mm range of distances from bregma, $F_{4,25} = 5.29$, $p < .05$. Post hoc analyses again confirmed that c-Fos positive cells at this level were significantly lower in the FOG group compared to the VEH group. At this level it was also found that c-Fos positive cells were significantly fewer in count in the MAW group compared to the VEH, see Figure 25b.

Results—Central nucleus of the amygdala

Several significant differences in c-Fos activity were detected in the CeA. There was an omnibus significant difference in c-Fos expression in the range of −1.82--1.94 mm from bregma, $F_{4,20} = 4.27$, $p < .05$. Post hoc analyses showed that there were significantly more c-Fos positive cells in the FOG group at this level compared to the VEH, PRO, and PWD groups (Figure 26a). There was also a significant omnibus difference among the treatment groups at the −2.06--2.18 mm distance range in the CeA, $F_{4,23} = 4.74$, $p < .05$. Post hoc analyses showed that there were significantly more c-Fos positive cells in the MAW group compared to the PRO and PWD groups at this level (Figure 26b).

25a


c-Fos Positive Cells
(mean ± SEM)
24
16
8
0
*
†
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

25b

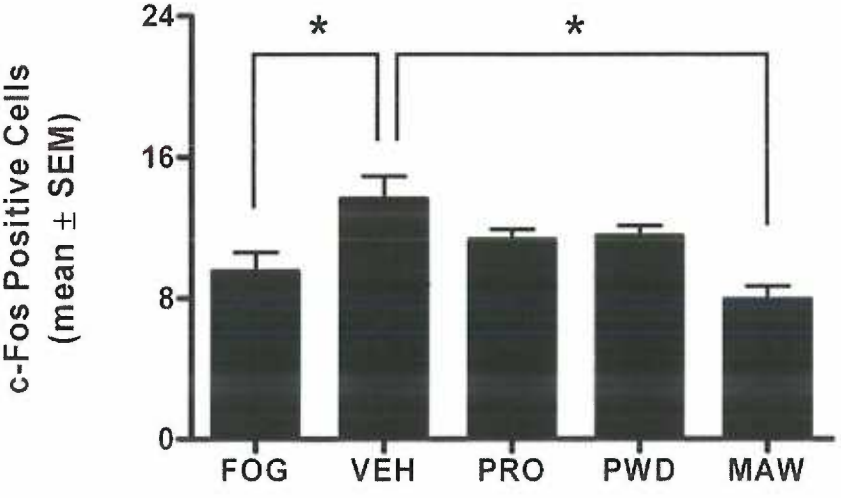
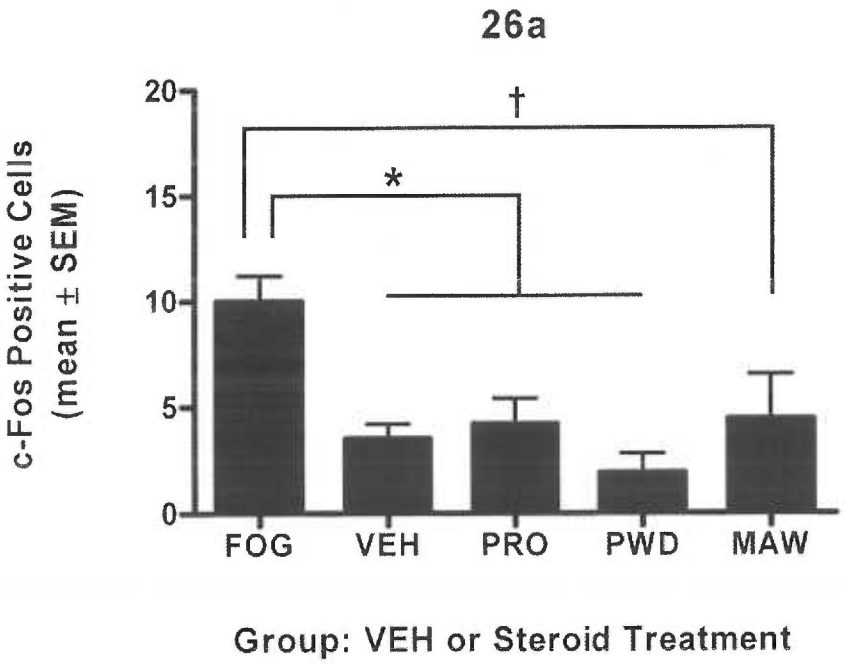

c-Fos Positive Cells
(mean ± SEM)
24
16
8
0
*
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 25. Mean (± SEM) number of c-Fos positive cells in the granule layer of the dentate gyrus at (a) −1.46--1.94 mm from bregma, and (b) −2.06--2.54 mm from bregma. (a) * $p < .05$ difference in immunoreactive cells between the VEH group and the FOG group. † $p < .10$ difference in immunoreactive cells between the VEH group and the MAW group. (b) * $p < .05$ difference in immunoreactive cells between the VEH group and each of the FOG and MAW groups. Final sample sizes in the −1.46--1.94 mm range were $n = 6$ for the FOG group (no usable sections were collected from two mice), $n = 7$ for the MAW group (no usable sections were collected from one mouse mouse), $n = 7$ for the PRO group (no usable sections were collected from one mouse), $n = 6$ for the PWD group (no usable sections were collected from two mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice). Final sample sizes in the −2.06--2.54 range were $n = 7$ for the FOG group (no usable sections were collected from one mouse), $n = 6$ for the MAW group (no usable sections were collected from two mice), $n = 7$ for the PRO group (no usable sections were collected from one mouse), $n = 5$ for the PWD group (no usable sections were collected from one mouse), and $n = 5$ for the VEH group (no usable sections were collected from three mice).


26a
c-Fos Positive Cells
(mean ± SEM)
20
15
10
5
0
†
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

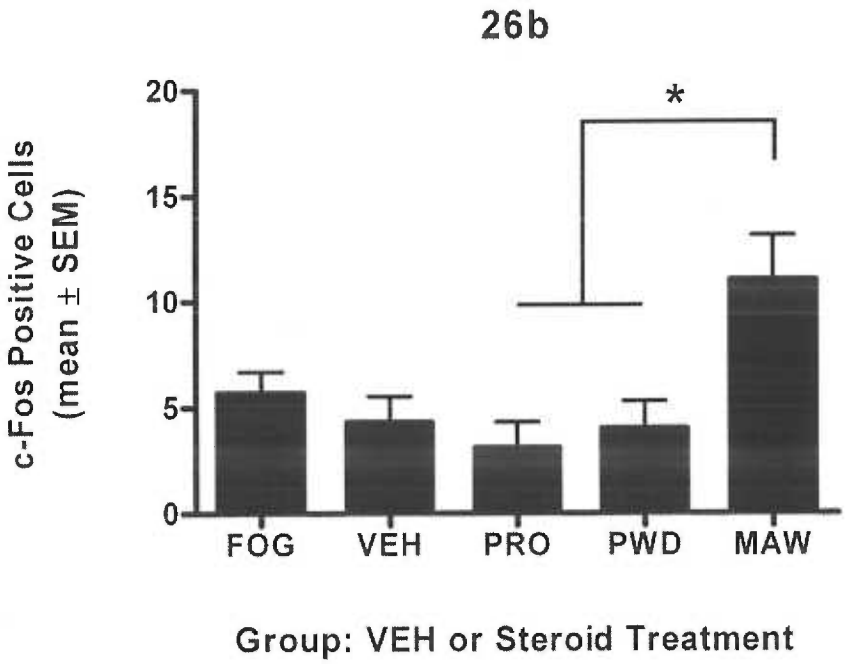

26b
c-Fos Positive Cells
(mean ± SEM)
20
15
10
5
0
*
FOG
VEH
PRO
PWD
MAW
Group: VEH or Steroid Treatment

Figure 26. Mean (± SEM) number of c-Fos positive cells in the central nucleus of the amygdala at (a) −1.82--1.94 mm from bregma, and (b) −2.06--2.18 mm from bregma. (a) * $p < .05$ difference in immunoreactive cells between the FOG group and each of the VEH, PRO, and PWD groups. † $p < .10$ difference in immunoreactive cells between the FOG group and the MAW group. (b) * $p < .05$ difference in immunoreactive cells between the MAW group and each of the PRO and PWD groups. Final sample sizes in the −1.82--1.94 mm range were $n = 3$ for the FOG group (no usable sections were collected from three mice), $n = 4$ for the MAW group (no usable sections were collected from one mouse), $n = 7$ for the PRO group (no usable sections were collected from one mouse), $n = 5$ for the PWD group (no usable sections were collected from two mice), and $n = 6$ for the VEH group (no usable sections were collected from two mice).

Results—Basolateral amygdala

There were no significant overall differences in c-Fos expression among the different treatment groups in either of the areas of the BLA that were measured. There was not a significant difference in c-Fos positive cells among the treatment groups in the −1.82--1.94 mm range from bregma, $F_{4,26} = 2.01$, *ns*. There was also no difference among the treatment groups in the −2.06--2.18 mm distance range, $F_{4,26} = 0.687$, *ns*. When these two spans were combined there was a significant difference among the treatment groups, $F_{4,28} = 3.41$, $p < .05$. Post hoc analyses revealed that there were significantly more c-Fos positive cells in the VEH group compared to each of the PRO and MAW groups (Figure 27).

Results—Periaqueductal grey

There were no significant overall differences in c-Fos expression among the different treatment groups in either of the spans of the PAG that were measured. There was not a significant difference in c-Fos positive cells in the −2.54--2.80 mm range from bregma, $F_{4,24} = 0.498$, *ns*. There was also no difference among the groups in the −2.92--3.52 mm distance range, $F_{4,23} = 0.718$, *ns*. When these two spans were combined there remained no significant differences among these treatment groups, $F_{4,26} = 0.685$, *ns* (Figure 28).

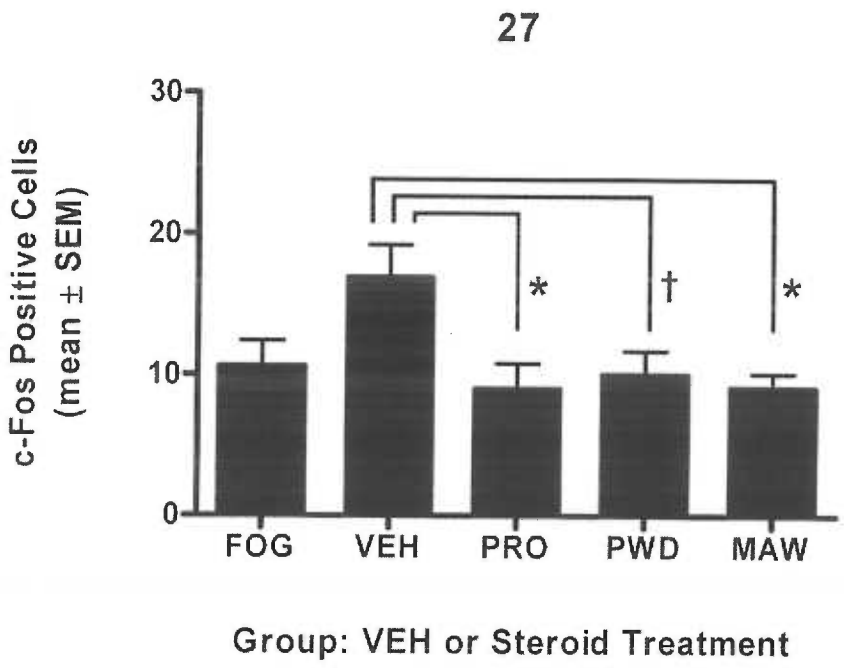


Figure 27. Mean (± SEM) number of c-Fos positive cells in the basolateral amygdala at −1.82--2.18 mm from bregma. * $p < .05$ difference between the VEH group and each of the PRO and MAW groups. † $p < .10$ difference between the VEH group and the PWD group. Final sample sizes were $n = 6$ for the FOG group (no usable sections were collected from two mice), $n = 6$ for the MAW group (no usable sections were collected from one mouse), $n = 7$ for the PRO group (no usable sections were collected from one mouse), $n = 7$ for the PWD group (no usable sections were collected from one mouse), and $n = 7$ for the VEH group (no usable sections were collected from one mouse).

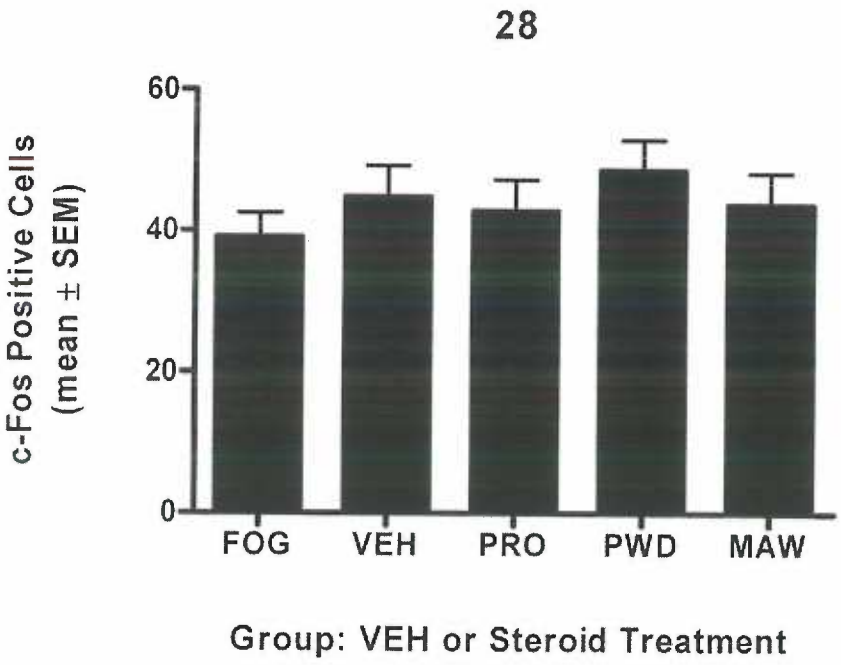


Figure 28. Mean (± SEM) number of c-Fos positive cells in the periaqueductal grey at −2.54--3.52 mm from bregma. Final sample sizes were n = 6 for the FOG group (no usable sections were collected from two mice), n = 6 for the MAW group (no usable sections were collected from two mice), n = 6 for the PRO group (no usable sections were collected from two mice), n = 7 for the PWD group (no usable sections were collected from one mouse), and n = 6 for the VEH group (no usable sections were collected from two mice).

Results—Ventral tegmental area

There were no significant overall differences in c-Fos expression among the different treatment groups in the VTA in the −2.54--2.80 mm range from bregma, $F_{4,23} = 0.726$, *ns* (Figure 29).

Results—Dorsal raphe nucleus

There were no significant overall differences in c-Fos expression among the treatment groups in the DR in the −4.36--4.84 mm range from bregma, $F_{4,24} = 2.23$, *ns* (Figure 30).

Results—Median raphe nucleus

There was a significant overall difference in c-Fos expression among the treatment groups in the MnR in the −4.36--4.84 mm range from bregma, $F_{4,25} = 2.91$, $p < .05$. Post hoc analyses revealed that there was higher c-Fos expression in the MAW group compared to the PWD group (Figure 31).

Results—Plasma corticosterone

There was a significant overall difference in plasma corticosterone concentrations among the different treatment groups, $F_{4,34} = 4.02$, $p < .05$. Post hoc analyses showed that plasma corticosterone was significantly higher in the PWD and MAW groups compared to the FOG group, see Figure 32.

Results—Immunohistochemistry and data summaries

Samples of immunohistochemical labeling for c-Fos are shown in Figure 33 and Figure 34. Mean and SEM c-Fos cell count values for each group in each brain region are shown in Table 22.

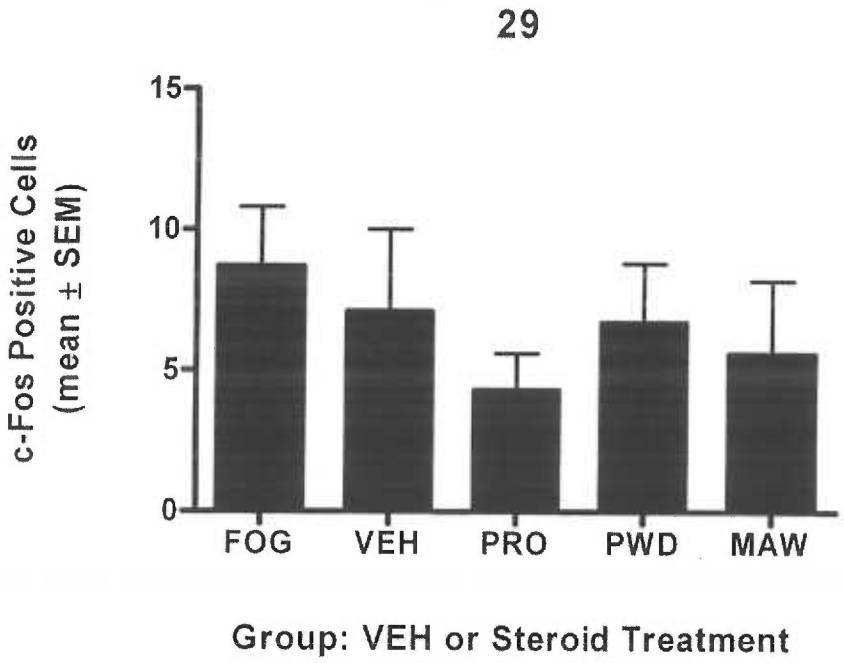


Figure 29. Mean (± SEM) number of c-Fos positive cells in the ventral tegmental area at −2.54--2.80 mm from bregma. Final sample sizes were $n = 6$ for the FOG group (no usable sections were collected from two mice), $n = 5$ for the MAW group (no usable sections were collected from three mice), $n = 6$ for the PRO group (no usable sections were collected from two mice), $n = 7$ for the PWD group (no usable sections were collected from one mouse), and $n = 5$ for the VEH group (no usable sections were collected from three mice).

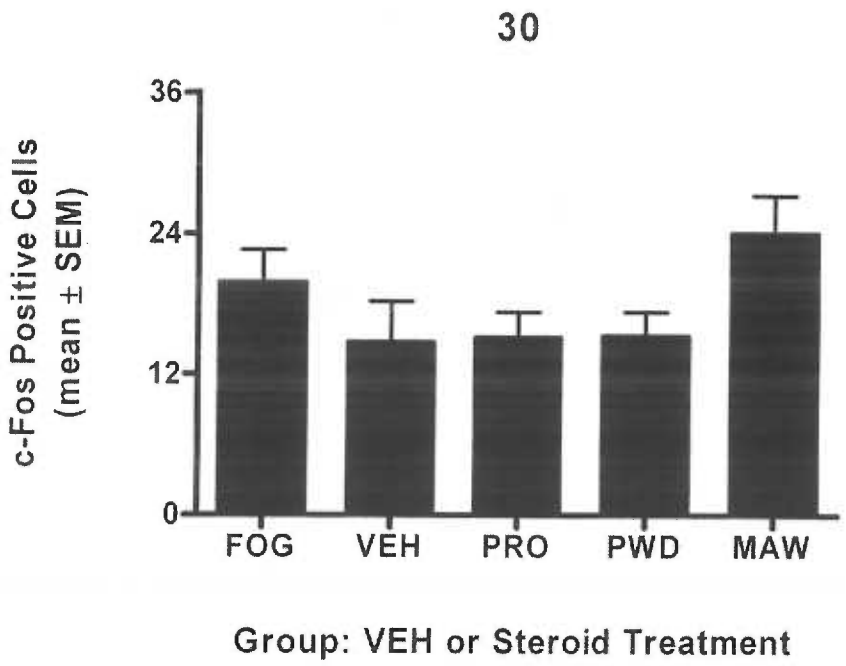


Figure 30. Mean (± SEM) number of c-Fos positive cells in the dorsal raphe nucleus at −4.36--4.84 mm from bregma. Final sample sizes were n = 6 for the FOG group (no usable sections were collected from two mice), n = 5 for the MAW group (no usable sections were collected from two mice), n = 5 for the PRO group (no usable sections were collected from three mice), n = 7 for the PWD group (no usable sections were collected from one mouse), and n = 5 for the VEH group (no usable sections were collected from three mice).

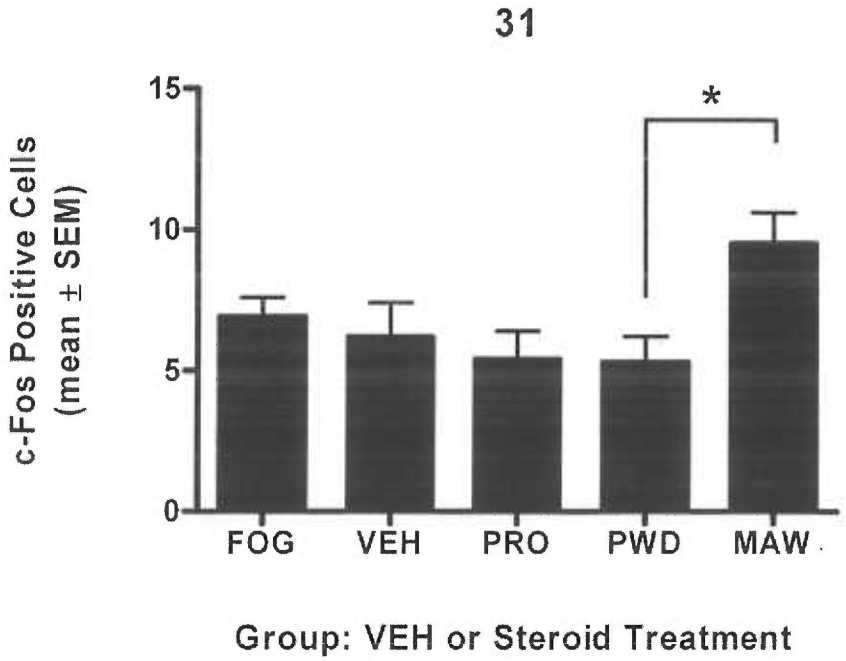


Figure 31. Mean (± SEM) number of c-Fos positive cells in the median raphe nucleus at −4.36--4.84 mm from bregma. * $p < .05$ difference between the MAW group and the PWD group. Final sample sizes were $n = 6$ for the FOG group (no usable sections were collected from two mice), $n = 5$ for the MAW group (no usable sections were collected from two mice), $n = 5$ for the PRO group (no usable sections were collected from three mice), $n = 7$ for the PWD group (no usable sections were collected from one mouse), and $n = 5$ for the VEH group (no usable sections were collected from three mice).

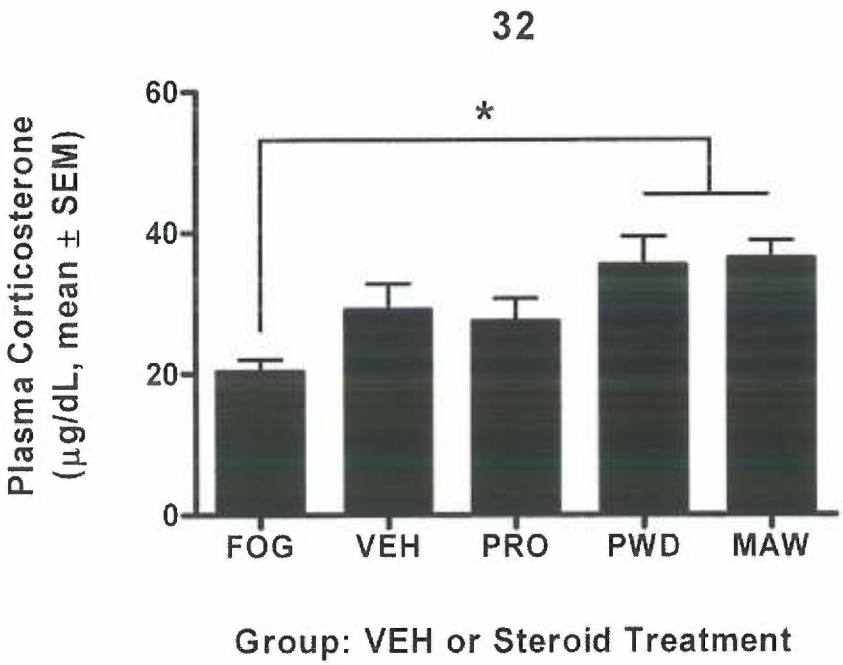


Figure 32. Mean (± SEM) plasma corticosterone concentration (µg/dL). * $p < .05$ difference between FOG group and each of PWD and MAW groups. Final sample sizes were $n = 8$ for each of the FOG, VEH, PRO, and PWD groups, and $n = 7$ for the MAW group (the counts for one mouse could not be fit to the standard curve).

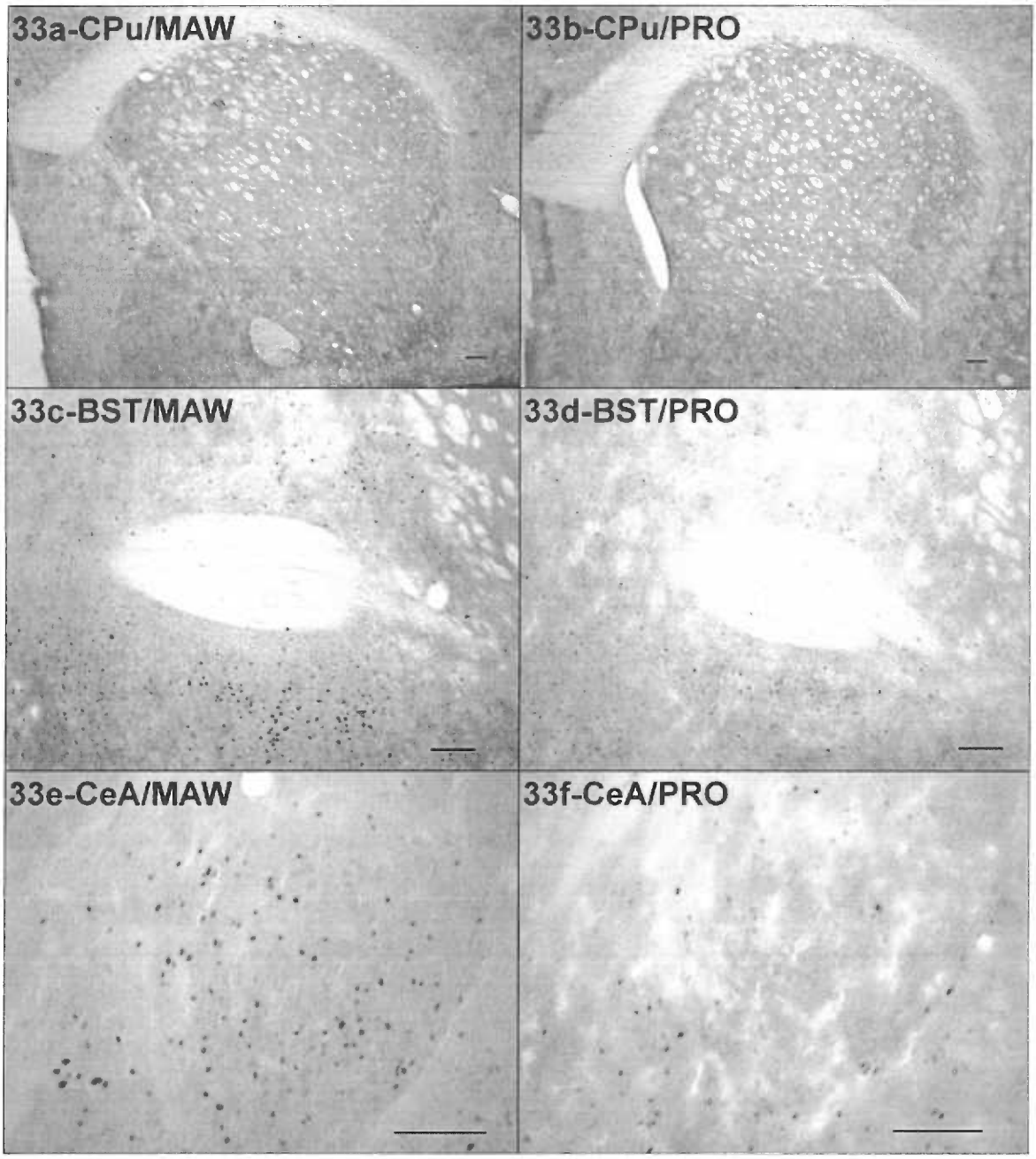


Figure 33. Immunohistochemical identification of c-Fos positive cells. (a-f) Example sections from brain regions and groups indicated in each panel. The magnification in each panel was chosen to include the whole brain region, but during counting all regions were viewed at the same magnification which made the sections appear approximately as large as they are reproduced in panels c and d, and which was

sufficient to identify c-Fos labeled nuclei. Bars set into the lower right corner of each panel are each 0.1 mm long.

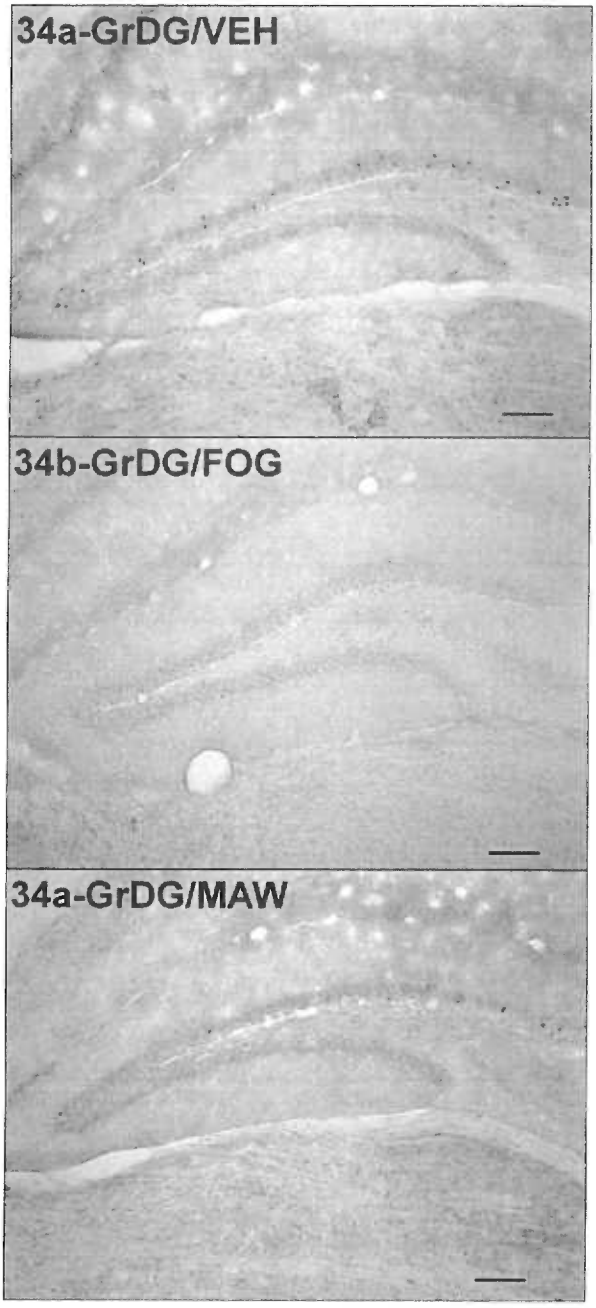


Figure 34. Immunohistochemical identification of c-Fos positive cells. (a-c) Example sections from the granule layer of the dentate gyrus in groups indicated in each panel. The magnification was chosen to include the whole brain region. Bars set into the lower right corner of each panel are 0.1 mm long.

Table 22: c-Fos Cell Counts (mean ± SEM) (Page 1)

		Group				
Brain region		FOG	VEH	PRO	PWD	MAW
CPu						
1.34-1.10	*	5.1±2.6	7.3±1.7	0.8±0.6	6.8±4.0	14.9±3.0
0.98-0.74	*	4.5±1.3	5.1±0.9	0.8±0.3	1.3±0.3	9.5±1.0
AcbSh						
1.34-1.10		16.3±2.6	23.4±4.2	24.5±2.2	18.2±3.4	23.3±4.0
0.98-0.74		17.7±1.4	25.1±4.7	16.6±3.6	14.8±6.1	20.7±1.6
1.34-0.74		17.3±1.4	24.8±4.3	18.0±3.3	16.3±4.0	22.2±1.4
LAcbSh						
1.34-1.10	*	4.6±1.1	3.1±0.9	10.4±1.4	3.5±1.1	3.9±1.1
0.98-0.74		5.2±1.3	4.6±1.3	5.0±2.1	2.5±1.3	5.3±0.8
AcbC						
0.98-0.74		5.8±1.1	6.4±1.2	7.0±0.0	4.5±2.2	9.4±1.7
LSD						
1.18-1.10		4.3±0.5	4.3±1.0	4.7±1.2	2.2±1.1	5.3±0.6
0.98-0.74		4.3±0.5	5.9±0.9	7.6±1.3	6.0±1.3	4.9±0.4
1.18-0.74		4.2±0.3	6.0±0.7	5.2±0.9	4.8±1.0	4.9±0.5

Table 22: c-Fos Cell Counts (mean ± SEM) (Page 2)

		Group				
Brain region		FOG	VEH	PRO	PWD	MAW
LSV						
1.18-1.10		12.3±2.8	9.7±1.9	10.9±4.0	7.1±0.4	20.3±5.1
0.98-0.74	*	19.5±3.9	16.3±3.5	7.9±1.9	19.4±5.4	22.9±1.1
BST						
0.26	*	25.0±3.4	26.8±5.0	12.3±1.4	27.4±3.9	37.0±5.4
0.14		25.3±6.1	21.4±7.0	24.4±3.2	22.0±5.3	28.3±7.5
GrDG						
−1.46--1.94	*	10.3±1.1	15.1±1.7	13.4±1.1	13.5±1.4	9.6±0.8
−2.06--2.54	*	9.5±1.1	13.6±1.3	11.3±0.6	11.5±0.6	7.9±0.8
CeA						
−1.82--1.94	*	10.0±1.2	3.5±0.7	4.2±1.2	1.9±0.9	4.4±2.1
−2.06--2.18	*	5.7±1.0	4.3±1.2	3.1±1.2	4.0±1.3	11.0±2.1
BLA						
−1.82--1.94		10.5±2.2	17.1±2.6	7.6±2.0	12.1±3.0	11.5±2.4
−2.06--2.18		8.8±1.6	14.5±3.9	9.0±2.0	8.7±3.6	9.3±2.3
−1.82--2.18	*	10.6±1.8	16.9±2.9	9.0±1.8	10.0±1.7	9.1±1.0

Table 22: c-Fos Cell Counts (mean ± SEM) (Page 3)

		Group				
Brain region		FOG	VEH	PRO	PWD	MAW
PAG						
−2.54--2.80		50.6±9.0	60.0±7.0	48.5±3.2	59.4±9.3	52.2±4.4
−2.92--3.52		34.0±3.7	35.3±3.2	34.8±5.2	40.6±3.8	32.3±3.7
−2.58--3.52		39.1±3.4	44.8±4.4	42.8±4.4	48.6±4.3	43.7±4.5
VTA						
−2.54--2.80		8.7±2.1	7.1±2.9	4.3±1.3	6.7±2.1	5.6±2.6
DR						
−4.36--4.84		19.8±2.8	14.7±3.5	15.1±2.2	15.2±2.1	24.0±3.3
MnR						
−4.36--4.84	*	6.9±0.7	6.2±1.2	5.4±1.0	5.3±0.9	9.5±1.1

Note. Pairwise statistical comparisons are omitted because the patterns are difficult to illustrate in tabular form. Asterisks (*) to the right of the bregma levels indicate that an omnibus significant difference was found. Detailed statistical information is given in the results section.

Chapter 6 Discussion

Previous attempts to examine c-Fos production during hormone withdrawal have not made a systematic examination of multiple brain regions simultaneously. At the same time, relevant research that has focused on specific brain regions (for example, microinjection of steroid compounds into brain regions such as the amygdala) have apparently been based mostly on the established role of these regions in emotional processes, but without any special relevance to reproductive depression. The current mapping project did not attempt an exhaustive examination of every brain region that could potentially be relevant to depression, but did make a concerted effort to consider many areas that could be involved in emotion or motivation. Therefore, the current experiment assessed brain regions that could be involved in emotion without being entirely biased only towards those regions that have already been studied.

Many of the brain regions that were studied showed no difference whatsoever among the different treatment conditions (AcbSh, AcbC, LSD, PAG, VTA, and DR). Some other brain regions had evidence of differential c-Fos expression in one rostrocaudal field (relative to bregma) but not in a second field that was studied (LAcbSh, LSV, BST), or only when levels were combined (BLA, MnR). Still other brain regions had one set of c-Fos expression patterns among groups within one field, but a very different set of c-Fos expression patterns among groups within a different field (CPu, CeA). These findings are very difficult to interpret. The α-level set for these studies was .05, which would suggest that at least some of these findings are not due to type-I errors, as more than 5% of the omnibus comparisons

met the criterion for significance. However, the lack of consistency between one field of a brain region and another field of the brain region prevents making very firm conclusions about the brain regions listed above.

The inconsistencies in the data make it difficult to match c-Fos expression patterns to the known behavioral differences observed among these groups. If there had been several brain regions where the MAW and PWD groups had similar expression patterns relative to the other groups, and especially compared to the VEH and PRO groups, these differences could have reflected the increases in forced swim test immobility observed in other experiments. Even differences in c-Fos expression only in the PWD group could be used to identify brain regions underlying forced swim test immobility. However, the only significant pairwise comparison involving the PWD group was a comparison with the FOG group. The FOG group was an additional control for making comparisons with the MAW group, but comparisons between the PWD and FOG groups do not have obvious scientific importance. Therefore, this project did not identify a convincing candidate region that might explain the increase in forced swim test immobility during progesterone withdrawal.

One reason why increased c-Fos expression was not found in the PWD group in different brain areas could be related to the marker for cell activity that was used. Each of the related transcription factors has a unique temporal pattern of expression relative to the stimuli that elicit their expression. c-Fos was used for several reasons, but one of the reasons was that c-Fos expression is maximal at approximately 120 min after a precipitating event (Kovács, 1998). Thus, the

interval between the final injection and tissue collection (120 min) was consistent with the interval between a subject's last injection and forced swim test that was used in most of the experiments described in this work. In most of the current research the withdrawal period that was used was approximately 3 d, but since immobility did not increase in the passive progesterone withdrawal experiment until day 8 (day 3 of withdrawal in Experiment 1) it was not known whether the brain changes responsible for this phenomenon only began on day 8 or if the brain changes began earlier but did not manifest behaviorally until later. Using a marker with a slower peak or using c-Fos but collecting tissue at a different time might better have captured changes in neuron activity from progesterone withdrawal.

This experiment complements data showing that metabolic allopregnanolone withdrawal increased forced swim test immobility (Experiment 2). It was hypothesized that allopregnanolone withdrawal would be associated with rebound hyperexcitability that would be detected in specific brain regions using c-Fos immunohistochemistry. Given the results of Chapters 3 and 4, it is possible that progesterone withdrawal is not related to $GABA_A$ receptor mechanisms and increased cellular activity or hyperexcitability. While all changes in behavior are presumably attributable to changes in neuronal activity on some level, it is possible that withdrawal of progesterone receptor activation produces changes in neuronal activity that are not detectable using c-Fos immunohistochemistry, because of the fact that the intracellular progesterone receptors use relatively-slow gene transcription mechanisms, while the $GABA_A$ receptors are inhibitory anion channels that mediate rapid changes in cell activity. Instead, perhaps another member of the

Fos family of transcription factors would be a better indicator of the long-term changes that might mediate the effect of progesterone withdrawal on forced swim test immobility. For example, FosB, Fra-1, and Fra-2 have a half-lives ranging from approximately 10-200 hr, compared to 2 hr for c-Fos (Kovács, 1998). Measuring one of these Fos family proteins that remain detectable for longer periods of time might reveal brain regions that have activation patterns that are consistent with the behavioral effects of progesterone withdrawal on forced swim test immobility.

The finding in Experiment 1 that three days of progesterone withdrawal were necessary to increase forced swim test immobility may indicate that there is a cascade of events and changes that must take place in the brain in order for the behavioral difference in depression-like behavior to emerge. Such a sequential process might never be amenable to the type of approach used here, so an entirely different approach to uncovering the neural substrates of progesterone withdrawal might have been more successful.

The only brain region studied where both fields showed similar patterns of expression appears to be the GrDG, where finasteride decreased cell activity (see also Figure 34). Since finasteride was shown to have an acute locomotor suppressing effect (Experiment 13), it is interesting to consider the results of the current experiment with reference to locomotor activity. Although the treatment procedures were extremely different, it should be noted that J. S. Rhodes and colleagues (2005) found a significant, positive correlation between c-Fos expression in the dentate gyrus and locomotor activity. However, in another study lesioning the granule cells of the dentate gyrus appeared to increase locomotor activity (Won

et al., 2003). Given the lack of consensus in the literature and that this experiment was not itself designed to test the role of the dentate gyrus in locomotor activity, firm conclusions cannot yet be made about the role of the dentate gyrus in locomotor behavior at this time.

An interesting result of the current experiment was that plasma corticosterone concentrations were elevated in the MAW and PWD groups compared to the FOG group. This finding is difficult to interpret, but it is intriguing as a preliminary piece of evidence because the MAW and FOG groups differ only by their history of exposure to progesterone. Taking only these two groups into consideration, one might suggest that the MAW group might have higher corticosterone levels because on the day of blood collection this group received progesterone and therefore had more circulating pro-hormone for many steroids. However, the PRO group also received a progesterone injection on this day but did not have elevated corticosterone levels, and the PWD group that did have elevated corticosterone levels did not receive progesterone on the day of blood collection. This finding may be a small indication that finasteride may have some unique effects that emerge only when it is administered together with progesterone. If this is the case then there may still be some merit to the allopregnanolone withdrawal hypothesis. Further research is needed in this area.

The fact that plasma corticosterone concentrations were elevated in the PWD and MAW groups raises the question of whether these groups would also have shown increased brain activity in the regions regulating adrenal axis function. One brain area that could play such a role is the paraventricular nucleus, which is the

hypothalamic component of the hypothalamic-pituitary-adrenal (HPA) axis that coordinates the function of the adrenal glands. Since there was a modest increase in plasma corticosterone in the PWD and MAW groups (compared to FOG), one positive control that could have improved the current experiment would have been to examine c-Fos expression in the paraventricular nucleus. The plasma corticosterone data would suggest that c-Fos activity might be similarly increased in the PWD and MAW groups in the paraventricular nucleus. At the same time, given previous studies of c-*fos* mRNA in postpartum animals (Lightman et al., 2001) it would also be reasonable to hypothesize that progesterone withdrawal would not be associated with an increase in paraventricular nucleus c-Fos expression.

The work with the by Doornbos and colleagues (2009b) should also be mentioned, as in their studies they reported that affective behaviors following hormone withdrawal did not correspond to periventricular nucleus or HPA axis variables. It is not entirely certain whether the authors had actually studied the *peri*ventricular nucleus as reported, or whether they actually had studied the *para*ventricular nucleus, but in either case their work adds to the evidence from Luckman (1995) and from Lightman and colleagues that the paraventricular nucleus and the HPA axis are not affected by hormone withdrawal.

Aside from the paraventricular nucleus, given the finding that corticosterone concentrations were elevated in the MAW and PWD groups, an additional positive control for the current experiment would have been to examine c-Fos expression in the urocortin-containing population of cells in the perioculomotor area (pIIIu, previously referred to as the Edinger-Westphal nucleus or the non-preganglionic

Edinger-Westphal nucleus). Not only has allopregnanolone has been shown to increase c-Fos expression in the pIIIu (Bachtell et al., 2002; but see also Murphy et al., 2006), which immediately raises the possibility that some of the actions of allopregnanolone or progesterone could be mediated by the pIIIu, but the pIIIu has also been implicated in regulating stress responses (Kozicz, 2007), which leaves open the possibility that the differences in corticosterone concentrations could be mediated by this brain region.

The most consistent finding across brain regions was that there were decreased c-Fos positive cell counts in the PRO group. The number of cells expressing c-Fos was significantly decreased in the PRO group compared to the MAW and VEH groups in the caudate putamen, compared to the MAW group in the ventral lateral septum, compared to the MAW group in the bed nucleus of the stria terminalis, compared to the FOG and MAW groups in the central nucleus of the amygdala, and compared to the VEH group in the basolateral amygdala (depending on the distances from bregma). Although not statistically significant, the PRO group also had among the lowest count of c-Fos positive cells in the ventral tegmental area, the dorsal raphe nucleus, and the median raphe nucleus. The important exception was where the number of c-Fos positive cells was increased in the PRO group compared to all other groups in the lateral nucleus accumbens shell (only from 1.34-1.10 mm from bregma).

In four out of the five brain regions where the PRO group had lowered immunoreactivity the comparison was significant between the PRO and MAW groups (see also Figure 33). Thus, the most consistent finding across all brain

regions was between these two groups. Mice in these two groups all received daily injections of progesterone on all days of the experiment, and they were the only two groups to receive progesterone all eight days. But while the PRO group received injections of vehicle on days 6-8, the MAW group received finasteride injections on these last three days of the experiment. Since progesterone is metabolized to allopregnanolone, and since allopregnanolone is a potent inhibitory neurosteroid, it makes sense that the PRO group would have decreased neuronal activity, reflected in decreased c-Fos protein expression. It is also reasonable that the MAW group, prevented by finasteride from forming allopregnanolone, would have relatively high neural activity, measured by c-Fos protein expression.

It was hypothesized that the PWD group would have similar c-Fos expression profiles across brain regions as the MAW group, since both treatments are anticipated to decrease allopregnanolone and increase cell activity. Earlier data showed that the general progesterone withdrawal procedure used in these experiments does decrease brain allopregnanolone content after three days of withdrawal, which is what the metabolic allopregnanolone withdrawal treatment is believed to do also. However, c-Fos expression in the various brain regions was often not similar between the PWD and MAW groups. Therefore, it is important to consider that the treatments were likely to have very different effects aside from their effects on allopregnanolone.

In the progesterone withdrawal group there is no direct steroid manipulation after day 5 of the experiment, meaning that the animals might conceivably return to physiological levels of all steroids by the test day. In contrast,

the metabolic allopregnanolone withdrawal group should have high levels of progesterone but low levels of the 5α-reduced steroids (see Figure 1). At the same time, progesterone is the precursor for many other steroids with a vast array of effects (glucocorticoids, mineralocorticoids, estrogens, androgens, and more). Since finasteride is expected to prevent the 5α-reductase pathway, progesterone metabolism is likely to be shunted to alternate metabolic pathways that increase some or all of these other neuroactive steroids. Therefore, it is not surprising that the PWD and MAW groups have different expression profiles.

The MAW group had elevated c-Fos expression in several brain regions. The metabolic allopregnanolone withdrawal treatment also increased forced swim test immobility, but based on locomotor activity data this effect on swim test immobility could be due to general sedation. One of the brain regions where c-Fos expression was increased in the MAW group was in the central nucleus of the amygdala, a brain region that has been implicated in many behavioral processes. For example, the amygdala is also implicated in anxiety-like behavior, which may be related to depression-like behavior. In particular, the amygdala is thought to be important for some of the learned aspects of anxiety-related behaviors (Roozendaal et al., 2009).

However, with regard to the current data, it is perhaps more relevant to consider the potential role of the amygdala in sleep behavior, because sleep behavior could potentially reconcile the findings that metabolic allopregnanolone withdrawal treatment increased c-Fos expression and decreased locomotion. Jha and colleagues (2005) found that some neurons in the central nucleus of the amygdala had increased firing rats during rapid eye movement (REM) sleep (but see

Sanford et al., 2006). Thus, it is possible that the increase in c-Fos expression in the MAW group compared to the PRO group is related to increases in activity of neurons that are involved in REM sleep. This could explain the differences in spontaneous locomotor activity that were observed in mice treated with finasteride (Experiment 13). Also, recall that Walf and coworkers (2006) performed intra-amygdala injections of finasteride to rats and observed increased forced swim test immobility. In the Chapter 4 it was suggested that finasteride increased forced swim test immobility by a general locomotor suppressant effect in the current studies. Taking into account the possible role of the amygdala in sleep or sedation, it is not surprising that Walf and coworkers observed increased swim test immobility in rats that received site-specific injections of finasteride into the amygdala.

Collectively, the current experiment revealed some interesting preliminary findings regarding the effect of finasteride on c-Fos expression in the dentate gyrus, and on the effects of metabolic allopregnanolone withdrawal and passive progesterone withdrawal on plasma corticosterone levels. In principle, the data are consistent with the hypothesis that allopregnanolone withdrawal would result in increased cell activity compared to mice that received just progesterone for all eight days, as observed in the caudate putamen, central nucleus of the amygdala, bed nucleus of the stria terminalis, and the ventral lateral septum. It is possible that one or more of these regions is involved in the locomotor depressant effects of finasteride, but at the same time this project did not achieve its goal of identifying a candidate brain region that might mediate the effect of progesterone withdrawal on forced swim test immobility.

Chapter 7: General Discussion

Allopregnanolone may have a limited role in progesterone withdrawal

A large body of research has demonstrated anti-anxiety and antidepressant roles of allopregnanolone in rodents or humans. It has also been proposed that $GABA_A$ receptors might be a common pathway in the etiology of depression and anxiety (Paul, 1988). However, while the experiments reported here had inconsistent results, taken as a whole they fail to support the allopregnanolone withdrawal hypothesis.

Some individual experiments supported the allopregnanolone withdrawal hypothesis. Administering the 5α-reductase inhibitor finasteride increased forced swim test immobility, and administering the $GABA_A$ receptor antagonist picrotoxin increased forced swim test immobility in some experiments. One experiment showed that administering picrotoxin with progesterone increased forced swim test immobility compared to administering picrotoxin with vehicle, and also compared subjectively to treatments in other experiments where mice received vehicle or progesterone without picrotoxin. These data therefore supported the idea that picrotoxin increased forced swim test immobility because $GABA_A$ receptors are involved in the progesterone withdrawal response, an effect presumably mediated by a GABAergic metabolite of progesterone such as allopregnanolone. Also, the timing of picrotoxin administration necessary to increase forced swim test immobility coincided with the acute increase in brain allopregnanolone following progesterone administration, which also supports the allopregnanolone hypothesis.

Passive allopregnanolone withdrawal did not increase forced swim test immobility. It is likely that this was due in part to the quick metabolism of allopregnanolone, which would have prevented neuroadaptation that could later be unmasked during the "withdrawal" phase. It is also possible that the relevant withdrawal phase for depression-like behavior following exogenous allopregnanolone injections is different from the withdrawal phase for exogenous progesterone administration. For example, anxiety-like behavior was increased at 3-4 hours after exogenous allopregnanolone injections in rats (Gulinello & S. S. Smith, 2003). However, it is also possible that allopregnanolone withdrawal simply does not increase depression-like behavior. In any case, the current data from the passive allopregnanolone withdrawal experiment do not support the allopregnanolone withdrawal hypothesis.

The finding that metabolic allopregnanolone withdrawal increased forced swim test immobility is confounded by general locomotor effects. Since finasteride administration was associated with a robust decrease in locomotor activity, it is possible that the increase in forced swim test immobility associated with finasteride was due to a general sedative effect rather than a true depression-like response.

These data present little evidence that allopregnanolone or $GABA_A$ receptors are critically involved in forced swim test immobility during progesterone withdrawal. Allopregnanolone is currently receiving much attention for its possible contributions to reproductive depression, including how its withdrawal might act to induce these symptoms. At the same time, there have been increasingly-conflicting reports in which allopregnanolone has been reported to have depressogenic,

anxiogenic, or other negative effects (e.g., Gracia et al., 2009; H. Shen et al., 2007; reviewed in Andréen et al., 2009), which contradicts some of the traditional thinking of allopregnanolone as an anxiolytic and possibly-antidepressant steroid (Longone et al., 2008).

There is too much evidence to dispute the idea that allopregnanolone can alter mood, but the complex and inconsistent nature of these effects makes it very difficult to distill any sort of fundamental principles describing these phenomena. Although the experiments with picrotoxin yielded mixed results, two of the experiments did show increases in forced swim test immobility in the expected direction. It is possible that allopregnanolone does influence forced swim test behavior, and there are at least two important possible explanations for why the current experiments yielded mixed results in spite of this possibility.

The first explanation can be viewed, depending on one's perspective, as a matter of effect size or sample size. It is a general rule of statistics that one's ability to detect a significant difference between two groups (statistical power) is in direct proportion to the strength of the effect (effect size) when other factors such as sample size and variance are held constant. However, there are countless small environmental factors that cannot be controlled from one experiment to the next, despite reasonable attempts to do so. Therefore, it is possible that environmental factors introduced variability into some of the current experiments but not others. Supposing a relatively small effect size and a degree of statistical power that was correspondingly marginal, it would not be surprising that some experiments would test "significant" while others would not. This first explanation may be true, but if it

were true then still the most that could be said would be that the $GABA_A$ receptors have a significant but weak effect on forced swim test immobility.

There is a second type of explanation that is perhaps more interesting from a theoretical perspective. This explanation is that it is possible that allopregnanolone withdrawal does increase forced swim test immobility, but that there are other internal factors at work that compete against the depressogenic effect of allopregnanolone withdrawal. In order to conceptualize the following examples it may be helpful to think of the expression of depression or depression-like behaviors as like a set of scales that must be tipped in one direction in order for the behaviors to manifest. One example of competing internal factors comes from the fact that $GABA_A$ receptors are ubiquitous in the brain. Therefore, it is conceivable that withdrawal of $GABA_A$ receptor activity in one brain region could "tip the scales" in the direction of depression, but that simultaneous withdrawal of $GABA_A$ receptor activity in another brain region could "tip the scales" back in the other direction.

A second example of how internal factors could compete with allopregnanolone withdrawal comes from the recognition that progesterone receptors, and therefore progesterone itself, were demonstrated to have robust influences in forced swim test immobility. Inhibiting activity of progesterone receptors using CDB-4124 or mifepristone resulted in a depression-like response, so it is possible that progesterone helps prevent a depression-like state. It is possible that picrotoxin treatment could have "tipped the scales" in the direction of depression-like behavior because of precipitated allopregnanolone withdrawal, but that the presence of progesterone and the accompanying activation of progesterone

receptors was also sometimes sufficient to "tip the scales" back in favor of normal performance in the forced swim test.

Progesterone receptors may play an important role in progesterone withdrawal

Experiments using the progesterone receptor antagonists CDB-4124 and mifepristone demonstrated robust increases in forced swim test immobility. This effect was not additive with picrotoxin, and did not appear to be dependent on acute changes in locomotor ability. Mifepristone is known to be a potent glucocorticoid antagonist, but CDB-4124 is considered to be more selective for progesterone receptors (see Table 23). Therefore, the similar effects of these drugs on forced swim test immobility are presumably owed to their similar antagonist characteristics at progesterone receptors. This finding is consistent with the report by Schmidt and colleagues (1991) that mifepristone treatment in women resulted in premenstrual syndrome-like symptoms.

No information could be found in the literature that indicates what other receptors, if any, have been studied with regard to CDB-4124 or picrotoxin, aside from the progesterone and $GABA_A$ receptors that these drugs (respectively) are generally agreed to specifically inhibit. The strength of the current data and conclusions is largely dependent on the presumption that that CDB-4124 and picrotoxin are selective antagonists for progesterone receptors and $GABA_A$ receptors, but it is also important to keep in mind that these compounds may have biological functions that are currently unknown. Still, the main strength of the current pharmacological experiments is that they used compounds that are (presumed to be) selective for specific targets in order to avoid the multiple effects

Table 23: Effects of Key Steroids and Picrotoxin on the Activity of Selected Receptor and Enzyme Targets

Compound	NPR	MPR	MRC	GAR	PXR	GCR	5AR
Progesterone	↑[a]	↑[b]	↑[c]	×[d]/↓[e]	↑[f]	↓[g]	P⟶[h]/↑*[i]/↓[j]
Allopregnanolone	↑[e]	×[k]	↑[l]	↑[l]/↓[m]	↑[n]	↓[o]	⟶A[h]/↑[p]
Mifepristone	↓[q]	↓[r]	×[s]	×[t]	↑[f]	↓[u]	↓[i]
CDB-4124	↓[q]	?	?	?	?	×[o]	?
17β-estradiol	↑*[v]	↓[w]	×[a]	↑[x]	↑[y]	↑*[z]	×[i]
Finasteride	×[aa]	?	?	?	?	?	↓[bb]/UK[cc]
Picrotoxin	?	?	?	↓[dd]	?	?	?

Note. Abbreviations used: NPR, nuclear progesterone receptors; MPR, membrane bound G-protein coupled progesterone receptors; MRC, progesterone membrane receptor components; GAR, $GABA_A$ receptor; PXR, pregnane X receptor; GCR, glucocorticoid receptor; 5AR, 5α-reductase enzyme. Up (↑) and down (↓) arrows indicate that the compound increases or decreases activity/function of the receptor

or enzyme or is an agonist or antagonist for these receptors. Where one cell says "UK" (UK) that indicates that the effect of finasteride on the type III isozyme of 5α-reductase is unknown. Question marks (?) indicate that no specific, relevant information about the activity of the compound/protein pair was located. An x-shape (×) indicates that the compound has little or no activity at the protein target (in some cases this is relative to physiological concentrations). The rightwards arrow from P (P→) indicates that progesterone is a substrate for this enzyme, while the rightwards arrow to A (→A) indicates that the production of allopregnanolone is catalyzed (upstream) by this enzyme. An up arrow with asterisk (↑*) indicates that the compound induces the expression of this protein. Note that this table is provided as an overview of the current literature, but that the quality of evidence is not equal for all compound/protein combinations, and that some of the reported effects may be limited in extent or circumstance. [a]Brinton et al., 2008. [b]Thomas, 2008. [c]Rohe et al., 2009. [d]Veleiro & Burton, 2009. [e]Beyer et al., 1995. [f]Kliewer et al., 1998. [g]Samra et al., 1984; Pedersen et al., 2003. [h]Mellon & Griffin, 2002. [i]Matsui et al., 2002. [j]Faredin et al., 1992. [k]Sleiter et al., 2009. [l]Viéro et al., 2006. [m]Belelli & Lambert, 2005. [n]Lamba et al., 2004. [o]Basta-Kaim et al., 2007. [p]Sánchez et al., 2008. [q]Attardi et al., 2004. [r]Chien et al., 2009. [s]Engmann et al., 2006. [t]Maguire & Mody, 2007. [u]Attardi et al., 2002. [v]Cui et al., 2005. [w]Mönkkönen et al., 2007. [x]Viero & Dayanithi, 2008. [y]Xue et al., 2007. [z]Ferrini et al., 1995. [aa]Sudduth et al., 1993. [bb]Finn et al., 2006. [cc]Uemura et al., 2008. [dd]Yoon et al., 1993.

of the endogenous steroids. For example, progesterone not only activates progesterone receptors, but also inhibits glucocorticoid receptors (see Table 23). Thus, using selective receptor antagonists can reveal the receptors involved in forced swim test immobility in a way that only manipulating the endogenous steroids cannot.

Limitations and future directions

Withdrawal. One of the chief limitations of the present work is that it did not rigorously address the *withdrawal* aspect of whether the depressogenic effect of progesterone receptor antagonists was dependent upon withdrawal from high levels of progesterone receptor activation. A simple test of this principle would be to see if progesterone administration was necessary for these drugs to increase immobility. If administering these drugs alone caused no increase in swim test immobility, then it could be argued that progesterone pre-exposure is necessary for an immobility-inducing effect, and that therefore these drugs increase swim test immobility by withdrawing (and not simply by blocking) the progesterone signal.

Genetic manipulations of progesterone receptors. Another limitation of the present work is that there was only one general strategy for testing the role of progesterone receptors in this behavior. Whereas the allopregnanolone withdrawal hypothesis was tested using a number of strategies (passive, metabolic, precipitated), the precipitated withdrawal/antagonist strategy was the only specific test of the progesterone withdrawal hypothesis. The first experiment with progesterone withdrawal (Experiment 1) cannot be considered a specific test of the progesterone withdrawal hypothesis because, as demonstrated in Experiment 3, it

simultaneously results in allopregnanolone withdrawal. The problem of not having multiple, specific tests of the progesterone withdrawal hypothesis could have been remedied by several different approaches. For example, a future direction for this research could be to use mice that have been genetically altered to be deficient of progesterone receptors. If passive progesterone withdrawal or progesterone receptor antagonists failed to produce immobility in progesterone receptor-deficient mice then this would support the hypothesis that these receptors are necessary for progesterone withdrawal to induce depression-like behaviors. This is a wide-open area for study for any researchers with access to this genotype. Strains of mice that are selectively deficient in the A or B isoform (Conneely et al., 2003) could be used to test the dependence of forced swim test immobility during progesterone withdrawal on specific nuclear receptor isoforms.

Multiple systems. The current experiments were almost entirely oriented towards the behavioral pharmacology of progesterone withdrawal as it relates to a mouse model of reproductive depression using a single model of behavioral depression or depressed mood. Focusing on a single behavioral outcome meant that many small experiments could be done in quick succession, but some generalizability was lost in exchange for being able to move from one design to the next. This is potentially an important point. The present experiments could be taken to suggest that allopregnanolone is not greatly involved in the behavioral effects of progesterone withdrawal, but that would be a hasty generalization because it is quite possible that allopregnanolone withdrawal could robustly induce all sorts of physiological and behavioral changes in mice that relate to other the

symptoms of reproductive depression. Given that there are many symptoms of reproductive depression that could easily be tracked in rodent models (for example, pain sensitivity, anxiety, eating and appetite changes, immune function), there is much room for expanding this model to include more aspects of reproductive depression.

Reversibility. If the progesterone withdrawal hypothesis is correct, then there should be a way to reverse the effect of progesterone withdrawal by restoring progesterone receptor activity. This was never directly tested within the same experiment, but comparing across the experiments and treating the 0 d WD group from Experiment 1 as the baseline, one could argue that three days of passive progesterone withdrawal increased swim test immobility but that replacement progesterone during these three days returned immobility to baseline.

Aside from studies that reiterate the temporal association, little actual human research has been performed to address the effects of progesterone withdrawal on depression. However, a relevant line of research has studied whether progesterone can be used as a treatment for reproductive depression such as premenstrual syndrome. Dalton championed using progesterone in this way (e.g., Dalton, 1977, 1979), but there is little empirical evidence to confirm (or reject) the benefit of progesterone for premenstrual syndrome (O. Ford et al., 2006, 2009). Also, no placebo-controlled, randomized studies of progesterone for postpartum depression have been published to date (Dennis et al., 2009).

Vulnerability. An important limitation of most theories of reproductive depression is that they fail to account for the fact that only a small portion of women

experience clinically-relevant symptoms. This is the case for the progesterone withdrawal theory of reproductive depression, because all women with normal fertility experience progesterone withdrawal, but only a subset have symptoms. Identifying one or more "vulnerability" factors that predisposes or permits a woman to experience reproductive depression has long been considered to be an unfilled need in these lines of research (Bancroft, 1993, 1995). There has been some important work that has shown that susceptibility to reproductive depression may be under genetic control (e.g., Doornbos et al., 2009a; Kendler et al., 1998), however these genetic approaches have not yet been incorporated into paradigms that study hormone withdrawal.

There was some behavioral variability within each of the treatment groups in each of the present experiments, including the passive progesterone withdrawal and precipitated progesterone withdrawal groups. Exploratory analyses of these groups revealed that the withdrawal groups typically contained a fair number of mice that spent very little time immobile despite the overall higher group mean. Thus, it is interesting to speculate whether some differences in sensitivity to progesterone withdrawal were captured in these experiments. If so, then perhaps environmental manipulations could be a fruitful way to research sensitivity to progesterone withdrawal.

The choice to use an inbred strain of mice imparts both unique benefits and limitations compared to using a genetically diverse strain of mice. A specific limitation is that data from one inbred strain cannot necessarily be generalized to other genotypes. However, inbred strains offer a tremendous opportunity to work

with a standardized genotype, which can be important in cases such as reproductive depression where there is evidence to suspect that vulnerability factors such as gene differences contribute to the disorders. For this reason, in the present work an inbred strain was used to maintain genetic consistency. All of the studies reported here employed the use of mice from the DBA/2J strain. The DBA inbred mouse strain was the first inbred strain of mice developed for scientific use (Beck et al., 2000), and the DBA/2 substrain was developed around 1930 (Holmes, 2003) and remains one of the most commonly used strains of mice for biomedical research, especially among inbred strains.

The DBA/2J strain was chosen for the current studies based in part on the work of Lucki and colleagues (2001) that assessed genetic contributions to forced swim test immobility. Among the strains of laboratory mice that they tested, their work showed that most strains exhibit decreased forced swim test immobility when administered antidepressant drugs, but that some strains do not. For example, the FVB/NJ strain exhibits almost no immobility behavior under control conditions, and it is therefore not surprising that antidepressant drugs do not further decrease this behavior. The DBA/2J strain was chosen for the present work because the data from Lucki and colleagues suggested that the DBA/2J strain was among the most sensitive strains to antidepressant manipulations.

In additional experiments (not reported in the present work) it was found that passive progesterone withdrawal did not induce increased forced swim test immobility in C57BL/6 inbred mice, and neither passive progesterone nor metabolic allopregnanolone withdrawal increased forced swim test in mice from

WSC1 or WSC2 outbred strains[4]. These studies in C57BL/6, WSC1, and WSC2 mice were not intended to be a rigorous study of the genetic contributions to sensitivity to progesterone withdrawal, but they do provide preliminary evidence that the DBA/2J strain of mice may be unusually sensitive to progesterone withdrawal. Since reproductive depression has been suggested to be a heritable trait, future research should take into consideration the possibility that different mouse strains may vary in their responses to steroid manipulations, especially in models of affective disorders and emotion-related behavior.

Neuroanatomy. Finally, a major limitation of the current studies is that the c-Fos mapping project (Experiment 17) failed to identify a convincing candidate brain region that mediates the effect of progesterone withdrawal on forced swim test immobility. As discussed in Chapter 6, there were some strategic choices that were made which might have prevented identifying such regions. More importantly, this experiment was conceived before the progesterone receptor antagonist and locomotor studies cast doubt upon a role for allopregnanolone and $GABA_A$ receptors in the increase in forced swim test immobility that is associated with progesterone withdrawal. Thus, part of the premise of this experiment was that allopregnanolone withdrawal might induce increased depression-like behavior by a mechanism of rebound hyperexcitability following its withdrawal. It was this

[4] WSC1 and WSC2 are replicate mouse strains derived from an eight-way cross of inbred strains and bred on a rotational mating scheme to minimize gene fixation. The "WSC" name indicates that they are "Withdrawal Seizure Control" lines that were bred using the same gene pool as for the selectively-bred "Withdrawal Seizure Prone" and "Withdrawal Seizure Resistant" mouse lines, but without any intentional selective pressures. The lines are first described by Crabbe et al., 1985.

supposed hyperexcitability that led to the project of mapping neural activity (and therefore c-Fos) in the first place.

Progesterone receptors may be more important for the increased immobility during progesterone withdrawal, but it is not certain whether progesterone withdrawal would have this effect because of overt hyperexcitability. For example, terminating the signal to progesterone receptors could cease production of gene products necessary for maintaining certain synaptic connections. Thus, during progesterone withdrawal it is hypothetically possible that cellular firing rates could remain unchanged but the postsynaptic target could become a slightly different population of cells due to synaptic remodelling. Therefore, the changes induced by progesterone would not be visible in a c-Fos mapping study, but could be tested using other approaches. Lesion or microinfusion techniques could be applied to regions that are generally implicated in affective behaviors. More brain regions could have been chosen for study, but the regions that were used were relevant brain regions. Aside from being regions that are thought to play roles in emotional and motivated behaviors, many of these brain regions do express nuclear progesterone receptors, at least in some species or at certain ages (see Table 24).

Table 24: Expression of Nuclear Progesterone Receptors in the Brain (Page 1)

Brain region	Subjects/Comments
Cerebellum	Expressed in juvenile and adult male rats[a]
Hippocampus	Expressed in juvenile and adult male rats[a]
Hypothalamus	Expressed in juvenile and adult male rats[a], expressed in domestic hens[b], expressed in female mink[e], expressed in female gray short-tailed opossum[f], expressed in male juvenile rats (in certain nuclei only) and neonatal male rats[g], expressed in female guinea pigs[h]
Frontal cortex	Expressed in juvenile and adult male rats[a]
Olfactory bulb	Expressed in juvenile and adult male rats[a]
Ventral midbrain	Expressed in embryonic and postnatal but not juvenile male and female mice[c]
Caudate putamen/ striatum	Expressed in cocaine-treated but not vehicle-treated adult male rats[d], expressed in embryonic and neonatal but not juvenile male rats[g]; also: membrane bound receptors expressed in striatum[n]
Nucleus accumbens	Expressed in domestic hens[b]
Lateral septum	Undetected in dorsal lateral septum and ventral lateral septum in adult rats[i]

Table 24: Expression of Nuclear Progesterone Receptors in the Brain (Page 2)

Brain region	Subjects/Comments
Bed nucleus of the stria terminalis	Expressed in female opossum (reported to be relatively low and variable)[f], expressed in neonatal and juvenile male rats[g], little or no expression in adult rats[j], expressed in male and female rats[m]
Amygdala	Expressed in neonatal and juvenile male rats[g], absent in female guinea pigs[h], moderate expression in cortical nucleus and amygdalopiriform transitional nucleus but little or no expression in most nuclei in adult rats[j], weekly expressed in male and female fetal sheep[l]
Dentate gyrus	Expressed in embryonic and neonatal but not juvenile male rats[g], absent in neuronal progenitor cells (NPGs) derived from dentate gyrus[i], little or no expression in adult rats[j], expressed in female adult rabbits[k]; also: membrane bound progesterone receptors expressed in NPGs derived from dentate gyrus[i]

Note. This table is only intended to give a survey of findings, but the quality of evidence is not necessarily equal across the reports. For example, depending on the

particular study, expression could refer to detection of progesterone receptor mRNA or immunoreactivity for progesterone receptor protein. Brain regions in Roman typeface are regions that were studied in the current c-Fos mapping experiment or that contain such a region; brain regions set in italics are included for additional information. In preparing the table preference was given to studies of rodents, but where such studies were unavailable other species were also included. [a]Guerra-Araiza et al., 2001. [b]Sterling et al., 1987. [c]Beyer et al., 2002. [d]H.-B. K. Wu et al., 2006. [e]Warembourg et al., 1998. [f]Vitazka et al., 2009. [g]Quadros et al., 2009. [h]Warembourg et al., 1986. [i]Liu et al., 2009. [j]Kato et al., 1994. [k]Camacho-Arroyo et al., 2007. [l]Roselli et al., 2006. [m]Auger & De Vries, 2002. [n]Zheng et al., 1996.

Validity of the forced swim test model of depression

The forced swim test was used in the present work to provide one assessment of the affective states of the mice, and to understand the treatments that affect these states. It is common to evaluate behavioral models, such as the forced swim test, as a way to establish how useful these tests are. One relevant issue in discussing the usefulness of a behavioral test is whether the test is reliable in the sense that results can be replicated. This is certainly a very important consideration from a pragmatic standpoint, and the forced swim test is generally viewed as a highly reliable test (Cryan et al., 2002), but the current section is intended to discuss the what interpretations can be drawn from swim test immobility across experiments, rather than to focus on how reliable individual studies should be assumed to be.

In order to determine what kind of interpretations can be made about forced swim test behavior it is necessary to consider its "validity" as a behavioral assay of depression. There are many forms of validity for any given test, and each type of validity relates to a certain dimension of similarity between the model and whatever phenomenon the model is meant to reflect (referred to here as the "target").

Face validity. One commonly discussed form of validity is called "face validity." Face validity refers to the similarity between phenomena that can be observed in a model to phenomena that can be observed in a target. For example, if a mouse injected with ethanol (drinking alcohol) exhibited decreased ability to walk along a balance beam, one might propose that the balance beam holds face validity for the incoordinating effects of ethanol in humans.

To determine whether the forced swim test has face validity for depression it must be considered whether adopting a posture of immobility in a tank of water is in any way analogous to the phenomena that are observed in people with depression. As with any analogy, one's task becomes to distinguish the important parts of the analogy from the parts where the analogy breaks down. Clearly, being left in a tank of water is not a situation that has any material relevance to depression. However, if immobility behavior is taken as a sign of "giving up," then in this respect the forced swim test may be relevant to humans. People with depression often have decreased activity, so the forced swim test could also be said to have face validity as a model of depression in that regard.

However, there are many aspects of the observed phenomena in the forced swim test that do not relate to symptoms of depression. One might propose that since behavior in the swim tank does not resemble changes in sleep patterns or appetite, that the forced swim test has poor face validity. Such criticisms are well justified, and Geyer and Markou (1995) made the general statement that face validity "is difficult to defend rigorously" (p. 790). In the same spirit as their discussion of face validity, the current work treats face validity as simply a good place to start when considering the validity of a model.

Construct validity. Another commonly discussed dimension of validity is "construct validity." The central issue of construct validity for a behavioral model is whether precipitating events that cause behavior are similar between the model and the target. It is not a requirement of construct validity that the behaviors in question are similar between the model and the target, so construct validity is

independent from face validity. However, construct validity is so-called because it is dependent on an individual's or a group's construct for understanding the target; consequently, construct validity is relative to the constructs that are held. For example, a laboratory assay of anxiety-related behavior in rodents could be said to have good construct validity in reference to the construct that anxiety is a response to a threatening situation. However, this similarity would not in itself support biological constructs of anxiety such as $GABA_A$ receptor involvement (e.g., Paul, 1988). Construct validity is often difficult to establish, not only because different stakeholders support different constructs, but because the constructs themselves are constantly undergoing revision or are insufficiently detailed to allow such analysis to occur (Belzung & Grielbel, 2001; Geyer & Markou, 1995). Thus, a model that has good construct validity at one point can lose construct validity if the old construct is revised or rejected. However, Geyer and Markou also note that it is a valuable endeavor to examine the construct validity of models for their continual refinement.

In the case of depression, there are many constructs that describe the underlying mechanisms of the disorder. The most common biological construct of depression is the idea that deficiencies in serotonin result in depression, so the observation that forced swim test immobility could be induced by dietary deprivation of tryptophan, a precursor for serotonin, provides an example of similar causes of behavioral changes between rats and humans (Jans et al., in press; but see the cited article for genetic differences). A more psychologically-oriented construct describing depression is that people learn to be depressed, termed "learned

helplessness." Just as people with depression may give up hope more quickly because they have learned, through past experience, that obstacles cannot be overcome, so might increases in forced swim test immobility within or between testing sessions indicate a learned response (Geyer & Markou, 1995; Porsolt et al., 1977a; 2001).

As discussed throughout the current work, progesterone withdrawal is hypothesized to elicit symptoms of depression in women. Therefore, although depression is certainly not exclusive to progesterone withdrawal, the idea that progesterone withdrawal is one mechanism that can induce depression is itself a construct of depression. Therefore, the findings that passive and precipitated progesterone withdrawal increased forced swim test immobility in the current data are themselves validations of construct validity for the forced swim test by the standard construct validation criteria.

Predictive validity. "Predictive validity" refers to the type of validity that is achieved when those predictions that come from a model prove true in the target. Tests such as the forced swim test are often conceived as screening tools for identifying new pharmacological compounds that may treat the disorder of interest. Thus, a novel compound that decreases forced swim test immobility would be predicted to improve symptoms of depression in the human, and if the prediction were confirmed then this correspondence of effects between the model and the target would support the model's predictive validity. Outside the realm of industry, these "predictions" are frequently made in a retrospective fashion. For example, a drug that is already known to be efficacious for depression is then tested in an

animal model, and if the model "predicts" the antidepressant effect of the drug the predictive validity of the model is supported.

Geyer and Markou (1995) proposed that predictive validity is both the only form of validity that is necessary and the only form of validity that is sufficient to support the general validity of an animal model. To the extent that this proposal can be defended, the forced swim test is perhaps the best-validated animal model of depression because its predictive validity has been supported in so many ways. In the initial description of the model (Porsolt et al., 1977b), antidepressant drugs of several classes showed immobility-reducing effects in the forced swim test. Among these drugs were three tricyclic antidepressants, a monoamine oxidase inhibitor, and three atypical antidepressants. Two benzodiazepine anxiolytics were tested with null results, and tranquillizers increased forced swim test immobility. The psychostimulants amphetamine and caffeine both decreased swim test immobility, but it is unclear whether this increase is due to a general increase in locomotor activity or if it is due specifically to the mood-elevating effects of such drugs.

Although many more antidepressant compounds have subsequently been tested, it is perhaps the experiments not involving antidepressant drugs that make the predictive validity of forced swim test so strong. Multiple non-pharmacological antidepressant therapeutic modalities have been modified to be applied to laboratory animals and have resulted in decreased swim test immobility, consistent with their antidepressant effects in humans. Electroconvulsive shock (Li et al., 2007; Porsolt et al., 1977a, 1977b, 1978), sleep deprivation (Lopez-Rodriguez et al., 2004; Porsolt et al., 1978), light exposure (Schulz et al., 2008), environmental

enrichment (Porsolt et al., 1978), and deep brain stimulation (Hamani et al., in press) are all related to antidepressant treatments in humans, and all have been reported to decrease forced swim test immobility. The findings that sleep deprivation decrease immobility are especially important, because these findings provide evidence that fatigue and swim test immobility are not necessarily correlated. Additionally, transcranial magnetic stimulation, which has been proposed as a treatment for depression and anxiety, decreases forced swim test immobility without inducing anxiolytic-effects in common animal models of anxiety (Hargreaves et al., 2005). Therefore, the forced swim test has positive predictive validity for this treatment modality, while the animal anxiety models that were tested did not have predictive validity for magnetic stimulation (these tests included the elevated plus maze, the social interaction test, and the predator avoidance test).

In addition to the pharmacological and non-pharmacological antidepressant treatments that result in corresponding decreases in immobility, the predictive validity of the forced swim test is also supported by a number of treatments that produce depression in humans and have similarly immobility-increasing effects in the swim test. As mentioned above, dietary tryptophan depletion, also used to experimentally-induce depression in humans, is effective at inducing forced swim test immobility in rats (Jans et al., in press). Furthermore, treatment with drugs that pharmacologically reduce catecholamine concentrations also increase forced swim test immobility (Nagakura et al., 2009; Porsolt et al., 1978). (For more examples of treatments that induce depression in humans and increase swim test in rodents, see Rygula et al., 2008; Mazarati et al., 2008; Stevenson et al., 2009; T. H. Wu & Lin,

2008.) As with construct validity, the current data also support the predictive validity of the forced swim test: progesterone withdrawal increases swim test immobility, which correctly predicts that progesterone withdrawal induces depression in women (e.g., Schmidt et al., 1991).

Overall assessment of validity. There are additional dimensions of validity that could be assessed. For example, convergent validity describes the characteristic of multiple models that yield comparable interpretations about the same manipulated variables. The forced swim test has convergent validity with the tail suspension test, since the two assays generally yield compatible results when testing anti-depressant drugs. However, face, construct, and predictive validity remain among the most important measures of validity for laboratory models of depression, and Geyer and Markou (1995) have argued vehemently that predictive validity is the only necessary type of validity for animal depression models. The forced swim test satisfies the requirements for these three forms of validity, and does so most strongly for predictive validity.

It is clear that there is some overlap between the evidence that is used to support one form of validity and another form of validity. For example, the strength of the predictive validity of the forced swim test comes, in part, from the findings that antidepressant medications decrease forced swim test immobility. Since some of the scientific community's notions about depression itself come from the mechanisms by which these drugs act, it cannot be avoided that some degree of circular reasoning appears in using this pharmacological evidence to support both the construct and the predictive validity. The same can be said of using

progesterone withdrawal data to support both the construct and predictive validity. In fact, this is the same problem that Geyer and Markou (1995) described in construct validity when they discussed that scientific constructs are dependent on scientific data. For this reason, Geyer and Markou proposed that predictive validity is the most important. Constructs about why certain treatments affect depression will change over time, but the facts that they do affect depression can be more clearly established and are less prone to revision. Therefore, although the construct and face validity of the forced swim test can be defended or criticized, it remains inarguable that the predictive validity of this assay is very strong. Judging by the most common criteria for animal models of depression, the forced swim test is a well-validated behavioral test of depression for laboratory animals.

Criticisms of the forced swim test model

To say that a model has been validated is not to say that it is free from limitations. For the current purposes, most criticisms that are discussed as limitations of the forced swim test can be grouped into one of two general categories. The first category of criticisms contains those that propose that forced swim test immobility may not actually indicate the state that it is claimed to indicate, while the second category contains those that propose that there are other behavioral measures and variables that are also valuable or are more valuable than swim test immobility.

In order to address these criticisms, it is first necessary to consider what forced swim test immobility might indicate. Throughout the present work, swim test immobility has been equated with a depression-like state, effect, response,

behavior, or symptom, but the way in which these states, effects, responses, behaviors, or symptoms are like depression has not yet been defined. It is not clear whether swim test immobility indicates the presence of one particular symptom of depression in the test subject, all of the depression symptoms collectively, or some mix of these symptoms. It is not even absolutely clear that putting the model-to-target comparison in terms of symptoms is the proper frame of reference, but given the predilection of psychiatry to focus on symptoms it is symptoms that usually receive attention.

The reason that the depression-like characteristics of immobility have not been more narrowly defined in terms of specific symptoms may be that there is no well-accepted, clear sense in which immobility is "like" depression. As discussed in the previous section, the forced swim test is a valid behavior for modeling depression, but the construct validation and predictive validation processes do not guide understanding the behavior any further. In order for construct validation to further guide understanding of what the forced swim test specifically represents, there would need to be clearer constructs (either psychological or biological) that explain specific symptoms of depression. Similarly, for predictive validation to inform the discussion of which symptoms swim test immobility reflects there would need to be symptom-specific phenomena in humans that could be specifically compared to the model.

If there was a drug that specifically treated some symptom of depression, (changes in cognition, for example), then that drug could be tested in the forced swim test, and either the drug would or would not alter swim test immobility.

Depending on the results of the experiment, swim test immobility might or might not be supported to have predictive validity for changes in cognition. Similarly, if there was a well-defined construct that described why people with depression experience cognitive changes, then the forced swim test could be compared against that construct. However, there are neither specific prediction nor construct criteria to further assess the predictive or construct validity of forced swim test immobility for specific symptoms.

In contrast, there are specific phenomena that can be used to determine the symptom-specific face validity of forced swim test immobility for depression. Although face validation may be difficult to defend rigorously (Geyer & Markou, 1995), it may at least provide a starting point for understanding what symptoms forced swim test immobility may represent. Immediately one can reject guilt, appetite, and suicidality from the possible symptoms that swim test immobility might represent on face level. (These can only be rejected in terms of face similarity; they cannot be rejected entirely.) However, there are symptoms that immobility might reasonably resemble on face level.

Activity. One possible criticism of forced swim test immobility is that the decrease in swimming may reflect a general suppression of activity. In some cases a treatment might interfere with activity in a way that is not germane to depression. For example, a neuromuscular blocker could acutely increase forced swim test immobility without affecting the emotional state of the animal. Despite such exceptions, on face level, immobility could directly represent the changes in physical activity that are associated with depressed mood. There is growing evidence in

humans that depression (or its related symptoms) and low levels of physical activity are reciprocally reinforcing (Hawkley et al., 2009; Pagoto et al., 2009; Roshanaei-Moghaddam et al., 2009). Furthermore, there is accumulating evidence that physical activity itself is a clinically- and experimentally-effective method for reducing depression or depressed mood (Blake et al., 2009; Weinstein et al., in press). Therefore, although it is expected that specific cases could exist where immobility does not truly represent an emotional state, decreases in physical activity in other cases could be very relevant to depression.

Successful coping. Another criticism of the forced swim test is that immobility may reflect a successful coping strategy. For example, rats that were exposed to trials where a ladder was lowered into the swim tank exhibited no statistically-significant decrease in a subsequent forced swim test compared to rats that had a similar pre-exposure to the swim tank but without the opportunity to escape (O'Neill & Valentino, 1982). This finding is superficially inconsistent with the predictions that the learned helplessness model would make about forced swim test immobility: If mice and rats increase their swim test immobility because they learn that they cannot escape the tank, then offering an escape should result in decreased swim test immobility. Because the opportunity to escape by a ladder did not decreased subsequent immobility, this finding has been used to argue that animals who exhibit swim test immobility must be using a successful coping strategy rather than learning to be helpless (West, 1990).

However, there are some key differences between the learned helplessness model and the study by O'Neill and Valentino that should be examined. In the

typical learned helplessness training procedure an animal must perform some arbitrary task (e.g., pushing a lever) to avoid a shock. In contrast, in the study by O'Neill and Valentino, the introduction of the escape ladder was not contingent on the rats performing any behavior. Therefore, this study is insufficient to test whether forced swim test immobility is akin to learned helplessness. New studies show that rats increase forced swim test immobility as a function of learning that a previously-available escape is no longer available (e.g., Huston et al., 2009), providing evidence that animals can learn whether the swim test is escapable or not, and that this learning affects subsequent performance in the test in the manner that would be predicted by learned helplessness theory.

Critics of the forced swim test will sometimes suggest that, if immobility behavior is not similar to learned helplessness, that it must instead reflect a successful coping strategy, for example to save energy (e.g., West, 1990). Studies such as that by Huston and coworkers (2009) provide evidence that rats do increase swim test immobility as a function of learning that escape is impossible, but leaving this evidence aside for the moment it is worth pursuing the idea that immobility may be a successful coping strategy. In fact, "learned helplessness" in humans could also reflect a successful coping strategy. It is a fact of life that people will face negative circumstances over which they have little control. When faced with such an incontrollable life event, it may be adaptive not to try to overcome these obstacles, and rather to conserve one's energy. The learned helplessness theory of depression focuses on the fact that what distinguishes people with depression is that people with depression tend to over-generalize and act as though all situations

are hopeless (a global attribution style, Alloy et al., 1984). Viewed in this light, it may be irrelevant whether swim test immobility is a successful energy conservation strategy. What may be more important are the factors that cause some animals to engage in this behavior more quickly than others, which may be related to the animal's predisposition to assess a situation as hopeless.

Fear and freezing. Two rodent models of human anxiety produce a freezing response that, on face level, resembles the immobility posture assumed during the forced swim test. The acoustic startle response test employs a loud noise to elicit a freezing or startle response from a laboratory animal such as a rat or mouse. When an arbitrary stimulus such as light cue is presented immediately prior to the noise during training, the light cue alone will also come to elicit the freezing response or will potentiate the freezing response during light plus noise trials (Richardson, 2000). In other protocols, an auditory stimulus or a context is used to signal the delivery of a footshock. The footshock itself may cause a jumping or other injury-related response, but on subsequent trials the auditory or context signal alone will elicit a freezing response (Chang et al., 2009). The two methods can have overlapping procedural features, as footshock is sometimes used to augment the acoustic startle response (Walker & Davis, 2002). Collectively these procedures are called "fear conditioning" procedures because of the presumed connection between freezing and fear, and the fact that freezing is a conditioned response to a previously-neutral stimulus. Regardless of which procedure or mix of procedures is used, a conditioned stimulus eventually elicits a freezing response in the animal. It

is this freezing response that appears similar, on face level, to the immobility adopted during forced swim testing.

Closer inspection calls into question whether freezing to a conditioned stimulus in these procedures is similar to forced swim test immobility beyond visual appearances. There are two key comparisons that must be made. First, animals will quickly acquire a freezing response to the fearful stimulus (e.g., Chang et al., 2009). Second, animals will undergo a process called extinction in which the response of freezing to the conditioned stimulus is diminished. During repeated exposures the animal forms the new learned association that this stimulus no longer predicts the original (unconditioned) fear-evoking stimulus (Quirk & Mueller, 2008). In contrast to these phenomena, in the forced swim test animals are slow to acquire the immobility response, but will allocate increasing proportions of their time to immobility (e.g., Porsolt et al., 1978). Therefore, although fear-evoked freezing and swim test immobility may appear similar, their learning functions may be very different.

One possible explanation for the discrepancy between extinction of freezing to a conditioned stimulus and increased swim test immobility across the test session could be that in extinction the animals are only being exposed to the conditioned stimulus, whereas the water, tank, or some other feature of the forced swim test could be viewed as an unconditioned stimulus for immobility. Therefore, it is also desirable to compare the unconditioned stimuli in fear conditioning experiments to the forced swim test environment, which could be a sort of unconditioned stimulus. In the case of acoustic startle animals can habituate to the unconditioned stimulus of

a loud, startling noise (Koch, 1999). No studies were found in the fear conditioning literature that explicitly tested for habituation to long-term shock, but the learned helplessness procedure itself is an analogous preparation. Following repeated inescapable shocks in learned helplessness procedures rats exhibit persistent deficits in escape-directed behaviors (e.g., Seligman et al., 1975).

Thus, comparing forced swim test immobility to freezing behavior following conditioned or unconditioned fear stimuli, swim test immobility is not similar in its patterns of expression to conditioned fear stimuli, nor to the unconditioned stimulus of an acoustic startle. The expression of forced swim test immobility is not entirely like that of freezing to an electrical shock, because freezing to an electrical shock occurs rapidly whereas swim test immobility takes minutes to develop. Still, both prolonged forced swimming and repeated electrical shock result in persistently suppressed escape-directed behavior so they might be considered similar in that regard. However, to say that swim test immobility resembles conditioned fear would beg the question that freezing represents fear, rather than another emotion such as depression.

One possible way to reconcile this possible similarity between conditioned fear and depression-like behavior in the forced swim test is simply to point out that antidepressant drugs do decrease forced swim test immobility and learned-helplessness behaviors (e.g., Giral et al., 1988; Kametani et al., 1983). In contrast, antidepressant drugs have been shown not to block conditioned freezing behavior (e.g., Davis, 1986; Spenatto et al., 2008). One final consideration is that immobility elicited by a fear-provoking stimulus is generally associated with sustained muscle

tension or "tonic" immobility (Moskowitz, 2004), whereas there is no indication of muscle tension or rigidity in mice that are immobile in the forced swim test. Taking all of the evidence collectively, there does not appear to be a strong case to support the hypothesis that forced swim test immobility is related to freezing behavior observed in response to fear-evoking stimuli.

Social interaction. The second category of criticisms about the forced swim test includes the aspects of depression that the forced swim test does not seem to address, regardless of its putative overall validity as a model of depression. One such aspect of depression is social interaction. As is common with many chronic illnesses, one overt phenomenon associated with depression is that people suffering from depression may choose to spend less time interacting with others, a behavior termed "social withdrawal" (Rigby et al., 1999). Since many chronic illnesses and other medical complications are associated with social withdrawal and with depression, it is not clear whether there is a direct link between social withdrawal and depression (e.g., Ahrens & Linden, 1996; Danker et al., in press; Boekaerts & Röder, 1999; Jost & Grossberg, 1996). Instead, social withdrawal may be a general feature of mental illness and not a specific symptom of depression (Winograd-Gurvich et al., 2006).

Still, social withdrawal is associated with depression and it is therefore important to recognize that the forced swim test does not specifically test behaviors that are thought to reflect social withdrawal. Therefore, one limitation to the face validity of the forced swim test is that forced swim test immobility does not resemble the decreased social interactions observed in people with depression.

However, social isolation procedures in mice have been demonstrated to increase forced swim test immobility in mice (Martin & Brown, in press), rats (Dandekar et al., 2009; Kuramochi & Nakamura, 2009) and prairie voles (Grippo et al., 2008). Therefore, although the forced swim test does not assess social withdrawal as a symptom, performance in this test is influenced by social interactions. These findings increase the construct of validity of the forced swim test as a model of depression with regard to the theories that propose that decreased social interaction and persistence of depression are reciprocally-reinforcing phenomena (a thorough discussion of the relevant theories is provided by Lara & Klein, 1999). Testing the effects of progesterone withdrawal on social interaction would be an interesting way to expand the current line of research.

Anhedonia. Anhedonia can be generally defined as the inability to receive joy, pleasure, or satisfaction from experiences in life. As a diagnostic criterion for depression it is usually defined in terms of lost or diminished interest in experiences, people, or objects that once brought joy or pleasure to a person. Anhedonia was once considered a cornerstone diagnostic feature of depression, but low positive affect or depressed mood supplanted anhedonia as the key feature of depression (Snaith, 1993). Even so, it should be noted that a small percentage of people with depression do not report sadness (Mouchet-Mages & Baylé, 2008); for these patients, the chief complain is often anhedonia.

The forced swim test does not appear to have face validity as a model of anhedonia. Although the diagnostic criterion of "lost interest" could be mistaken to resemble an immobile animal's "lost interest" in escaping the swim tank, the

similarity seems insubstantial. A model with better face validity for anhedonia would include measures of sucrose or saccharin consumption, since these behaviors clearly have face validity for the receipt of pleasure from an activity. Yet a lack of face validity does not necessarily indicate that the forced swim test does not model some aspect of anhedonia. Several recent studies have shown that treatments that alter forced swim test immobility cause corresponding changes in sucrose or saccharin consumption (e.g., Banasr & Duman, 2008; Mazarati et al., 2008; Prendergast & Kay, 2008).

As discussed previously, in order to assess the symptom-specific construct validity of a model one would need symptom-specific constructs that describe the etiology of the disorder in the target. Similarly, in order to assess the symptom-specific predictive validity of a model one would require manipulations that selectively modulate that symptom in the target (e.g., a drug that in humans improves anhedonia without affecting other symptoms of depression). These constructs and manipulations are not available, so it is not possible to determine whether the agreement between forced swim tests and sucrose or saccharin consumption tests is due to the different tests measuring different symptoms that are all present in the same animals, or whether the various tests are sensitive to general features of the overall disorder. Nonetheless, the concordance of results from forced swim tests and from sucrose or saccharin consumption at least supports the convergent validity of these two tests as models of depressive disorders. A recent finding that is important for the purposes of the current work is that a hormone-simulated pregnancy regimen that had previously been shown to

increase forced swim test immobility has also been shown to decrease sucrose consumption in female rats (A. D. Green et al., 2009). Based on this finding, one might hypothesize that the progesterone withdrawal procedures used in the current studies would also decrease sucrose or saccharin consumption.

Mechanisms for progesterone withdrawal-induced forced swim test immobility

The current work supports the hypothesis that progesterone withdrawal increases forced swim test immobility without the involvement of other steroids such as allopregnanolone. Since passive progesterone withdrawal increased forced swim test immobility, and since both of the progesterone receptor antagonists tested (CDB-4124 and mifepristone) increased forced swim test immobility, these data indicate that either passive or active termination of the progesterone signal increased swim test immobility.

The best-studied class of progesterone receptors is the nuclear progesterone receptor family (also called the classic progesterone receptors or the intracellular progesterone receptors). These receptors have different physiological effects depending on how they dimerize before binding to DNA to direct gene transcription, and they form at least three dimeric species between the A and B receptor isoforms (A:A, A:B, and B:B) (Mani, 2008). Not much is known yet about the C, M, S, and T isoforms (Samalecos & Gellersen, 2008), including the extent of their expression in brain or the dimer species that they form. Few studies have attempted to identify the specific genes that are regulated by progesterone, but from the studies that have been performed it is clear that progesterone regulates a vast number of genes in the brain, including genes for proteins involved in neurotransmission, cell survival, and

signal transduction (Auger et al., 2006; A. P. Reddy & Bethea, 2005). Despite the relative few studies that have sought to identify genes regulated by progesterone, given the power of gene microarray techniques these studies have identified large numbers of candidate genes that are potentially regulated by progesterone (over 10,000 gene probes in Bethea & Reddy, 2008). Thus, it is beyond the scope of the present work to consider every candidate gene for its potential role in forced swim test during progesterone withdrawal. However, as an example, a recent study with monkeys reported that progesterone plus estrogen treatment increased mRNA for superoxide dismutase (SOD1, a protein that increases cell survival by scavenging free radicals) in neurons from the dorsal raphe nucleus, compared to monkeys treated with only estrogen (Bethea & Reddy, 2008).

Since the dorsal raphe nucleus is a key region for serotonin signaling, the finding that progesterone treatment significantly increases mRNA for a cell survival protein (SOD1) could indicate that progesterone helps maintain normal dorsal raphe function and, therefore, helps to maintain normal emotional states. Since the current data indicate that progesterone withdrawal increases depression-like symptoms in mice, one potential mechanism could be through the loss of factors like SOD1 and corresponding dysfunction in emotion-regulation centers. Thus, although the field of determining what genes are regulated by progesterone is still in its infancy, there are some reasonable candidate genes that have been identified as being regulated by progesterone in brain regions relevant to emotional regulation and function.

The intent of discussing the effects of progesterone on SOD1 in the raphe nucleus in monkeys is not to draw specific parallels between the work of Beathea and Reddy (2008) and the present studies. Indeed, in the present work no significant changes in c-Fos expression were observed in the dorsal raphe nucleus. Rather, the purpose is to consider the sorts of mechanisms that could by hypothesized to connect progesterone withdrawal to depression. Bethea and Reddy observed increased SOD1 mRNA in the dorsal raphe nucleus, but they did not test wether the same mRNA is also increased by progesterone in other brain regions. On the other hand, in the present experiment no change in c-Fos expression was observed in the dorsal raphe nucleus at the end of the withdrawal period, but that does not mean there were no differences in SOD1 expression. Therefore, future progesterone withdrawal research should address some of these candidate mechanisms.

Concluding remarks

Taken as a whole, the experiments described in the present work support a role for progesterone receptors in increased depression-like behavior during progesterone withdrawal. This effect does not seem to be dependent upon acutely suppressed locomotor activity. In contrast, the neuroactive steroid allopregnanolone and its chief target receptor, the $GABA_A$ receptor, seem not to play a major role in this phenomenon. There are important limitations to these studies, but they should provide an impetus for reconsidering the role of progesterone in reproductive depression. Progesterone receptors should be considered as candidate targets for pharmacological therapies for reproductive depression.

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