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Activity of Protein Kinase C is Important for 3 α ,5 α -THP's Actions at Dopamine Type 1-like and/or GABA_A receptors in the Ventral Tegmental Area for Lordosis of Rats

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Abstract

In the ventral tegmental area, progestogens facilitate sexual receptivity of rodents via actions at dopamine type 1-like and/or γ -aminobutyric type A receptors and activation of downstream signal transduction molecules. In the present study, we investigated whether effects of progesterone's metabolite, 3 α ,5 α -THP, to enhance lordosis via actions at these receptors in the ventral tegmental area requires phospholipase C-dependent protein kinase C. The objective of this study was to test the hypothesis that: if progestogens' actions through dopamine type 1-like and/or γ -aminobutyric type A receptors in the ventral tegmental area for lordosis require protein kinase C, then inhibiting protein kinase C in the ventral tegmental area should reduce 3 α ,5 α -THP-facilitated lordosis and its enhancement by dopamine type 1-like or γ -aminobutyric type A receptor agonists. Ovariectomized, E₂ (10 μ g s.c. at hr 0)-primed rats were tested for their baseline lordosis responses and then received a series of three infusions to the ventral tegmental area: first, bisindolylmaleimide (75 nM/side) or vehicle; second, SKF38393 (100 ng/side), muscimol (100 ng/side), or vehicle; third, 3 α ,5 α -THP (100, 200 ng) or vehicle. Rats were pre-tested for lordosis and motor behavior and then tested for lordosis after each infusion and 10 and 60 mins after the last infusion. Rats were tested for motor behavior following their last lordosis test. As has been previously demonstrated, 3 α ,5 α -THP infusions to the ventral tegmental area increased lordosis and effects were further enhanced by infusions of SKF38393 and muscimol. Infusions of bisindolylmaleimide to the ventral tegmental area attenuated 3 α ,5 α -THP-, SKF38393-, and/or muscimol-facilitated lordosis. Effects on lordosis were not solely due to changes in general motor behavior. Thus, 3 α ,5 α -THP's actions in the ventral tegmental area through membrane receptors may require activity of protein kinase C.

1. Introduction

Progesterone (P) has "genomic" actions via progestin receptors and "non-genomic" actions on neuronal membranes to mediate the onset and duration of reproductive behavior of estradiol (E₂)-primed rodents through actions in the ventromedial hypothalamus (VMH) and midbrain

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ventral tegmental area (VTA) [27]. In the VMH, P binds to cognate intracellular progesterin receptors to initiate lordosis, the female-typical mating posture of rodents [81]. In the midbrain VTA, P has rapid, membrane-mediated effects to modulate the duration and intensity of sexual receptivity [16], [17], and [18]. Progesterone facilitates lordosis through its actions in the VTA even when the few intracellular progesterin receptors in the VTA are blocked [20] and [29]. As P has its actions independent of cognate progesterin receptors in this region, the mechanisms by which progestogens in the VTA influence lordosis are of interest. Given that progestogens' role in the VTA to mediate lordosis are well-understood [9], a research approach we have used successfully to reveal functionally-relevant membrane actions of P in the midbrain VTA involves manipulating progestogens' actions in this region and examining subsequent effects on lordosis, as a behavioral bioassay [13].

A critical step in the actions of P in the VTA to mediate the quality and duration of lordosis behavior of rodents is formation of 5α -pregnan- 3α -ol-20-one ($3\alpha,5\alpha$ -THP). In the VTA, P is readily converted by actions of the 5α -reductase enzyme to dihydroprogesterone, which is subsequently metabolized by the 3α -hydroxysteroid dehydrogenase enzyme to form $3\alpha,5\alpha$ -THP [14]. Inhibiting or enhancing the actions of these metabolism enzymes in the VTA, respectively, reduces [30] and enhances [25] lordosis. Moreover, $3\alpha,5\alpha$ -THP is a neurosteroid that is produced in the brain from biosynthesis that occurs independent of peripheral glands [8]. Mating induces $3\alpha,5\alpha$ -THP biosynthesis in the midbrain VTA [21]. Preventing and or amplifying biosynthesis of $3\alpha,5\alpha$ -THP in the VTA, respectively, attenuates and increases lordosis [22], [23], and [24]. Given the essential role of $3\alpha,5\alpha$ -THP in the midbrain VTA in mediating lordosis, the mechanisms for these effects is of great interest.

$3\alpha,5\alpha$ -THP, like other neurosteroids, can exert rapid, nongenomic actions [42] and [51]. In physiological concentrations, $3\alpha,5\alpha$ -THP is devoid of affinity for intracellular progesterin receptors [71]. Specific targets for neurosteroids in plasma membranes have been postulated, but not elucidated. It is established that $3\alpha,5\alpha$ -THP can have actions through a number of identified neurotransmitter substrates including receptors for GABA_A [52], glutamate [63] and [83], nicotine [5], dopamine [34] and [60], norepinephrine [3] and [48], opiates [72] and [82] and/or sigma [57]. The diversity of substrates through which $3\alpha,5\alpha$ -THP can have its actions suggest that there may be common factors downstream of neurotransmitter receptors that are important for $3\alpha,5\alpha$ -THP's mechanism.

A common action of $3\alpha,5\alpha$ -THP that could be initiated at various neurotransmitter receptors may involve second messenger pathways. A well-established nongenomic action of $3\alpha,5\alpha$ -THP is its allosteric modulation of γ -aminobutyric Type A (GABA_A) receptors [49] and [58]. Infusions of GABA_A receptor agonists to the VTA enhance progesterogen-facilitated lordosis [16], whereas, infusions of GABA_A receptor antagonists to the VTA inhibit progesterogen-facilitated lordosis [19] and [29]. Although it is well-known that GABA_A receptors are coupled to chloride channels, evidence is emerging that some of their actions may be mediated by second messengers [12]. D₁ and D₂ receptors are coupled to the adenylyl cyclase pathway with D₁ mediating the stimulation and, D₂ the inhibition, of adenylyl cyclase. Notably, infusions of D₁ and D₂ receptor agonists to the VTA facilitate and inhibit, respectively, progesterogen-facilitated lordosis [38] and [78]. Manipulations to the VTA to inhibit G-proteins, adenylyl cyclase and/or 3'-5'-cyclic adenosine monophosphate (cAMP), can attenuate the enhancing effects of GABA_A or D₁ receptor agonists on progesterogen-facilitated lordosis [32], [33], [34], [35], [36], [65], and [66]. Moreover, D₂ receptors are also coupled negatively to the phosphatidylinositol (PI) signal transduction pathway and $3\alpha,5\alpha$ -THP may have actions via this pathway [38].

Hormones and neurotransmitters (as well as growth factors) can activate the PI signal transduction pathway through receptor-triggered hydrolysis of phosphoinositides by

phospholipase C (PLC). The activation of G-proteins coupled to PLC is an early event in the PI signal transduction pathway leading to increases in free calcium in the cytosol and inositol 1,4,5, triphosphate (InsP(3)) formation. A number of distinct PLC isozymes have been identified in the midbrain of the neonatal and adult rat [74]. Members of the PLC family catalyze the hydrolysis of PI to activate several secondary mediators such as intracellular calcium and protein kinase C (PKC). Progesterone induces total PLC activity in the uterus and facilitates oxytocin-induced mobilization of the PI pathway [71] and [73]. Progesterone can increase PKC activity in an amphibian oocyte preparation [44] and [45]. This second messenger pathway may also mediate some of the *in vivo* effects of progestogens. Administration of kinase blockers attenuates progestogen-facilitated lordosis [39] and [53]. Activation of PKC enhances lordosis of ovariectomized rats [46] and [59]. Infusions of PLC or PKC inhibitors to the VTA of ovariectomized hamsters subcutaneously administered E₂ and P-reduces lordosis and its facilitation by D₁ and GABA_A agonists [66] and [67]. The objective of the present study was to investigate whether PKC activity in the VTA is essential for 3 α ,5 α -THP-facilitated lordosis and its modulation by D₁ and/or GABA_A receptor activity. In this study, we tested the hypothesis that: if PKC is essential for progestogens' actions in the VTA for lordosis, then inhibiting PKC in the VTA will attenuate 3 α ,5 α -THP- or 3 α ,5 α -THP plus D₁- or GABA_A-mediated increases in lordosis of ovariectomized E₂-primed rats.

2. Materials and Methods

All procedures utilized in the present study were done with approval from The University at Albany Institutional Animal Care and Use Committee and in accordance with The National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.1. Animals

Rats were bred (original breeders from Taconic Farms, Germantown, NY) and raised in The University at Albany- Laboratory Animal Care Facility in the Social Sciences Building. Experimental subject were adult (approximately 55 days of age, 200-250 grams) female Long-Evans rats (N=53). Rats were group-housed (4-5 per cage), under reversed-lighting conditions (lights off between 0800 and 20:00 hrs), with *ad libitum* access to rodent chow and water in their home cages.

2.2. Surgery

Rats were ovariectomized to remove the primary peripheral source of E₂ and progestogens, and implanted with bilateral guide cannulae in one surgical session. Rats were anesthetized with xylazine (12 mg/kg; intraperitoneal (IP) Bayer Corp., Shawnee Mission, KS, USA) and ketamine hydrochloride (80 mg/kg, IP; Fort Dodge Animal Health, Fort Dodge, IA, USA). The VTA coordinates used for stereotaxic surgery in all experimental subjects were adapted from Paxinos and Watson (from bregma, AP = -5.3, ML = $\pm$ 0.4, DV = -7.0[64]). Cannulae were modified 23-gauge thin-walled stainless steel needles with 30-gauge removable inserts aimed at the VTA.

Rats had a one-week recovery period following surgery before weekly (for three weeks) E₂-priming and behavioral testing commenced. Before inclusion in the study and during the weekly test sessions, recovery of rodents from surgery and evaluation of effects of drug infusions (i.e. animal becoming sick) were assessed. Rodents were evaluated for their ability to cage climb, right themselves, and respond to external stimuli, groom themselves, and gain weight [54]. In the present study, two rats did not meet these criteria for continued inclusion in the study.

2.3. Hormone-administration

Ovariectomized rats were hormone-primed with 17β -E₂ (10 μ g/ 0.2 ml in corn oil vehicle; Sigma Chemical Company, St. Louis, MO, USA). E₂ was administered via subcutaneous injections to the nape of the neck.

2.4. Drugs and Infusions

Rats were infused with the PKC inhibitor (bisindolylmaleimide), D₁ agonist (SKF38393), and GABA_A agonist (muscimol). All were obtained from Sigma Chemical Company (St. Louis, MO, USA). Drugs were dissolved in saline and bilaterally infused in 1 μ l. SKF38393 and muscimol were administered at a concentration of 100 ng/ μ l, as has been previously shown to enhance progesterone-facilitated lordosis of E₂-primed hamsters and rats within 30, and up to 180, mins after administration [21], [34], [35], [36], [65], [66], and [67]. Bisindolylmaleimide, was administered at a concentration of 75 nM/ μ l. This bisindolylmaleimide regimen was based upon our pilot data and other published reports [1], [12], and [77].

Rats were administered infusions of 3 α ,5 α -THP (100 or 200 ng/ μ l in 25% β -cyclodextrin vehicle; Sigma Chemical Company, St. Louis, MO, USA) to the VTA. These regimen have been utilized to investigate progesterone's mechanisms in the VTA, which may involve D₁ and GABA_A receptors and their downstream effectors, by our laboratory [34], [35], [36], and [37].

Drugs were infused to rats under minimal hand-held restraint. Infusions were accomplished by hand by inserting a 30-gauge needle, connected to a 5 μ l Hamilton Syringe (Reno, NV, USA) with PE-20 tubing, into the guide cannulae and then administering drug (or vehicle) at a rate of 1 μ l/min. The infusion needle was left in place for 60 secs to minimize infusate displacement and the whole procedure was repeated for the other cannula.

2.5. Assessment of Reproductive Behavior

For assessment of sexual receptivity, lordosis responses of experimental rats following mounting by a sexually-experienced male conspecific were examined in a 50 $\times$ 25 $\times$ 30 cm mating chamber. The percentage of times female rats exhibit lordosis in response to mounting by a male during a 10-minute test period, or after ten mounts were made (lordosis quotients; LQs), were used to quantify sexual receptivity of female rats [29]. Lordosis responses were also rated (0= no lordosis to 3=maximum dorsiflexion) according to the scale described by Hardy and Debold [41]. Experimental rats were vaginally-masked to minimize possible mating-induced changes in sexual behavior [69] and 3 α ,5 α -THP levels in the VTA [34].

2.6. Assessment of Motor Activity

To assess general motor activity, experimental rats were placed in a 39 $\times$ 39 $\times$ 30 cm Digiscan Optical Animal Activity Monitor (Accuscan Instruments Inc., Columbus, OH, USA) for 5 mins. The number of horizontal beam breaks that were made by experimental animals were mechanically recorded and utilized as an index of general motor activity [34].

2.7. Procedure

Rats were randomly-assigned to their first (bisindolylmaleimide or vehicle) and second (SKF38393, muscimol, or vehicle) infusion condition. Rats were tested once a week for 3 weeks until they received each 3 α ,5 α -THP dosage (0, 100 or 200 ng/side) once. There were 8-9 rats in each experimental condition. Each week for three weeks, experimental rats were subcutaneously injected with 17β -E₂ at hr 0. At hour 44, rats were pre-tested for motor activity and lordosis to verify that there were no baseline differences in rats before they received infusions to the VTA. In the present study, no differences were observed in rats' pre-testing

motor behavior or lordosis. After this pre-testing, rats were bilaterally infused with bisindolylmaleimide or vehicle and then were re-tested for lordosis immediately afterward. After this second test for lordosis, rats were infused with SKF38393, muscimol, or saline vehicle and were re-tested for lordosis immediately afterward. Rats were then infused with 3 α ,5 α -THP or β -cyclodextrin saline vehicle and were tested for lordosis 10 and 60 mins later. After the last lordosis test, motor activity of rats was assessed. Rats were administered 3 α , 5 α -THP to the VTA after infusions of the inhibitors and receptor agonists to follow-up on previous studies in our laboratory [32]. These present experiment extends previous investigations in hamsters that were administered P₄ subcutaneously 4 hours before infusions of inhibitors or agonists to the VTA and behavioral testing [32]. Unlike rats which will display low levels of lordosis following E₂-priming alone that can be facilitated with progestogens infused to the hypothalamus and/or VTA, hamsters require effects of P in the hypothalamus via PRs (which typically take hours) and rapid effects of progestogens (which typically take minutes) in the VTA for lordosis to be observed. As such, it was not possible to determine the specificity (timing, active metabolite, brain region target) of progestogens' effects in hamsters and this question could be addressed more adequately in the present study in rats.

2.8. Perfusion and Histological Analyses

Experimental rats were deeply anesthetized with sodium pentobarbital (150 mg/kg or to effect; IP) and bodies were perfused with 0.9% saline, followed by 10% formalin. Brains were removed from the skull and then stored in 10 % formalin followed by 30% sucrose-saline until they were frozen and sliced on a cryostat at 40 μ m at the level of the VTA. Brain slices were stained with cresyl violet. A researcher, who was blind to the experimental condition and behavioral data of the animals, examined infusion location by light microscopy. Infusion sites located dorsal to the mammillary peduncle, ventral to the red nucleus, medial to the medial lemniscus or substantia nigra and lateral to the interfascicular nucleus were considered to be within the VTA. Although all rats were intended to have infusions aimed at the VTA, and the same coordinates were used for each rat during stereotaxic surgery, there were three rats that, as determined with histological analyses, had infusions outside of the VTA (i.e. substantia nigra). Data from these rats were excluded from statistical analyses and their data is included in Table 1.

2.9. Statistical analyses

Analysis of the effects of all infusion conditions at each dosage of 3 α ,5 α -THP, and at each lordosis test, and interactions between these factors, demonstrated that behavioral effects were observed following 3 α ,5 α -THP infusions. Rats infused with bisindolylmaleimide (33% decrease compared to pre-testing E₂-priming) or vehicle (no change) did not have significantly altered lordosis. No significant differences in lordosis were observed in E₂-primed rats that were administered SKF38393 (35% increase compared to pre-testing E₂-priming), muscimol (94% increase compared to E₂-only control), or saline vehicle (no change). Rats were then infused with 100 or 200 ng 3 α ,5 α -THP or β -cyclodextrin saline vehicle and were tested for lordosis 10 (100 ng: 193% and 200 ng: 190% increase compared to pre-testing E₂-priming) minutes and 60 (100 ng: 253% and 200 ng: 248% increase compared to pre-testing E₂-priming) minutes following infusions and a similar pattern of effects was observed at these time points. We have previously demonstrated that infusions of 3 α ,5 α -THP to ovariectomized, E₂-primed rats produce similar effects on lordosis 10-120 minutes later [37]. Furthermore, rats were tested for motor activity on two occasions, i.e. before infusions and lordosis testing when they were E₂-primed only and immediately after their last lordosis test. No differences were observed between groups in the pre-test for motor behavior, or when the differences between the pre-test and post-test were compared (data not shown). No differences were observed between groups in the pre-test for motor behavior, or when the differences between the pre-test and

post-test were compared (data not shown). As such, the lordosis data from the 60 mins post-3 α ,5 α -THP infusion are presented in the Results section.

Lordosis quotients and motor activity (i.e. number of horizontal beam breaks made), 60 mins after 3 α ,5 α -THP infusions, were analyzed with ANOVAs with two between- (PKC inhibitor vs. vehicle; muscimol, SKF38393, vs. vehicle) and one within- (3 α ,5 α -THP concentration- 0, 100, or 200 ng) subjects variables. Significant main effects (where $p < 0.05$) were further analyzed with Fisher's PLSD comparisons to determine group differences. In the case of significant main effects of between- and within-subjects variables for measures, results presented are of the interaction between these variables, as is the case for lordosis quotients.

3. Results

Forty-eight rats received bilateral VTA infusions and 3 rats unintentionally received bilateral substantia nigra infusions. As such, there were 8 rats in each experimental condition that received bilateral infusions to the VTA. Data from rats that received missed site infusions to the substantia nigra were excluded from statistical analyses. Data from these rats are included in Table 1. Although there were few observations, the pattern of effects in these subjects, and in previous reports [37], show that infusions to the substantia nigra do not produce commensurate effects as do infusions to the VTA.

Infusions of 100 ng (253% increase compared to pre-testing E₂-priming) or 200 (248% increase) ng 3 α ,5 α -THP to the VTA of ovariectomized, E₂-primed rats significantly increased lordosis quotients 60 minutes following infusions, compared to vehicle (no change). 3 α ,5 α -THP-facilitated lordosis was enhanced by co-administration of SKF38393 (100 ng 3 α ,5 α -THP: 216%, 200 ng 3 α ,5 α -THP: 357% increase compared to pre-testing E₂-priming) or muscimol (100 ng 3 α ,5 α -THP: 269%, 200 ng 3 α ,5 α -THP: 300% increase compared to pre-testing E₂-priming) to the VTA. There was a significant interaction between all variables for lordosis quotients $F(4,126) = 2.5$, $p < 0.04$. Infusions to the VTA of the PKC inhibitor, bisindolylmaleimide, but not vehicle, attenuated effects of intra-VTA 3 α ,5 α -THP (vehicle: 33%, 100 ng 3 α ,5 α -THP: 53%, 200 ng 3 α ,5 α -THP: 31% decreased compared to pre-testing E₂-priming)-, SKF38393- (vehicle: 1% increased, 100 ng 3 α ,5 α -THP: 7% decreased, 200 ng 3 α ,5 α -THP: 64% increased compared to pre-testing E₂-priming), and muscimol- (vehicle: 30% decreased, 100 ng 3 α ,5 α -THP: 53% increased, 200 ng 3 α ,5 α -THP: 21% decreased compared to pre-testing E₂-priming), to facilitate lordosis (Figure 1).

Infusions of 100 ng 3 α ,5 α -THP to the VTA increased total beam breaks made in the activity monitor, compared to vehicle or 200 ng 3 α ,5 α -THP infusions $F(2,126) = 4.1$, $p < 0.01$ (Table 2); however, there were no main effects of bisindolylmaleimide infusions, agonist infusions, or interactions between these variables for motor activity of rats.

4. Discussion

The current findings support our hypothesis that, in the VTA, D₁- and/or GABA_A receptor-mediated increases in progestogen-facilitated lordosis of E₂-primed rats involve activity of PKC. Infusions of the PKC inhibitor, bisindolylmaleimide, prevented the enhancing effects of subsequent infusions of SKF38393, muscimol, and/or 3 α ,5 α -THP on lordosis of E₂-primed rats. Notably, motor behavior data collected from rats do not suggest that LQs were solely a function of general motor behavior of rats. There was a statistically significant effect of 100, but not 200, ng 3 α ,5 α -THP to increase beam breaks made in the activity monitor. Although there were no statistically significant effects of infusions of bisindolylmaleimide to the VTA, there were apparent differences in some groups, such that those infused with bisindolylmaleimide alone made fewer, and those administered bisindolylmaleimide,

SKF38393, and 100 ng 3 α ,5 α -THP made more, beam breaks in the apparatus. This inconsistent pattern of effects in the activity monitor, but not lordosis, suggest that differences in lordosis are not likely only due to differences in general motor behavior of rats. Thus, inhibiting PKC in the VTA can prevent progesterogens' actions for lordosis as well as block D₁- or GABA_A-receptor-mediated increases in progesterogen-facilitated lordosis of rats.

Results of these experiments confirm and extend previous research that suggests, in the VTA, progesterogens' actions for lordosis involve D₁- and/or GABA_A receptors and second messenger signaling downstream of these receptors. The observations from the present experiment that infusions of the D₁ agonist, SKF38393, or the GABA_A receptor agonist, muscimol, to the VTA increase 3 α ,5 α -THP's actions for lordosis are consistent with previous reports from our laboratory that demonstrate progesterogen-facilitated lordosis is enhanced by activating D₁ or GABA_A receptors in the VTA [34]. Moreover, the findings that bisindolylmaleimide infusions to the VTA inhibited the facilitative effects of progesterogens, D₁ and/or GABA_A receptor agonists on lordosis extend our current understanding of how second messengers in the VTA are involved in progesterogens' actions for lordosis of rodents. We have previously shown that inhibiting G-proteins, adenylyl cyclase, cAMP, cAMP-dependent protein kinase, protein kinase A (PKA), in the VTA reduces P or 3 α ,5 α -THP-facilitated lordosis of E₂-primed hamsters and rats [21], [32], [34], [35], [36], [65], and [66]. Effects of peripheral or intraventricular manipulations, which would have global effects in the brain, the adenylyl cyclase pathway on progesterogen-facilitated lordosis have been previously reported [4] and [39]. Effects of intra-VTA manipulations in the present and previous studies from our laboratory support the notion that the VTA is an important brain target for these membrane-mediated effects of progesterogens, which rapidly initiate downstream signaling pathways, for lordosis.

The results of the current experiment reveal that the PI second messenger pathway may also be important in mediating progesterogen-facilitated lordosis. Here we found that blocking PKC in the VTA produced specific effects to inhibit progesterogen-, D₁- and GABA_A receptor-facilitated lordosis. These findings build upon prior results from other laboratories which demonstrate that the PI pathway may be involved in lordosis. Inhibiting PLC, which is upstream of PKC, in the VTA attenuates progesterin-, D₁ and GABA_A receptor mediated lordosis [32]. Systemic administration of phorbol esters, which can activate PKC, facilitate lordosis of E₂-primed rats [46] and [52]. The present findings are congruent with the recent proposition that P and 3 α ,5 α -THP may use different signaling mechanisms for facilitating lordosis [34]. According to this, P may utilize the PKA cascade, while 3 α ,5 α -THP acts through the PKC system and dihydroprogesterone may be capable of actions through both pathways [34]. Our findings are congruous with the latter hypothesis. We have found that lordosis facilitated by systemic P, infusions of 3 α ,5 α -THP, D₁ and/or GABA_A agonists to the VTA are attenuated when inhibitors of either the adenylyl cyclase or the PI pathways are applied to the VTA. Given that in the VTA, 3 α ,5 α -THP is robustly secreted and labile, such that it readily back converts to dihydroprogesterone [40], this may explain why we have observed that infusions to the VTA of inhibitors of either the adenylyl cyclase and/or the PI pathway can produce similar decrements in lordosis. Moreover, signal transduction systems, such as adenylyl cyclase and PI, are richly complex with a variety of isoforms for each target, resulting in diverse and flexible systems, a key characteristic necessary for behavioral modulation [47]. From the present data, the interaction of these pathways for 3 α ,5 α -THP actions in the VTA cannot be elucidated. Indeed, we find similar attenuation of 3 α -5 α -THP-, SKF38393-, and muscimol-facilitated lordosis of E₂-primed rats with infusions of a PKA [68] and PKC (present study) inhibitor to the VTA. Future studies could investigate this question by co-administering PKA and PKC inhibitors with 3 α ,5 α -THP to the VTA and determining the effects on lordosis. The involvement of the adenylyl cyclase and PI signal transduction pathways certainly does not preclude involvement of other signal transduction pathways and another consideration to make

is that there may be a mediator of these effects that are downstream of both PKC and PKA. Indeed, progestogens may also have actions through other second messenger-mediated pathways, such as cGMP [7] and/or mitogen-activated protein kinase (MAPK) [33] and [39], to mediate progestogen-facilitated lordosis or have other functional effects [50]. An important consideration to make in future investigations on this topic is the relationship of these signaling cascades for the functional effects of progestogens in the VTA.

PKC has many downstream effects and how each of these PKC-dependent intracellular events alters progestogen-facilitated lordosis has not been fully elucidated. However, there is evidence that PKC can modulate progestogen biosynthesis/neurosteroidogenesis in a number of model systems. In support, P production is stimulated in luteal cells of primates through the activation of PKC [6]. Additionally, PKC may target activity of the steroidogenic acute regulatory (StAR) protein which mediates transfer of cholesterol from the outer to the inner mitochondrial membrane, where it is converted by P450 side chain cleavage (P450_{scc}) enzyme to pregnenolone (i.e. the rate-limited step in steroid biosynthesis). Inhibition of PKC decreases steroid production and StAR gene transcription in R2C Leydig tumor cells [43]. Indeed, pharmacological manipulations that alter neurosteroidogenesis in the VTA produce robust effects on lordosis of naturally-receptive or hormone-primed hamsters or rats. Decreasing neurosteroid biosynthesis in the VTA with the mitochondrial benzodiazepine receptor (MBR) antagonist, PK-11195, reduces, and enhancing steroid production with the MBR agonist, FGIN-27, increases, midbrain 3 α ,5 α -THP concentrations and sexual behavior of hamsters and rats [23]. Similarly, infusions of a non-specific P450_{scc} inhibitor, digitoxin, which can have many effects, including altering cell survival, calcium uptake, and cAMP-dependent pathways, to the VTA has clear effects to reduce midbrain 3 α ,5 α -THP concentrations and attenuate lordosis of naturally-receptive rats [67]. These findings, and others indicating levels of 3 α ,5 α -THP in the midbrain VTA positively correlate with lordosis [27], suggest that the extent to which the PKC inhibitor infusions utilized inhibited 3 α ,5 α -THP production need to be considered. The same PKC inhibitor regimen utilized in the present study reduced effects of systemically-administered P-mediated increases in lordosis when administered to the VTA, but not the substantia nigra, of E₂-primed hamsters [32]. Furthermore, in the present study, we found that PKC inhibitor-induced decrements in lordosis were not prevented by subsequent administration of 3 α ,5 α -THP, which produces physiological 3 α ,5 α -THP levels akin to that observed in naturally-receptive rats [26]. As such, it seems unlikely that an inhibitory effect of bisindolylmaleimide on 3 α ,5 α -THP concentrations could account for all of its effects on lordosis. Together, these data suggest that the experimental manipulations with PKC that we have utilized enable us to tap into the mechanisms of progestogens in the VTA for lordosis, rather than merely producing non-specific changes in neurosteroidogenesis.

In summary, these findings support the notion that, in the VTA, progestogens' membrane-mediated actions for lordosis that involve D₁ and/or GABA_A receptors require PKC. The role of other signal transduction processes and protein kinases, such as MAPK, for progestogens' actions for lordosis also need to be explored. Lordosis, as a progestogen-dependent behavior, is an effective bioassay to use to determine mechanisms underlying progestogens' action as well as effects of manipulating signal transduction pathways. In addition to 3 α ,5 α -THP [31], [75], and [80] and neurotransmitter systems [11] and [61], signal transduction factors (i.e. MAPK, PKA, PLC, and PKC) are important molecular targets to consider in the etiology and/or treatment of several neuropsychiatric conditions [2], [10], [55], [56], and [62]. For example, acute treatment with a selective serotonin reuptake inhibitor, fluoxetine, which is not typically associated with symptom reduction among depressed people, upregulated PKC and other kinases in whole rat brain [70]; the opposite pattern of effects was observed with chronic treatment, in a timeframe more consistently associated with symptom improvement. Moreover, a common side effect of pharmacological treatment for these neuropsychiatric disorders is sexual dysfunction. Indeed, these findings suggest that having a better understanding of the

mechanisms of progestogens through neurotransmitter systems and their downstream effectors, such as PKC, for lordosis is not only important for determining basic mechanisms of progestogens, but is clinically-relevant.

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Figure 1. SKF38393- or muscimol-mediated increases in 3α,5α-THP-facilitated lordosis of E₂-primed rats are attenuated by infusions of the PKC inhibitor, bisindolylmaleimide (BIS), to the VTA

Figure depicts lordosis quotients (LQs) of E₂-primed rats, 60 minutes following 3α,5α-THP infusions, and pretreatment with vehicle or BIS pre-treatment, and vehicle (open), SKF38393 (stippled), or muscimol (solid) at each 3α,5α-THP dosage (0, 100 or 200 ng). One asterisk above bars indicates significantly lower LQs due to BIS infusion ($p < 0.05$) vs. vehicle infusions at that 3α,5α-THP dosage. Two asterisks indicates significantly increased LQs due to agonist infusions ($p < 0.05$) vs. vehicle infusions at that 3α,5α-THP dosage. # indicates significantly increased LQs due to 3α,5α-THP infusions ($p < 0.05$) vs. vehicle infusions.

Lordosis quotients (LQs) and total number of beam breaks of E₂-primed rats that received bilateral infusions to the substantia nigra, rather than the intended infusion site, the ventral tegmental area

Data from rats administered bisindolylmaleimide (BIS)/vehicle (n=1), BIS/SKF38393 (n=1), or BIS/muscimol (n=1) and 3 α ,5 α -THP to the substantia nigra, at the test 60 mins after 3 α ,5 α -THP infusions. Because of problems with cannulae patency, data from the rats that received infusions of BIS and agonists and 200 ng 3 α ,5 α -THP were not collected.

Table 1

First Infusion	Second Infusion	LQ			Total Number of Beam Breaks		
		Infusions of 3 α ,5 α -THP (ng)			Infusions of 3 α ,5 α -THP (ng)		
BIS		0	100	200	0	100	200
	Vehicle	25	20	17	1063	481	715
	SKF38393	20	0	-	233	1469	-
	Muscimol	20	17	-	274	936	-

Effects of infusions of vehicle or bisindolylmaleimide (BIS) and vehicle, SKF38393, or muscimol to the VTA of E₂-primed rats for motor activity

Means (+ SEM) of total number of beam breaks made in the activity chamber are depicted. Data were collected 60 mins after infusions of 3 α ,5 α -THP.

First Infusion	Second Infusion	n	Total number of Beam Breaks			
			Infusions of 3 α ,5 α -THP (ng)			
			0	100	200	
Vehicle	Vehicle	8	741 $\pm$ 176	745 $\pm$ 151 *	535 $\pm$ 63	
	SKF38393	8	733 $\pm$ 188	691 $\pm$ 145 *	584 $\pm$ 156	
	Muscimol	8	534 $\pm$ 141	760 $\pm$ 104 *	606 $\pm$ 83	
BIS	Vehicle	8	370 $\pm$ 66	874 $\pm$ 164 *	555 $\pm$ 163	
	SKF38393	8	669 $\pm$ 140	1095 $\pm$ 128 *	572 $\pm$ 130	
	Muscimol	8	480 $\pm$ 99	556 $\pm$ 140 *	696 $\pm$ 127	

An * demonstrates that 100 ng 3 α ,5 α -THP is significantly increased compared to 0 or 200 ng 3 α ,5 α -THP (p<0.05).