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Membrane Actions of Progestins at Dopamine Type 1-like and GABA_A Receptors Involve Downstream Signal Transduction Pathways

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Abstract

In the ventral tegmental area (VTA), progestins facilitate lordosis via rapid actions at membrane dopamine Type 1-like (D₁) and/or GABA_A receptors (GBRs), rather than via cognate, intracellular progestin receptors. Downstream signal transduction pathways involved in these effects were investigated using lordosis as a bioassay. If progestins' actions at D₁ and/or GBRs in the VTA require activation of G-proteins, adenylyl cyclase, cyclic AMP-dependent protein kinase A (PKA), phospholipase C (PLC), and/or PKC, then pharmacologically blocking these pathways would be expected to attenuate progestin-facilitated lordosis and its enhancement by D₁ and GBR activity. Ovariectomized, estradiol-primed rats were infused first with vehicle or signal transduction inhibitor, and second with vehicle, a D₁ or GBR agonist, and then with vehicle or progestins to the VTA. Rats were tested for lordosis following infusions. Results indicated that initiation of G-proteins, adenylyl cyclase, PKA, PLC, or PKC in the VTA is required for rapid effects of progestins through D₁ and/or GBRs to facilitate lordosis. As well, progestins' actions at n-methyl-d-aspartate receptors (NMDARs) may modulate activity at D₁ and/or GBRs and mitogen activated protein kinase may be a common signaling pathway. Findings from a microarray study demonstrated that there was upregulation of genes associated with steroid metabolism, GBRs, D₁, NMDARs and signal transduction factors in the midbrain VTA of naturally-receptive mated compared to non-mated rats. Thus, in the VTA, progestins have rapid membrane-mediated actions via D₁, GBRs, NMDARs and their downstream signal transduction pathways.

Keywords

neurosteroids; midbrain; 5 α -pregnan-3 α -ol-20-one; allopregnanolone; ventral tegmental area

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Introduction

Our research focuses on elucidating the effects and mechanisms of progestins for mediating reproductive and non-reproductive behavior. To investigate the former, progestins' actions are manipulated in brain regions that are targets of progestins' actions for sexual receptivity in rodents, such as the ventromedial hypothalamus (VMH) and ventral tegmental area (VTA). The resulting effects on lordosis, the stereotypic posture that female rodents assume to enable mating to occur (given appropriate hormonal and environmental stimulation), are then used as a bioassay of progestins' actions. Results of lesions or progestin administration to the VMH and/or VTA of ovariectomized (ovx) rodents primed with 17 β -estradiol (E₂) indicate that progesterone (P₄) has effects to initiate and modulate lordosis respectively in these regions [1–7]. In addition to different consequences of P₄'s actions in these regions, there is emerging evidence that P₄ has different mechanisms in the VMH and VTA to mediate lordosis.

P₄'s actions in the VMH for lordosis

P₄'s effects in the VMH to initiate lordosis can occur via traditional actions at intracellular progestin receptors (PRs) and induction of gene transcription. Classic intracellular PRs are highly expressed in the VMH of rodents and are upregulated by E₂ and/or P₄ coincident with lordosis [8–10]. Blocking actions at PRs in the VMH inhibits lordosis of naturally-receptive or ovx, hormone-primed rats [11–16]. Administration of PR ligands to ovx, E₂-primed rats [15], but not PR knockout mice (PRKO), facilitates lordosis [17–18]. The latency for P₄ to enhance lordosis when applied to the VMH of ovx, E₂-primed rodents is typically several hours [6,19–20]. Moreover, free P₄, but not actions of P₄ relegated to cell membranes, enhance lordosis when applied to the VMH [6,19–20]. Thus, in the VMH, P₄ may initiate lordosis in part via actions at cognate PRs.

P₄'s actions in the VTA for lordosis

In the VTA, P₄ mediates lordosis through non-classical (“non-genomic”), rapid actions at neuronal membranes. Evidence that intracellular PRs in this region are not required for P₄'s actions are as follows. First, there are very few intracellular PRs in the VTA of adult rodents and those that are localized to the VTA are not E₂-induced [9,13,21]. Second, actions of P₄ at PRs in the VTA are not required for facilitation of lordosis. P₄ can enhance lordosis when applied to the VTA of PRKO mice and the efficacy of progestins to facilitate lordosis when applied to the VTA is independent of their capacity to bind PRs [22,23]. Third, membrane actions of P₄ in the VTA are sufficient to enhance lordosis. Free P₄ and P₄ bound to large macromolecules (P₄:BSA, P₄:HRP) have similar effects to rapidly enhance lordosis when applied to the midbrain VTA [6,19–20]. Fourth, P₄ has rapid effects in the midbrain VTA to facilitate lordosis. Within 60 secs of application to the VTA, P₄ increases cell firing, and P₄ enhances lordosis within 5 mins [24]. These rapid effects may be faster than actions of steroids to bind cognate PRs and initiate gene transcription and/or translation [25]. Together, these data suggest that P₄ has PR-independent, membrane-mediated, and rapid effects in the VTA to facilitate lordosis.

Role of the P₄ metabolite and neurosteroid, 3 α ,5 α -THP, for lordosis

Some of P₄'s effects in the midbrain VTA to facilitate lordosis may be due in part to actions of 3 α -hydroxy-5 α -pregnan-20-one (3 α ,5 α -THP). P₄ and 3 α ,5 α -THP levels are high in the midbrain of naturally-receptive or hormone-primed rats. Levels of 3 α ,5 α -THP, rather than P₄, more positively correlate with the initiation of lordosis responses of naturally-receptive mice [22]. 3 α ,5 α -THP is the most effective of all progestins at facilitating lordosis when applied to the VTA of E₂-primed rodents [22]. The midbrain VTA is one of the brain regions with the highest levels of expression/activity of P₄ metabolism enzymes, such as 5 α reductase and 3 α

hydroxysteroid dehydrogenase, which convert P_4 to dihydroprogesterone and subsequently $3\alpha,5\alpha$ -THP [26]. Inhibitors of these enzyme can rapidly attenuate lordosis of naturally-receptive or ovx, hormone-primed rodents and reduce midbrain $3\alpha,5\alpha$ -THP when administered systemically or to the VTA. Notably, these decrements in lordosis and $3\alpha,5\alpha$ -THP levels produced by metabolism inhibitors can be reversed by subsequent infusions of $3\alpha,5\alpha$ -THP to the midbrain VTA [27–29]. Upregulating the activity of these P_4 converting enzymes facilitates lordosis and increases $3\alpha,5\alpha$ -THP levels [30]. Thus, P_4 's metabolism to $3\alpha,5\alpha$ -THP in the midbrain VTA is essential for lordosis.

Another source of $3\alpha,5\alpha$ -THP in the midbrain VTA is biosynthesis in glial cells. Peripheral benzodiazepine receptors (PBRs) enhance the transport of cholesterol from the outer to the inner mitochondrial membrane forming pregnenolone, which is subsequently metabolized to P_4 and $3\alpha,5\alpha$ -THP [31]. This *de novo* synthesis of pregnenolone in the midbrain VTA occurs independent of peripheral gland secretion and in response to reproductively-relevant stimuli. For example, mating rapidly increases in $3\alpha,5\alpha$ -THP in the midbrain VTA and this is observed among intact, naturally-receptive rodents, as well as ovx, and/or adrenalectomized E_2 -primed rodents [32–33]. Increasing or decreasing neurosteroidogenesis of $3\alpha,5\alpha$ -THP in the VTA with infusions of PBR ligands, respectively, enhances and attenuates lordosis of hormone-primed or naturally-receptive rodents [34–35]. Inhibiting P450scc activity in the midbrain VTA produces decrements in $3\alpha,5\alpha$ -THP and lordosis [34–35]. Thus, another source of $3\alpha,5\alpha$ -THP in the midbrain VTA is from central biosynthesis.

GBRs as a target for $3\alpha,5\alpha$ -THP's actions in the VTA

γ -aminobutyric acid type ($GABA_A$)/benzodiazepine receptor complexes (GBRs) may be a target for $3\alpha,5\alpha$ -THP's actions to facilitate lordosis (GBRs; [32–33]). $3\alpha,5\alpha$ -THP is a potent, positive allosteric modulator of GBRs [36]. In nanomolar concentrations, $3\alpha,5\alpha$ -THP potentiates the activity of GABA to increase the duration of chloride channel opening, and in higher concentrations, can exert these effects in the absence of GABA. GABAergic interneurons and GBRs have been localized to the VTA [37]. Progestins can enhance the function of GABAergic neurons by increasing the number, density, and/or affinity of GBRs [38–39]. In the midbrain VTA, levels of GABA are higher when $3\alpha,5\alpha$ -THP levels are increased during behavioral estrous and/or mating [32–33]. As well, less GABA is needed to displace 50% of 3H muscimol in a competitive binding assay using midbrain VTA tissues from naturally-receptive rats that have high levels of $3\alpha,5\alpha$ -THP in the midbrain VTA, as compared to diestrous, non-receptive rats [22,32–33]. Inhibiting GABA formation or actions at GBRs in the midbrain VTA attenuates progestin-facilitated lordosis of rodents [13–14]. Increasing GABA levels, by reducing breakdown of GABA, or enhancing the activity of GBRs, in the midbrain VTA enhances lordosis of rodents [13–14,22,32–33,40–41]. Progestins vary in their activity at GBRs and $3\alpha,5\alpha$ -THP is the most potent endogenous progestin at enhancing GBRs function. The actions of progestins to facilitate lordosis when applied to the midbrain VTA, corresponds with their ability to enhance GBR function in the midbrain VTA [22]. Thus, $3\alpha,5\alpha$ -THP may have actions in the midbrain VTA to facilitate lordosis through altering GABAergic function.

Dopamine receptors as a target for $3\alpha,5\alpha$ -THP's actions in the VTA

Dopaminergic neurons in the midbrain VTA may be another substrate through which $3\alpha,5\alpha$ -THP has actions to facilitate lordosis. Dopamine cell bodies are localized to the midbrain VTA and activity of dopamine type 1-like (D_1) receptors can regulate their function [43]. Progestins increase D_1 density in the striatum and dopamine levels in the midbrain [24,44]. D_1 antagonists attenuate, and D_1 agonists enhance, progestin-facilitated lordosis when administered to the

VTA [45–46]. Thus, these data support a role of dopamine signaling for $3\alpha,5\alpha$ -THP-facilitated lordosis.

The role of signal transduction cascades for progestins' actions

It has been proposed that signal transduction may be a common important pathway for steroids' actions [47]. D_1 receptors are well-known metabotropic receptors [48]. Emerging evidence suggest that GBRs may also have actions involving signal transduction pathways. For example, $3\alpha,5\alpha$ -THP's effects to prolong the opening of GBR chloride channels *in vitro* can be inhibited with antagonists of G-proteins, protein kinase C (PKC), or cAMP-dependent protein kinase [49]. Further, systemic administration of P_4 increases levels of cAMP in the prefrontal cortex in a $3\alpha,5\alpha$ -THP- and GBR-dependent manner [32–33,50]. Thus, together these data suggest that activity of signal transduction factors may underlie functioning of progestins at downstream targets, such as GBRs and D_1

Signal transduction targets of $3\alpha,5\alpha$ -THP, D_1 , and GBRs in the VTA

We hypothesized that if progestins' actions at D_1 and/or GBRs in the VTA [51] require activation of G-proteins, adenylyl cyclase, cAMP-dependent PKA, phospholipase C (PLC), and/or PKC, then pharmacologically inhibiting these factors in the VTA should attenuate lordosis. The primary approach that we have used to investigate this involves ovx Long Evans rats that are implanted with bilateral guide cannulae to the midbrain VTA. Rats are E_2 -primed (10 μ g/0.2cc) and forty-four hours later are tested for their baseline lordosis responses with a sexually-experienced male rat and general motor behavior in an activity monitor. Rats then receive bilateral infusions of vehicle or inhibitors of signal transduction molecules, followed by infusions of vehicle, a GBR agonist (muscimol; 100 ng/ μ l), or a D_1 agonist (SKF38393; 100 ng/ μ l). A half hour after each of these infusions, rats are tested for lordosis behavior. Rats are then bilaterally infused with vehicle or 100 ng/ μ l $3\alpha,5\alpha$ -THP and are tested for lordosis 10 minutes later and lordosis and motor behavior 60 minutes later (See Figure 1). Drug and $3\alpha,5\alpha$ -THP manipulations that were utilized did not alter general motor behavior, suggesting that drug infusions did not produce non-specific effects on behavior (data not shown). Furthermore, effects observed were specific to $3\alpha,5\alpha$ -THP because manipulation to the VTA of rats primed with E_2 alone did not alter lordosis. When all testing was complete, rats were perfused, and fixed brain tissues were frozen, sliced, and stained with cresyl violet so that implant location could be determined by light microscopy. Infusions outside of the VTA did not produce the same pattern of effects as did infusions to the VTA. Thus, only data from rats that received bilateral infusions to the VTA are considered in data analyses.

The results of this series of experiments supported our hypothesis (Table 1). In each experiment, compared to ovx, E_2 -primed control rats, infusions of $3\alpha,5\alpha$ -THP increased lordosis and this was enhanced by infusions of muscimol or SKF38393 to the VTA. First, whether activation of G-proteins is necessary for progestin-facilitated lordosis and its enhancement by actions at GBRs and/or D_1 in the VTA was investigated by administering a G-protein inhibitor (GDP- β S; 50 μ M/side). Blocking G-protein activity in the VTA attenuated effects of $3\alpha,5\alpha$ -THP, muscimol and/or SKF38393 to enhance lordosis [52]. Second, whether progestins' ability to enhance lordosis via actions at GBRs and/or D_1 in the VTA is dependent upon activation of adenylyl cyclase and PKA was investigated by administering infusions of an adenylyl cyclase (2',5'-dideoxyadenosine (DDA; 12 microM/side) or PKA (Rp-cAMP (100 ng/side) inhibitor to the VTA. Infusions of DDA or Rp-cAMP, but not vehicle, to the VTA attenuated $3\alpha,5\alpha$ -THP-, muscimol-, and SKF38393- facilitated lordosis [53–54]. Third, whether progestins' ability to enhance lordosis via actions at GBRs and/or D_1 in the VTA is contingent upon activation of PLC and PKC was investigated by administering infusions of a PLC (U73122; 400 nM/side) or PKC (bisindolylmaleimide; 75 nM/side) inhibitor to the VTA. $3\alpha,5\alpha$ -THP-,

muscimol-, and/or SKF38393-facilitated lordosis was attenuated by infusions of a PLC or PKC inhibitor, but not vehicle, to the VTA [55]. Together, data from this series of experiments suggest that, in the VTA, $3\alpha,5\alpha$ -THP's actions for lordosis via D_1 and/or GBRs require activity of G-proteins, adenylyl cyclase, PKA, PLC, and/or PKC.

Other downstream targets of progestins in the VTA- NMDARs and MAPK

An important consideration to make in interpreting these results is the circuitry of the midbrain VTA involving GABAergic, dopaminergic, and glutamatergic cells (See Figure 2).

GABAergic terminals, which have D_1 , synapse on dopaminergic cell bodies that contain GBRs and NMDARs [36]. Increased GABA input onto GBRs that are located on GABAergic interneurons in the VTA reduces the inhibitory actions of these cells on dopamine-containing cell bodies and increases somatodendritic dopamine release [56], which may underlie enhancements in lordosis. In the VTA, excitation of D_1 receptors on GABAergic afferents enhances GABA release [57]. Indeed, inhibiting GBRs attenuates progestin-facilitated lordosis and its enhancement by a D_1 agonist [58]. In addition to actions involving GBRs and D_1 , NMDARs in the VTA may also be involved in progestins' actions in the VTA. GABAergic terminals, which have D_1 , synapse on dopaminergic cell bodies that contain GBRs and NMDARs [37,59]. $3\alpha,5\alpha$ -THP reduces glutamate's excitatory effects [60] and increases glutamate levels in the midbrain VTA [32]. Infusions of a NMDAR antagonist, MK-801, to the VTA, enhance $3\alpha,5\alpha$ -THP-facilitated lordosis of rodents [61]. Thus, progestins' actions, in the VTA, involving GBRs, D_1 , and/or NMDARs may facilitate lordosis by reducing tonic inhibition of dopamine neurons.

We have begun further investigation of the downstream targets, such as mitogen activated protein kinase (MAPK), of progestins at these neurotransmitter substrates. MAPK is the primary intracellular signaling cascade that is activated by NMDAR phosphorylation [62]. In this study, ovx, E_2 -primed rats were administered P_4 systemically (100 or 250 μ g; SC) and then were infused with a MAPK (MEK-ERK) inhibitor, PD98059 (3 μ g/side; dissolved in 10% DMSO) or vehicle, bilaterally to the VTA. As expected, P_4 dose-dependently increased lordosis. However, compared to vehicle infusions, infusions of PD98059 to the VTA significantly decreased lordosis of rats administered 100 or 250, but not 0 μ g, P_4 (See Figure 3). Together, these data suggest that MAPK may be an important downstream regulator of progestins' actions in the midbrain VTA for lordosis.

Genes upregulated in the VTA of mated vs. non-mated rats

In the experiments described thus far, lordosis has been used as a behavioral endpoint to investigate the mechanisms for progestins' effects. One criticism of these experiments could be that pharmacological manipulations had overriding effects on physiologically-relevant processes. Although we did not observe evidence of this, we have used a microarray approach to ascertain genes in the midbrain VTA that are altered by mating in naturally-receptive rats that do not have central manipulations. As such, intact female rats that were naturally-receptive were mated or were just placed in a mating chamber without mating. Immediately thereafter, midbrain VTA tissues were collected and stored and subsequently sent to one of two microarray core facilities (Center for Functional Genomics, Albany, NY; University of California- Los Angeles, Los Angeles, CA) to be analyzed for changes in gene expression. Results of changes in gene expression, described below, reflect those that were a minimum two-fold change and were corroborated between the two core facilities.

In naturally-receptive mated rats, genes that were upregulated in the midbrain VTA were those that our pharmacological studies have demonstrated are involved in progestins' actions for lordosis (Figure 4). Mating induces $3\alpha,5\alpha$ -THP biosynthesis [63–66] and, of ~50 genes that were upregulated in the midbrain VTA of mated vs. non-mated rats, many were those involved

in steroid metabolism. Of the upregulated genes in the midbrain VTA of mated vs. non-mated rats, many were related to GABA, dopamine, and glutamate function and signal transduction. Genes involved in cell proliferation and cell death and housekeeping genes were also upregulated in the midbrain VTA of mated vs. non-mated rats. Thus, these findings suggest that natural actions of progestins in the midbrain VTA associated with mating may involve GBRs, D₁, NMDARs, and signal transduction pathways.

Conclusions

Steroids produce diverse functional effects and mechanisms, which can present a challenge when trying to elucidate steroids' actions. The approach we utilize of using lordosis, a behavior that is reliably modulated by progestins, as a bioassay has revealed many features of progestins' actions that are clearly physiologically-relevant and involve rapid signaling. Altering 3 α 5 α -THP, GBRs, and/or D₁ receptors and their downstream signal transduction factors (G-proteins, cAMP/adenylyl cyclase, PKA, PLC, and PLC) in the VTA alter lordosis. In addition to these pharmacological manipulations, behavioral manipulations (i.e. mating) alters expression of genes associated with progestin metabolism enzymes, GBRs, D₁, NMDARs and their downstream signal transduction pathways. These data demonstrate that non-classical actions of progestins in the midbrain VTA to facilitate lordosis involve GBRs, D₁, receptors (and NMDARs, MAPK) and their downstream signal transduction factors. Moreover, these studies have revealed the complexity of progestin actions in the midbrain VTA, which are functionally-relevant and meaningful. Ongoing studies in our laboratory are investigating these mechanisms in the VTA as well as brain areas, such as the hippocampus, using the same type of approach for learning and memory, affective behavior, and neurodegeneration.

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Figure 1.
Timeline depicting when rats receive experimental manipulations and testing.

Figure 2.
Circuitry of the midbrain VTA for putative effects of progestins, such as $3\alpha,5\alpha$ -THP, at dopaminergic, GABAergic and glutamatergic substrates.

Figure 3.

Lordosis quotients (mean $\pm$ sem) of ovariectomized, E₂-primed rats administered SC injections of P₄ (0µg, n=10; 100µg, n=14, or 250 µg, n=13) and vehicle or P₄ (0 µg, n=11; 100 µg, n=13, or 250 µg, n=9) and a MEK/ERK inhibitor to the midbrain VTA (PD98059). In brief, rats were pre-tested 44 hours after E₂-priming for normative lordosis and motor behavior responses in an activity monitor. No differences were observed for motor behavior. As such data are not shown. Immediately after this pre-test, rats were infused with DMSO vehicle or PD98059 to the VTA then injected with 0, 100, or 250 µg progesterone thirty minutes later. Three and a half hours following P₄ administration, rats were tested for lordosis and motor behavior. +

indicates a significant increase compared to 0 μ g P₄. * indicates a significant decrease compared to vehicle infusions to the VTA.

Figure 4.

Microarray results of the distribution of upregulated genes in the midbrain VTA of naturally-receptive mated vs. non-mated rats. Converging evidence from data collected from both laboratories in which the microarray analyses were done demonstrate that 48 genes were upregulated at least 2-fold in the midbrain of naturally-receptive, mated (n=6) compared to non-mated (n=6) rats at both sites. Rats were mated with a sexually-experienced male rat in what is known as a pseudo-naturalistic environment, i.e. a chamber in which she can pace or control the timing of sexual contacts made with the male. Non-mated controls were exposed to the empty testing chamber. Collected midbrain tissues were analyzed using an Affymetrix platform with a Rat Genome 230 2.0 Array.

Table 1

Summary of results of experiments in ovx, E₂-primed rats administered vehicle or a signal transduction inhibitor to the midbrain VTA followed by infusions of vehicle, a GBR agonist (muscimol), or D₁ agonist (SKF38393) before infusions to the VTA of 100 ng 3 α ,5 α -THP and lordosis testing. Lordosis data are represented as a % change from E₂-administered rats administered vehicle only to the midbrain VTA (100%).

Signal transductiontarget/inhibitor infused	Infusions to the VTA									
	Signal transduction inhibitor	-	-	-	-	-	-	-	-	-
	3 α ,5 α -THP infusion	+	+	+	+	+	+	+	+	+
	Muscimol	-	+	+	+	+	+	+	+	+
	SKF38393	-	-	-	-	-	-	-	-	-
G-proteins/GDP- β -S		375 $\pm$ 12 *	500 $\pm$ 10 **	487 $\pm$ 10 **	75 $\pm$ 7 ^	100 $\pm$ 3 ^	102 $\pm$ 5 ^	125 $\pm$ 7 ^	85 $\pm$ 5 ^	95 $\pm$ 4 ^
Adenylyl Cyclase/DDA		370 $\pm$ 10 *	487 $\pm$ 13 **	487 $\pm$ 9 **	100 $\pm$ 3 ^	100 $\pm$ 2 ^	102 $\pm$ 5 ^	125 $\pm$ 7 ^	85 $\pm$ 5 ^	95 $\pm$ 4 ^
PKA/Rp-cAMP		375 $\pm$ 10 *	460 $\pm$ 12 **	400 $\pm$ 7 **	100 $\pm$ 2 ^	100 $\pm$ 2 ^	102 $\pm$ 5 ^	125 $\pm$ 7 ^	85 $\pm$ 5 ^	95 $\pm$ 4 ^
PLC/U73122		323 $\pm$ 12 *	450 $\pm$ 12 **	453 $\pm$ 10 **	91 $\pm$ 8 ^	91 $\pm$ 8 ^	85 $\pm$ 5 ^	110 $\pm$ 7 ^	82 $\pm$ 5 ^	95 $\pm$ 4 ^
PKC/bisindolymaleimide		300 $\pm$ 8 *	400 $\pm$ 12 **	398 $\pm$ 9 **	85 $\pm$ 3 ^	85 $\pm$ 3 ^	110 $\pm$ 7 ^	110 $\pm$ 7 ^	82 $\pm$ 5 ^	95 $\pm$ 4 ^

* indicates significant increase from E₂-administered control rats.

** indicates significant increase from E₂-administered, 3 α ,5 α -THP -infused rats.

^ indicates significant decrease from rats that received infusions of vehicle and 3 α ,5 α -THP and/or muscimol or SKF38393.