Increasing $3\alpha,5\alpha$ -THP following inhibition of neurosteroid biosynthesis in the ventral tegmental area reinstates anti-anxiety, social, and sexual behavior of naturally receptive rats

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Abstract

The progesterone metabolite and neurosteroid, 5α -pregnan- 3α -ol-20-one ($3\alpha,5\alpha$ -THP), has actions in the midbrain ventral tegmental area (VTA) to modulate lordosis, but its effects on other reproductively relevant behaviors are not well understood. Effects on exploration, anxiety, and social behavior resulting from inhibition of $3\alpha,5\alpha$ -THP formation, as well as $3\alpha,5\alpha$ -THP enhancement, were investigated in the midbrain VTA. Naturally sexually receptive, female rats ($n=8-10$ /group) received infusions aimed at the midbrain VTA of vehicle, PK11195 (an inhibitor of neurosteroidogenesis), and/or indomethacin (an inhibitor of $3\alpha,5\alpha$ -THP formation from prohormones), and were subsequently infused with vehicle or FGIN 1-27 (a neurosteroidogenesis enhancer). The rats were then assessed in a behavioral battery that examined exploration (open field), anxiety (elevated plus maze), social (social interaction), and sexual (paced mating) behavior. Inhibition of $3\alpha,5\alpha$ -THP formation decreased exploratory, anti-anxiety, social, and sexual behavior, as well as midbrain $3\alpha,5\alpha$ -THP levels. Infusions of FGIN 1-27 following $3\alpha,5\alpha$ -THP inhibition restored these behaviors and midbrain $3\alpha,5\alpha$ -THP levels to those commensurate with control rats that had not been administered inhibitors. These findings suggest that $3\alpha,5\alpha$ -THP formation in the midbrain VTA may influence appetitive, as well as consummatory, aspects of mating behavior.

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Introduction

Ovarian hormones, such as 17β -estradiol (E_2) and progesterone (P_4), have coordinated actions at multiple brain sites to influence the expression of female sexual behavior in rats (typically operationalized as lordosis). In the ventromedial hypothalamus (VMH), E_2 and P_4 have actions via cognate, intracellular progesterin receptors (PRs) to initiate lordosis. In the midbrain ventral tegmental area (VTA), P_4 mediates the intensity and duration of lordosis, but these effects appear to be independent of actions at PRs. Application of P_4 to the VTA rapidly increases lordosis and these effects are not attenuated when P_4 actions are membrane limited or when the few intracellular PRs in the VTA are blocked by antisense oligonucleotides (Frye 2001a, 2001b). In the VTA, P_4 's actions involve formation of 5α -pregnan- 3α -ol-20-one ($3\alpha,5\alpha$ -THP), which is devoid of affinity for PRs, and has effects through GABA_A, NMDA, and/or dopamine-like type 1 receptors, and subsequent downstream signal transduction processes to influence lordosis (Dohi *et al.* 2008, Frye & Walf 2008). Thus, progesterin's actions in these regions influence the expression of female sexual behavior of rodents.

In the midbrain VTA, there are dynamic effects of progesterins to mediate lordosis, and for progesterin

formation to be altered by female sexual behavior. The VTA richly expresses all enzymes necessary to catalyze the formation of $3\alpha,5\alpha$ -THP from cholesterol (Frye 2001a, 2001b, Mellon *et al.* 2001). This process of neurosteroidogenesis, which occurs in the brain independent of ovarian sources, may play an important role in progesterin-mediated reproductive behaviors. Neurosteroidogenesis is increased following the activation of mitochondrial benzodiazepine receptors (MBRs), which facilitate the transport of cholesterol across mitochondrial membranes (Brown & Papadopoulos 2001). This increases the availability of cholesterol for conversion to pregnenolone via the P450 side-chain cleavage enzyme, one of the rate-limiting steps in steroid synthesis (Hall 1985, Otto *et al.* 1992). $3\alpha,5\alpha$ -THP is then formed from pregnenolone via sequential enzymatic steps involving the enzymes, 3β -hydroxysteroid dehydrogenase, 5α -reductase, and 3α -hydroxysteroid dehydrogenase (Plassart-Schiess & Baulieu 2001). Prior reports have demonstrated that activating MBRs with *N,N*-diethyl-2-(4-fluorophenyl)-indole-3-acetamide (FGIN 1-27) enhances lordosis of rats and hamsters concomitant with increases in midbrain $3\alpha,5\alpha$ -THP levels (Frye & Petralia 2003a, 2003b). Conversely, decreasing activity of MBRs in the VTA with

1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) inhibits lordosis and midbrain $3\alpha,5\alpha$ -THP concentrations (Frye & Petralia 2003a, 2003b). Naturally receptive or E_2 -primed rats that lack all peripheral sources of progestins have elevated levels of $3\alpha,5\alpha$ -THP in the midbrain VTA following mating (Frye 2001a, 2001b, Frye & Rhodes 2006a, 2008, Frye *et al.* 2007). Thus, neurosteroidogenesis in the midbrain VTA may play a dynamic role in mediating female sexual behavior of rodents.

Beyond lordosis, neurosteroidogenesis in the midbrain has implications for functional effects that may be reproductively relevant. $3\alpha,5\alpha$ -THP and the hypothalamic–pituitary–adrenal (HPA) stress axis interact, such that $3\alpha,5\alpha$ -THP is altered by, and can mitigate, stress responsiveness (Engel & Grant 2001). Exposure to extreme stressors, such as cold water swim, ether, or footshock, increases biosynthesis of pregnane and androstane neurosteroids (Purdy *et al.* 1991, Erskine & Kornberg 1992, Drugan *et al.* 1994). Following secretion in response to a stressor, $3\alpha,5\alpha$ -THP can modulate HPA response to restore parasympathetic tone. In support, administration of P_4 or $3\alpha,5\alpha$ -THP attenuates stress-induced increases in adrenocorticotropin and corticosterone, as well as transcription of CRH mRNA in the hypothalamus of stressed rats (Patchev *et al.* 1994, 1996). Perhaps, in part through its HPA effects, $3\alpha,5\alpha$ -THP can positively modulate affective behavior. P_4 or $3\alpha,5\alpha$ -THP reduces behavioral stress responses evoked by predator odor, forced swim, or footshock (Walf & Frye 2003, Walf *et al.* 2006), and can enhance antinociception, exploratory, anti-anxiety, and affiliative behavior (Finn *et al.* 2003, Frye & Rhodes 2006a, 2006b, 2008, Frye *et al.* 2006, Engin & Treit 2007). Given that successful mating requires suppression of fear, pain responses, and/or neophobia, rapid effects of $3\alpha,5\alpha$ -THP may underlie approach toward stimuli that were previously avoided.

The present study investigated the role of neurosteroidogenesis in the midbrain on appetitive (exploratory, anti-anxiety, social behavior) and consummatory (sexual) aspects of mating behaviors. We hypothesized that if biosynthesis of $3\alpha,5\alpha$ -THP in the midbrain VTA is important for modulating exploratory, anti-anxiety, social, and sexual behaviors, then decreasing and reinstating neurosteroidogenesis of $3\alpha,5\alpha$ -THP in the midbrain VTA should attenuate and enhance exploratory, anti-anxiety, social, and sexual behaviors respectively.

Results

Endocrine measures

Estrogen, progesterone, and dihydroprogesterone

There was no effect of central inhibition of $3\alpha,5\alpha$ -THP formation or FGIN 1-27 administration on the concentrations of plasma corticosterone or E_2 , P_4 , or DHP in plasma, midbrain, hippocampus, cortex, or striatum (Table 1).

$3\alpha,5\alpha$ -THP

There was an interaction between $3\alpha,5\alpha$ -THP inhibitors and FGIN 1-27 administration on midbrain $3\alpha,5\alpha$ -THP levels ($F(3,67)=4.71$, $P<0.05$), which was due to an increase in $3\alpha,5\alpha$ -THP levels following FGIN 1-27 administration in rats administered PK11195 and/or indomethacin, but not vehicle (Fig. 1, top). Plasma $3\alpha,5\alpha$ -THP levels were not altered by $3\alpha,5\alpha$ -THP inhibition in the brain or FGIN 1-27 infusion (Fig. 1, bottom).

Behavioral measures

Open field

Inhibition of $3\alpha,5\alpha$ -THP significantly decreased central entries in the open field ($F(3,67)=2.70$, $P=0.05$). Indomethacin, alone or in conjunction with PK11195, significantly reduced central square entries. Midbrain levels of $3\alpha,5\alpha$ -THP also significantly predicted central entries ($t(69)=2.96$, $P<0.05$) and accounted for a significant proportion of central entry variance ($R^2=0.11$, $F(1,69)=8.74$, $P<0.05$).

There was no effect of inhibition of $3\alpha,5\alpha$ -THP or FGIN 1-27 administration on peripheral or total entries in the open field (Table 2).

Elevated plus maze

There were significant interactions between inhibition of $3\alpha,5\alpha$ -THP formation and FGIN 1-27 administration on the time spent on the open ($F(3,67)=2.78$, $P<0.05$) and closed ($F(3,67)=5.50$, $P<0.05$) arms of the elevated plus maze. This was due to increased time spent on the open arms of the plus maze (Fig. 2, top), and decreased time spent on the closed arms (Table 2), among rats administered inhibitor followed by infusions of FGIN 1-27 but not vehicle. Midbrain levels of $3\alpha,5\alpha$ -THP also significantly predicted open arm time ($t(69)=2.82$, $P<0.05$) and accounted for a significant proportion of open arm time variance ($R^2=0.10$, $F(1,69)=7.97$, $P<0.05$).

There was a main effect for rats receiving infusions of inhibitor to make fewer open arm entries in the elevated plus maze than those administered vehicle or FGIN 1-27 without inhibitor infusions ($F(3,67)=5.70$, $P<0.05$; Table 2). No effects of central manipulation were observed for closed or total arm entries (Table 2).

Social interaction

Although there was an apparent effect of indomethacin, with or without PK11195, to decrease the duration of time spent in social interaction with a conspecific, this effect was not significant. Administration of FGIN 1-27 increased social interaction of rats, overall ($F(1,69)=3.80$, $P<0.05$; Fig. 2, bottom). Midbrain levels of $3\alpha,5\alpha$ -THP also significantly predicted the time spent in social interaction ($t(69)=2.45$, $P<0.05$) and accounted for a significant

Table 1 Neuroendocrine measures of female rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27 to the ventral tegmental area of the midbrain (mean $\pm$ S.E.M.).

Infusate #1 Infusate #2 Infusate #3	Vehicle Vehicle Vehicle	Vehicle Vehicle FGIN 1-27	PK11195 Vehicle Vehicle	PK11195 Vehicle FGIN 1-27	Vehicle Indomethacin Vehicle	Vehicle Indomethacin FGIN 1-27	PK11195 Indomethacin Vehicle	PK11195 Indomethacin FGIN 1-27
Corticosterone								
Serum (μ g/dl)	3.9 $\pm$ 1.3	3.5 $\pm$ 1.2	2.0 $\pm$ 0.5	3.1 $\pm$ 0.7	2.9 $\pm$ 1.2	2.3 $\pm$ 0.7	3.3 $\pm$ 0.9	3.4 $\pm$ 1.1
E ₂ ^a								
Serum (pg/ml)	18.3 $\pm$ 3.3	13.2 $\pm$ 3.1	10.3 $\pm$ 2.4	14.6 $\pm$ 3.6	11.6 $\pm$ 2.8	14.3 $\pm$ 2.9	13.2 $\pm$ 2.9	12.7 $\pm$ 3.4
Midbrain (pg/g)	1.4 $\pm$ 0.1	1.2 $\pm$ 0.4	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2	1.6 $\pm$ 0.2	1.1 $\pm$ 0.2	1.4 $\pm$ 0.2
Hippocampus (pg/g)	1.3 $\pm$ 0.3	1.0 $\pm$ 0.3	1.8 $\pm$ 0.3	1.6 $\pm$ 0.3	1.4 $\pm$ 0.7	1.4 $\pm$ 0.4	0.7 $\pm$ 0.2	1.7 $\pm$ 0.2
Cortex (pg/g)	1.5 $\pm$ 0.3	1.3 $\pm$ 0.4	1.8 $\pm$ 0.3	1.6 $\pm$ 0.3	1.3 $\pm$ 0.3	1.5 $\pm$ 0.4	1.4 $\pm$ 0.3	1.8 $\pm$ 0.3
Diencephalon (pg/g)	1.1 $\pm$ 0.2	1.2 $\pm$ 0.3	1.3 $\pm$ 0.3	1.5 $\pm$ 0.3	1.7 $\pm$ 0.3	1.4 $\pm$ 0.4	1.4 $\pm$ 0.3	1.8 $\pm$ 0.5
Interbrain (pg/g)	0.7 $\pm$ 0.2	1.3 $\pm$ 0.2	0.9 $\pm$ 0.2	1.3 $\pm$ 0.3	1.0 $\pm$ 0.2	0.7 $\pm$ 0.2	0.9 $\pm$ 0.2	1.1 $\pm$ 0.1
P ₄ ^b								
Serum (ng/ml)	19.6 $\pm$ 2.9	14.3 $\pm$ 3.6	17.1 $\pm$ 2.6	17.1 $\pm$ 4.0	14.6 $\pm$ 3.4	19.6 $\pm$ 3.7	15.0 $\pm$ 3.5	17.4 $\pm$ 4.5
Midbrain (ng/g)	1.9 $\pm$ 0.4	1.4 $\pm$ 0.4	1.4 $\pm$ 0.5	1.5 $\pm$ 0.5	1.8 $\pm$ 0.3	1.4 $\pm$ 0.4	1.2 $\pm$ 0.3	1.4 $\pm$ 0.4
Hippocampus (ng/g)	2.3 $\pm$ 0.4	1.5 $\pm$ 0.5	1.4 $\pm$ 0.4	1.9 $\pm$ 0.4	2.4 $\pm$ 0.8	2.9 $\pm$ 0.4	1.8 $\pm$ 0.4	1.9 $\pm$ 0.5
Cortex (ng/g)	1.8 $\pm$ 0.5	1.8 $\pm$ 0.7	2.9 $\pm$ 0.7	2.9 $\pm$ 0.8	2.7 $\pm$ 0.4	2.8 $\pm$ 0.4	2.5 $\pm$ 0.4	2.4 $\pm$ 0.6
Diencephalon (ng/g)	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5	1.9 $\pm$ 0.2	1.9 $\pm$ 0.3	1.9 $\pm$ 0.3	2.0 $\pm$ 0.2	2.1 $\pm$ 0.2	1.7 $\pm$ 0.4
Interbrain (ng/g)	1.4 $\pm$ 0.4	1.3 $\pm$ 0.5	1.7 $\pm$ 0.4	1.7 $\pm$ 0.3	2.1 $\pm$ 0.5	2.1 $\pm$ 0.4	1.5 $\pm$ 0.5	1.3 $\pm$ 0.3
DHP ^c								
Serum (ng/ml)	20.4 $\pm$ 4.0	14.6 $\pm$ 5.2	10.5 $\pm$ 4.5	10.4 $\pm$ 4.4	16.7 $\pm$ 3.6	15.1 $\pm$ 5.0	14.5 $\pm$ 4.2	19.9 $\pm$ 3.4
Midbrain (ng/g)	8.0 $\pm$ 0.6	6.7 $\pm$ 1.2	6.3 $\pm$ 1.0	7.2 $\pm$ 1.1	8.3 $\pm$ 1.0	7.2 $\pm$ 1.2	5.7 $\pm$ 0.4	7.2 $\pm$ 1.2
Hippocampus (ng/g)	10.7 $\pm$ 0.7	7.7 $\pm$ 1.7	7.2 $\pm$ 1.6	9.6 $\pm$ 1.4	10.0 $\pm$ 4.0	8.0 $\pm$ 2.3	7.5 $\pm$ 0.8	6.9 $\pm$ 2.0
Cortex (ng/g)	4.6 $\pm$ 0.4	4.7 $\pm$ 0.7	3.9 $\pm$ 1.0	4.8 $\pm$ 0.8	3.5 $\pm$ 0.6	4.2 $\pm$ 0.7	4.5 $\pm$ 0.6	5.1 $\pm$ 1.0
Diencephalon (ng/g)	4.6 $\pm$ 0.3	4.3 $\pm$ 0.3	3.5 $\pm$ 0.6	4.4 $\pm$ 0.7	4.2 $\pm$ 0.6	4.7 $\pm$ 0.4	3.9 $\pm$ 0.2	4.8 $\pm$ 0.9
Interbrain (ng/g)	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1	1.1 $\pm$ 0.2	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2	1.3 $\pm$ 0.2	1.2 $\pm$ 0.2	1.0 $\pm$ 0.2
3 α ,5 α -THP								
Serum (ng/ml)	17.4 $\pm$ 7.2	12.4 $\pm$ 8.1	21.4 $\pm$ 8.8	31.6 $\pm$ 10.2	25.5 $\pm$ 9.1	27.5 $\pm$ 10.9	24.8 $\pm$ 9.0	19.1 $\pm$ 9.4
Hippocampus (ng/g)	18.4 $\pm$ 8.0	12.5 $\pm$ 5.9	13.3 $\pm$ 7.1	19.6 $\pm$ 7.8	20.0 $\pm$ 6.5	27.0 $\pm$ 9.8	20.2 $\pm$ 6.1	21.9 $\pm$ 6.8
Cortex (ng/g)	8.4 $\pm$ 1.6	6.4 $\pm$ 2.6	3.4 $\pm$ 1.5	6.0 $\pm$ 2.4	5.2 $\pm$ 1.2	5.1 $\pm$ 1.9	4.9 $\pm$ 1.7	5.3 $\pm$ 1.9
Diencephalon (ng/g)	5.9 $\pm$ 1.7	4.8 $\pm$ 1.8	2.4 $\pm$ 1.3	4.2 $\pm$ 1.7	5.8 $\pm$ 1.6	4.9 $\pm$ 1.8	4.5 $\pm$ 1.3	6.5 $\pm$ 2.1
Interbrain (ng/g)	5.5 $\pm$ 2.9	5.8 $\pm$ 2.1	4.1 $\pm$ 1.7	5.8 $\pm$ 2.3	5.8 $\pm$ 2.2	4.6 $\pm$ 2.4	4.4 $\pm$ 2.0	2.3 $\pm$ 1.6

^aEstrogen. ^bProgesterone. ^cDihydroprogesterone.

proportion of social interaction variance ($R^2=0.08$, $F(1,69)=6.01$, $P<0.05$).

Sex behavior

There was a significant interaction between 3 α ,5 α -THP inhibition and FGIN 1-27 administration on lordosis quotients ($F(3,67)=3.18$, $P<0.05$; Fig. 3, top) and lordosis ratings ($F(3,67)=3.34$, $P<0.05$; Fig. 3, bottom). These interactions were due to FGIN 1-27, but not vehicle, administration increasing lordosis quotients and ratings of rats infused with inhibitors. Midbrain levels of 3 α ,5 α -THP also significantly predicted lordosis quotient ($t(69)=3.74$, $P<0.05$) and lordosis rating ($t(69)=4.05$, $P<0.05$) and accounted for a significant proportion of variance in lordosis quotients ($R^2=0.17$, $F(1,69)=14.00$, $P<0.05$) and lordosis ratings ($R^2=0.19$, $F(1,69)=16.43$, $P<0.05$).

FGIN 1-27 administration also significantly interacted with inhibitor condition on the percentage of proceptive behaviors displayed per sexual contact ($F(3,67)=3.53$, $P<0.05$). FGIN 1-27 infusions following inhibitors increased proceptivity quotients among vehicle- and PK11195-infused rats, but not among the rats infused with indomethacin alone or in conjunction with PK11195 (Fig. 4, top). Vehicle infusions following inhibitors did not

alter proceptivity and proceptivity could not be predicted by midbrain 3 α ,5 α -THP levels.

Percentage of aggression in response to sexual contacts appeared to be the greatest among indomethacin-infused rats; however, this was not significant (Fig. 4, bottom). Percentage of exits from the male to the neutral compartment of the paced mating chamber also appeared to decrease among females infused with indomethacin, with or without PK11195, but this was not significant (Table 3). Neither aggression quotients nor percentage of exits was predicted by midbrain 3 α ,5 α -THP levels.

Discussion

The results of the present study supported our hypothesis that manipulating neurosteroidogenesis of 3 α ,5 α -THP in the midbrain VTA would alter exploratory, anti-anxiety, and reproductive behavior of female rats. First, administration of PK11195, either alone or in conjunction with indomethacin, decreased central square entries in the open field (albeit this did not reach statistical significance), decreased time spent on the open arms of the plus maze, and decreased lordosis quotients and ratings concomitant with decreased levels of 3 α ,5 α -THP in the midbrain compared with vehicle administration. Second, FGIN 1-27 administration increased time spent on the open arms of

Figure 1 Midbrain (top) and plasma (bottom) $3\alpha,5\alpha$ -THP levels (mean $\pm$ S.E.M.) among rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27.

^Significant decrease among inhibitor-infused rats compared with rats infused only with vehicle ($P<0.05$). *Significant enhancement following FGIN 1-27 compared with infusion of inhibitor followed by vehicle infusions ($P<0.05$).

the elevated plus maze, time spent in social interaction, and enhanced lordosis as well as increasing levels of $3\alpha,5\alpha$ -THP in the midbrain VTA. Together, these data suggest that neurosteroidogenesis of $3\alpha,5\alpha$ -THP in the midbrain VTA is an important factor for modulating lordosis and other reproductively relevant behaviors.

The present findings confirm previous reports that manipulating neurosteroidogenesis can alter female sexual behavior. In support, blocking P450 side-chain cleavage decreased proceptive behaviors of ovariectomized/adrenalectomized E_2 -primed rats (Micevych *et al.* 2007). Furthermore, administration of PK11195 or FGIN 1-27 to the midbrain VTA respectively decreases and

Table 2 Motor behavior in open field and elevated plus maze tasks of female rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27 (mean $\pm$ S.E.M.).

	Vehicle/ vehicle	PK11195/ vehicle	Vehicle/ indomethacin	PK11195/ indomethacin
Total entries in open field				
Vehicle	382 $\pm$ 31	271 $\pm$ 42	329 $\pm$ 41	255 $\pm$ 51
FGIN 1-27	357 $\pm$ 27	344 $\pm$ 20	329 $\pm$ 27	369 $\pm$ 52
Peripheral entries in open field				
Vehicle	265 $\pm$ 23	199 $\pm$ 31	253 $\pm$ 23	195 $\pm$ 27
FGIN 1-27	246 $\pm$ 13	241 $\pm$ 11	261 $\pm$ 28	272 $\pm$ 33
Central entries in open field*				
Vehicle	117 $\pm$ 15	72 $\pm$ 14	76 $\pm$ 22*	61 $\pm$ 16*
FGIN 1-27	111 $\pm$ 12	102 $\pm$ 13	68 $\pm$ 10	97 $\pm$ 22
Closed arm time in elevated plus maze†				
Vehicle	111 $\pm$ 13	222 $\pm$ 18	226 $\pm$ 16†	227 $\pm$ 11†
FGIN 1-27	149 $\pm$ 26	121 $\pm$ 15	178 $\pm$ 19	165 $\pm$ 23
Total arm entries in elevated plus maze				
Vehicle	20 $\pm$ 3	13 $\pm$ 3	12 $\pm$ 3	12 $\pm$ 1
FGIN 1-27	17 $\pm$ 1	16 $\pm$ 2	12 $\pm$ 2	14 $\pm$ 2
Open arm entries in elevated plus maze*				
Vehicle	10 $\pm$ 2	4 $\pm$ 1*	5 $\pm$ 1*	4 $\pm$ 1*
FGIN 1-27	8 $\pm$ 1	6 $\pm$ 1	5 $\pm$ 1	6 $\pm$ 1
Closed arm entries in elevated plus maze				
Vehicle	10 $\pm$ 2	8 $\pm$ 2	8 $\pm$ 1	8 $\pm$ 1
FGIN 1-27	9 $\pm$ 1	10 $\pm$ 1	8 $\pm$ 1	8 $\pm$ 1

*Significant main effect for inhibitor-infused groups to make fewer entries than vehicle/vehicle-infused groups ($P<0.05$). †Significant interaction for FGIN 1-27 infusions to decrease closed arm time among inhibitor-infused, but not vehicle/vehicle-infused, rats ($P<0.05$).

increases lordosis of rats and hamsters concomitant with changes in the midbrain VTA levels of $3\alpha,5\alpha$ -THP (Frye & Petralia 2003a, 2003b). Similarly, the present study demonstrates that decreasing $3\alpha,5\alpha$ -THP levels in the midbrain VTA with the administration of PK11195 results in decreased expression and quality of lordosis. We have extended these prior data to show that administration of FGIN 1-27 can overcome the effects of PK11195 on $3\alpha,5\alpha$ -THP concentrations and lordosis. Administration of FGIN 1-27 attenuated deficits in lordosis induced by PK11195, such that lordosis was comparable with that of vehicle-administered rats. Thus, neurosteroidogenesis of $3\alpha,5\alpha$ -THP appears to play a critical role in mediating sexual behavior of female rats.

The present results also extend previous reports to suggest that neurosteroidogenesis of $3\alpha,5\alpha$ -THP in the midbrain VTA can modulate other reproductively relevant behaviors. Prior reports have shown that increasing neurosteroidogenesis in the hippocampus with FGIN 1-27 enhances anti-anxiety behavior in the elevated plus maze (Bitran *et al.* 2000). In the present experiment, PK11195 decreased midbrain levels of $3\alpha,5\alpha$ -THP and decreased exploratory and anti-anxiety behaviors while increasing $3\alpha,5\alpha$ -THP levels in the midbrain with FGIN 1-27 attenuated the effects of PK11195 on anti-anxiety behavior. Interestingly, social behavior was seemingly unaffected by decreasing activation of MBRs, but was enhanced following stimulation of MBRs. These data suggest that neurosteroidogenesis in the midbrain VTA

Figure 2 Time (s) spent on the open arms of an elevated plus maze (top, mean + S.E.M.) and time (s) spent interacting with a conspecific (bottom, mean + S.E.M.) among rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27. ^Significant decrease among inhibitor-infused rats compared with rats infused only with vehicle ($P < 0.05$). *Significant enhancement following FGIN 1-27 compared with infusion of inhibitor followed by vehicle infusions ($P < 0.05$).

may play an important role in modulating behaviors beyond lordosis, and that there may be different effects of manipulating neurosteroidogenesis depending upon behaviors examined.

The results of the present study are intriguing as they suggest that rapid formation of 3 α ,5 α -THP in the midbrain VTA may be critically important for mediating consummatory reproductive behaviors, as well as appetitive reproductive behaviors important for successful initiation of mating (exploration, anti-anxiety, social interaction). Of note, the effects of manipulations of neurosteroidogenesis in the midbrain VTA were not consistent across all behaviors examined, suggesting that other neuroendocrine factors may also play an important role. This notion is further supported by evidence from the regression analyses that revealed that 3 α ,5 α -THP midbrain levels explained between 8 and 19% of the variance in most behaviors studied. 3 α ,5 α -THP accounted for most variance in sexual

Figure 3 Percentage (top) and intensity (bottom) of lordosis (mean + S.E.M.) among sexually contacted rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27. ^Significant decrease among inhibitor-infused rats compared with rats infused only with vehicle ($P < 0.05$). *Significant enhancement following FGIN 1-27 compared with infusion of inhibitor followed by vehicle infusions ($P < 0.05$).

behavior as opposed to appetitive behaviors examined, which may be, in part, due to the fact that all subjects in the present study engaged in paced mating prior to tissue collection, which enhances 3 α ,5 α -THP concentrations in the midbrain (Frye & Rhodes 2006b). Enhancement of 3 α ,5 α -THP via FGIN 1-27 appeared to increase exploratory, anti-anxiety, social, and sexual behavior overall, but inhibition of 3 α ,5 α -THP did not necessarily attenuate these behaviors to the same degree. Exploration in the open field and social interaction were modestly decreased by inhibitors but social interaction was significantly enhanced by FGIN 1-27 infusion, whereas only after PK11195 did FGIN 1-27 reinstate exploratory behavior in the open field. These results of inhibitors on behavior further suggest that FGIN 1-27 most readily reinstated neurosteroidogenesis-related decrement via PK11195 compared with infusions that included indomethacin. This was most saliently observed on the measure of proceptivity wherein there

Figure 4 Percentage of proceptive (top; hopping, darting, ear wiggling) and aggressive behaviors (bottom; vocalizing, threatening, attacking) in response to sexual contact (mean $\pm$ S.E.M.) among rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27. *Significant enhancement following FGIN 1-27 compared with infusion of inhibitor followed by vehicle infusions ($P < 0.05$).

was a stair-step effect for FGIN 1-27 to enhance proceptivity greatly among the rats receiving only vehicle infusions, slightly less among the rats infused only with PK11195, and not at all among indomethacin-infused rats. Together, these findings suggest that exploratory, anti-anxiety, and sexual behavior may be particularly sensitive to the effects of neurosteroidogenesis.

In the present study, we examined the effects of manipulating neurosteroidogenesis in the midbrain VTA. While $3\alpha,5\alpha$ -THP was found to be enhanced in this investigation, recent findings suggest that progesterone can also have actions at novel membrane-bound receptors (Dohi *et al.* 2008). That attenuation of $3\alpha,5\alpha$ -THP formation in the present study reduced appetitive and consummatory sexual behaviors, yet $3\alpha,5\alpha$ -THP content accounted for, at most, 19% of variance in behavior, may suggest that $3\alpha,5\alpha$ -THP plays a critical role underlying the maintenance of other novel

Table 3 Percentage of pacing behavior displayed in a paced mating task by female rats infused with vehicle, PK11195, and/or indomethacin with or without subsequent infusions of vehicle or FGIN 1-27 (mean $\pm$ S.E.M.).

	Vehicle/ vehicle (%)	PK11195/ vehicle (%)	Vehicle/ indomethacin (%)	PK11195/ indomethacin (%)
Percentage of pacing in paced mating				
Vehicle	9 $\pm$ 6	10 $\pm$ 5	4 $\pm$ 2	6 $\pm$ 3
FGIN 1-27	8 $\pm$ 3	9 $\pm$ 5	5 $\pm$ 3	1 $\pm$ 1

neuroendocrine substrates that are important for these behaviors. Distribution and functional effects of novel substrates and targets of action are not yet clear but pose intriguing new mechanisms for future study. Given the effects on various aspects of appetitive behavior that were revealed, future investigations will focus on the role of neurosteroidogenesis in other CNS sites known to be important for mediating exploratory, anti-anxiety, social, and sexual behaviors, as well as novel progesterin targets.

Materials and Methods

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

Animals

Adult, intact, Long-Evans female rats ($n=71$) were bred in the Life Sciences Laboratory Animal Care Facilities at The University at Albany from the stock obtained from Charles River (Germantown, NY, USA). Rats were group housed in polycarbonate cages (45 $\times$ 24 $\times$ 21 cm) in a temperature-controlled room ($21 \pm 1^\circ\text{C}$) in the Laboratory Animal Care Facility. The rats were maintained on a 12:12 h reversed light cycle (lights off 0800 h) with continuous free access to Purina Rat Chow and tap water in their home cages.

Surgery

Rats were stereotactically implanted with bilateral guide cannulae aimed at the medial aspect of the VTA (from bregma: AP = -5.3 , ML = ± 0.4 , DV = -7.0) under xylazine (12 mg/kg) and ketamine (70 mg/kg) anesthesia. Guide cannulae consisted of modified 23 gauge thin wall stainless steel needles with 30 gauge removable inserts. Following surgery, animals were monitored for loss of weight, righting response, flank stimulation response, and/or muscle tone (Marshall & Teitelbaum 1974). No rats failed these assessments.

Hormonal milieu

Endogenous

Vaginal epithelium was examined daily (between 0700 h and 0800 h) to determine the phase of the estrous cycle, as per previous methods (Long & Evans 1922, Frye *et al.* 2000). Rats

were cycled through two normal estrous cycles (4- to 5-day cycle) prior to testing. The rats were tested on the evening of proestrus, when E₂ levels are declining, but progesterin levels are high relative to other phases of the estrous cycle (Feder 1984, Frye & Bayon 1999).

Exogenous

Rats received bilateral infusions of either indomethacin (10 μ g/ μ l, 10-min incubation), PK11195 (400 ng/ μ l, 20-min incubation), or an equal volume of β -cyclodextrin vehicle to the VTA. We have found that this dose of indomethacin is sufficient to attenuate lordosis when infused to VTA of naturally receptive or hormone-primed rats or hamsters (Frye & Vongher 2001). Dosing of PK11195 was based on the past findings that identified 400 ng as the minimum dose needed to reliably attenuate lordosis among sexually receptive female rats in a dose-response regimen (Bitran *et al.* 2000, Frye & Petralia 2003a, 2003b). Following the first infusion, the rats that received PK11195 next received infusions of indomethacin while rats in other groups received a second infusion of β -cyclodextrin vehicle. Ten minutes later, the rats received infusions of FGIN 1-27 (5 μ g/ μ l) or an equal volume of saline vehicle. This dosage of FGIN 1-27 is sufficient to enhance lordosis of estrogen- and progesterone-primed ovariectomized rats to the levels that are commensurate with naturally, sexually receptive rats, compared with lower or higher doses (Bitran *et al.* 2000, Frye & Petralia 2003a, 2003b). These manipulations yielded the following eight groups: vehicle/vehicle/vehicle ($n=10$), vehicle/vehicle/FGIN 1-27 ($n=8$), PK11195/vehicle/vehicle ($n=9$), PK11195/vehicle/FGIN 1-27 ($n=9$), vehicle/indomethacin/vehicle ($n=8$), vehicle/indomethacin/FGIN 1-27 ($n=9$), PK11195/indomethacin/vehicle ($n=10$), and PK11195/indomethacin/FGIN 1-27 ($n=8$). Central infusions were administered at a rate of 1 μ l/min through a 30 gauge needle attached to PE-20 tubing and a 5 μ l Hamilton syringe. The infusion needle was left in place for 60 s following infusions to reduce possible displacement of infusate. The rats were tested in the behavioral battery described below.

Behavioral testing

All rats were tested individually through the following test battery of exploratory, anxiety, social, and sexual behavior in the order described below. All testing occurred in a single room with testing apparatus brightly lit from above with three fluorescent bulbs (32 W each). The rats were tested sequentially through tasks, with no breaks between individual tasks, except the time required to clean the apparatus and transition rats from one task to another. As such, assessment in the entire battery took about 45–50 min for each rat. We have utilized these behavioral measures in the past as individual tasks (Frye *et al.* 2007), small batteries of anxiety, social, or sexual measures only (Frye *et al.* 2007), or as a single battery of testing (Frye & Rhodes 2006b). We find that behavioral and neuroendocrine status is not significantly affected by exposure to any task other than mating, which is the last task in the battery described below (Frye *et al.* 2007).

Behavioral data were collected with the ANY-Maze data collection program (Stoelting Co., Wheat Dale, IL, USA), and by an observer blind to the condition of experimental rats and the hypothesized outcome of the study. There was a 97% concordance rate between data that were collected by ANY-Maze and that collected by the uninformed observer. As such, the data collected via ANY-Maze were utilized in behavioral analyses.

Open field

Behavior in the open field is used as a measure of exploration, anxiety, and locomotor behavior (Blizard *et al.* 1975, Frye *et al.* 2000). The open field (76 $\times$ 57 $\times$ 35 cm) has a 48-square grid floor (6 $\times$ 8 squares, 9.5 cm/side): there is an overhead light illuminating the central squares (all but the 24 perimeter squares were considered central). As per previous methods, rats were placed in the open field and the path of their exploration was recorded for 5 min. The number of squares entered by rats in the center or periphery of the grid was calculated and these data were added together to yield the total number of squares entered. Prior reports indicate that total square entries in this task are robustly modulated by hormonal status of female rats and by steroid sensitive manipulations (Birke & Archer 1975, Walf & Frye 2007, Frye & Rhodes 2008). Other motor measures, such as rearing, may not be so sensitive to manipulations that alter hormonal milieu (Avitsur *et al.* 1995, Renard *et al.* 2007, Liang *et al.* 2008). Since the present study utilized a sample of female rats that were all matched on the phase of estrous cycle, motor differences were expected to be minimized. Thus, central square entries were utilized as an index of anti-anxiety, and total square entries as an index of thigmotaxis and motor behavior.

Elevated plus maze

Behavior in the elevated plus maze is also utilized to assess exploration, anxiety, and motor behavior (File 1990, Frye *et al.* 2000). The elevated plus maze consists of four arms, 49 cm long and 10 cm wide, elevated 50 cm off the ground. Two arms were enclosed by walls 30 cm high and the other two arms were exposed. As per previous methods, rats were placed at the juncture of the open and closed arms and the number of entries into, and the amount of time spent on, the open and closed arms was recorded during a 5-minute test. Time spent on the open arms was used as an index of anxiety and the total number of arm entries was used as a measure of motor activity since it has been found to positively correlate with other measures of motor activity including rearing behavior (Cruz *et al.* 1994, Rodgers & Johnson 1995).

Social interaction

The social interaction task was used to assess exploratory and anxiety behavior associated with interacting with a novel conspecific (File 1980, Frye *et al.* 2000). Each member of a pair of rats (one experimental, one stimulus) was placed in the opposite corners of an open field (76 $\times$ 57 $\times$ 35 cm). The total duration of time that experimental rats engaged an ovariectomized stimulus rat in crawling over and under partner, sniffing of partner, following with contact, genital investigation of partner, tumbling, boxing, and grooming was recorded during a 5-minute test (Frye *et al.* 2000). An ovariectomized rat was utilized as the stimulus

animal in order to avoid the exposure of experimental rats to vaginocervical stimulation, which might occur if a male had been used as the stimulus animal. The duration of time spent interacting with a conspecific is an index of anxiety behavior.

Paced mating

Paced mating was utilized over standard mating because of its greater ethological relevance and procedures were carried out as reported previously (McClintock & Adler 1978, Erskine 1985, Frye & Erskine 1990, Gans & Erskine 2003). Paced mating tests were conducted in a chamber (37.5×75×30 cm), which was equally divided by a partition that had a small (5 cm in diameter) hole in the bottom center, to allow a female free access to both sides of the chamber, but which prevented the larger stimulus male from moving between sides. Females were placed in the side of the chamber opposite the stimulus male. Rats were behaviorally tested for an entire ejaculatory series. Behaviors recorded were the frequency of mounts and intromissions that preceded an ejaculation. As well, the frequency (lordosis quotient = incidence of lordosis/number of mounts) and intensity (lordosis rating) of lordosis, quantified by rating of dorsiflexion on a scale of 0–3 (Hardy & DeBold 1972) was recorded. The percentage of proceptive behaviors (i.e. hopping, darting, ear wiggling) that occurred prior to sexual contacts was recorded for report as a proceptivity quotient. The percentage of aggressive behaviors (i.e. vocalizations, kicks, attacks) females displayed prior to sexual contacts was also recorded and reported as an aggression quotient. Pacing measures included the percentage of times the female left the compartment containing the male after receiving a particular copulatory stimuli (% exits after mounts, intromissions, and ejaculations) and latencies in seconds to return to the male compartment after these stimuli. The normal pattern of pacing behaviors for the percentage of exits and return latencies to be longer after more intensive stimulation (ejaculations > intromissions > mounts) was observed in the present study.

Tissue collection

Immediately following testing, whole brains and trunk blood were collected for later measurement of corticosterone, E₂, P₄, DHP, and 3 α ,5 α -THP. Trunk blood was centrifuged at 3000 *g* for 10 min, and serum was stored in 1.5 ml aliquot tubes at –80 °C. The brains were rapidly frozen on dry ice and stored at –80 °C prior to RIA.

Tissue preparation

Serum was thawed on ice and steroids extracted as described below. Because all brains were used for RIA, histological analyses were not possible. The brains were thawed and, during dissection, were visually examined for cannulae placement, which was commensurate with past placement confirmed by histology. In the past, we have administered 3 α ,5 α -THP to VTA or nearby sites, including substantia nigra or central gray (Frye & Rhodes 2006a, 2006b, 2008), and found that only administration to the VTA modulates exploratory, anxiety, and socio-sexual behavior. Following this, midbrain, hippocampus, striatum, cortex, and a control region (interbrain) were dissected and steroids were extracted as described below.

RIA for steroid hormones

E₂, P₄, DHP, 3 α ,5 α -THP, and corticosterone concentrations were measured as described below, using previously reported methods (Frye *et al.* 1996, Choi & Dallman 1999, Frye & Bayon 1999).

Radioactive probes

³H E₂ (NET-317, 51.3 Ci/mmol), P₄ (NET-208, specific activity = 47.5 Ci/mmol), 3 α ,5 α -THP (used for DHP and 3 α ,5 α -THP; NET-1047, specific activity = 65.0 Ci/mmol), and corticosterone (NET 182, specific activity = 48.2 Ci/mmol) were purchased from Perkin–Elmer (Boston, MA, USA).

Extraction of steroids from serum

E₂, P₄, DHP, and 3 α ,5 α -THP were extracted from serum with ether following incubation with water and 800 c.p.m. of ³H steroid (Frye & Bayon 1999). Corticosterone was extracted from serum by heating at 60 °C for 30 min (Choi & Dallman 1999). After snap-freezing twice, test tubes containing steroid and ether were evaporated to dryness in a Savant vacuum speed drier. Dried-down tubes were reconstituted with phosphate assay buffer to the original serum volume. Between 90 and 95% of steroid was recovered from plasma.

Extraction of steroids from brain tissues

E₂, P₄, DHP, and 3 α ,5 α -THP were extracted from the brain tissues following homogenization with a glass/glass homogenizer in 50% MeOH and 1% acetic acid. Tissues were centrifuged at 3000 *g* and the supernatant was chromatographed on Sepak cartridges equilibrated with 50% MeOH:1% acetic acid. Steroids were eluted with increasing concentrations of MeOH (50% MeOH followed by 100% MeOH). Solvents were removed using a speed drier. Samples were reconstituted in 300 μ l assay buffer. Between 86 and 94% of steroid was recovered from brain tissue.

Set-up and incubation of RIAs

The range of the standard curves was 0–1000 pg for E₂, 0–8000 pg for P₄, DHP, and 3 α ,5 α -THP, and 0–4 ng for corticosterone. Standards were added to assay buffer followed by the addition of the appropriate antibody (described below) and ³H steroid. Total assay volumes were 800 μ l for E₂ and P₄, 950 μ l for DHP, 1250 μ l for 3 α ,5 α -THP, and 900 μ l for corticosterone. All assays were incubated overnight at 4 °C, except for E₂ and corticosterone, which incubated at room temperature for 50 and 60 min respectively.

Antibodies

The E₂ antibody (E#244, Dr G D Niswender, Colorado State University, Fort Collins, CO, USA) was used in a 1:40 000 dilution, which generally binds between 40% and 60% of [³H]E₂ (Frye & Bayon 1999), and bound 48% in the present study. This E₂ antibody has negligible (<1%) cross-reactivity with other steroid hormones, including estrone, 17 α -estradiol, P₄, and 17-hydroxyprogesterone. The P₄ antibody (P#337 from Dr G D Niswender, Colorado State University), used in a 1:30 000 dilution, typically binds between 30% and 50% of [³H]P₄ (Frye & Bayon 1999), and bound 43% in the present study. The P₄

antibody has very low levels (<4%) of cross-reactivity with DHP and 3 α ,5 α -THP (Niswender 1973). The DHP (X-947) and 3 α ,5 α -THP antibodies (#921412-5, purchased from Dr Robert Purdy, Veterans Medical Affairs, La Jolla, CA, USA) were used in a 1:5000 dilution, typically bind between 40 and 60% of [³H]3 α ,5 α -THP (Frye & Bayon 1999), and bound 51% in the present study. The DHP antibody cross-reacts with 3 α ,5 α -THP (100%), 5 α -pregnan-3,20-dione (50%), 4-pregnen-3 α -ol-20-one (50%), and P₄ (17%; Purdy *et al.* 1990). The 3 α ,5 α -THP antibody cross-reacts with 3 α -hydroxypregn-4en-20-one (84%) and DHP (11%), and its β -isomer (7%), P₄ (6%), and pregnenolone (<2%; Purdy *et al.* 1990, Finn & Gee 1994). The corticosterone antibody (#B3-163, Endocrine Sciences, Tarzana, CA, USA), which typically binds 40–60% of [³H]corticosterone, was used in a 1:20 000 dilution and bound 45% in the present study.

Termination of binding

Separation of bound and free steroid was accomplished by the rapid addition of dextran-coated charcoal. Following incubation with charcoal, samples were centrifuged at 3000 g and the supernatant was decanted into a glass scintillation vial with 5 ml scintillation cocktail. Sample tube concentrations were calculated using the logit-log method of Rodbard & Hutt (1974), interpolation of the standards, and correction for recovery with 'AssayZap' interpolation software published by Biosoft (1994). The inter- and intra-assay reliability coefficients were: E₂, 0.09 and 0.10; P₄, 0.12 and 0.13; DHP, 0.12 and 0.14; 3 α ,5 α -THP, 0.13 and 0.15; and corticosterone, 0.04 and 0.07.

Statistical analyses

Each subject was tested once through the behavioral battery described yielding a factorial, between-group experimental design. Two independent variable conditions were examined: (1) 3 α ,5 α -THP inhibitor condition – which had four levels (vehicle, PK11195, indomethacin, or PK11195+indomethacin) and (2) FGIN 1-27 condition – which had two levels (vehicle or FGIN 1-27). Two-way analyses of variance (ANOVA) were utilized to examine the effects of 3 α ,5 α -THP inhibition and/or enhancement of 3 α ,5 α -THP formation on endocrine and behavioral endpoints. Since engaging in paced mating is also expected to enhance biosynthesis of 3 α ,5 α -THP (Frye & Rhodes 2006a, Frye *et al.* 2007), simple regression analyses were also utilized to determine the extent to which brain concentrations of 3 α ,5 α -THP may account for the aspects of behavioral responses (which occurred prior to sexual contact). α -level for statistical significance was $P < 0.05$. ANOVAs were followed by Fisher's protected least significant difference *post hoc* tests to ascertain group differences.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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