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Midbrain Microinfusions of Prolactin Increase the Estrogen-Dependent Behavior, Lordosis

Abstract. *Microinfusions of rat prolactin into the dorsal midbrain of estrogen-treated, ovariectomized rats increased lordosis behavior. Midbrain microinfusions of antiserum to prolactin into rats displaying maximum lordosis had the opposite effect. The distribution of a prolactin-like substance in the brain was studied immunocytochemically. The results suggest that a hypothalamic neuronal system projecting to the midbrain contains a prolactin-like substance that plays a role in facilitating this behavior and therefore may mediate some of the effects of estrogen on the brain. These data, together with others from studies of the prolactin gene and its regulation, indicate that it may be possible to analyze a sequence of molecular events in the brain that facilitate a behavioral response.*

Since estrogen alters brain functions, one can postulate the existence of specialized nerve cells that respond to changes in circulating estrogen concentrations and transduce this information into a form recognizable by other nerve cells. A useful model of an estrogen-modulated brain function is lordosis, a behavior displayed by sexually receptive rats. Much of the neural circuitry controlling lordosis has been determined (*1*). Cells in the mediobasal hypothalamus facilitate lordosis (*2*) and partially mediate the effect of estrogen on this behavior (*3*). Some of the estrogenic effect appears to be mediated by the synthesis of messenger RNA and protein (*4*). Projections from cells in the mediobasal hypothalamus to the dorsal midbrain are critical for lordosis (*5*). Some of these hypothalamic cells have midbrain projections and respond directly to estrogen (*6*), but the chemical nature of the neuroactive substances produced and released by these cells is unknown. Some cells within this anatomical area contain a prolactin-like immunoreactive substance (*7*). We show here that hypothalamic cells releasing a prolactin-like substance in the midbrain may facilitate lordosis.

Rats were ovariectomized and fitted with intracranial guide cannulas as described (*8*), but with two changes being made in the procedure: (i) the rats did not receive implanted Silastic capsules containing estradiol and (ii) the stereotaxic coordinates were 1.3 mm rostral to lambda and 4.5 mm below the dura; the cannulas, with tips 2.5 mm apart, were lowered with the top angled rostrally 10° from verticle. Infusions into control sites were made with these same coordinates, except the cannulas were lowered 1.5 mm below the dura. The microinfusions were 1 μl in volume, given over 2 minutes simultaneously on both sides of the brain.

The day after cannulation, each rat received 10 μg of estradiol benzoate (Sigma) subcutaneously. A week later, the rats were given an estrogenic regimen designed to produce low levels of lordotic responsiveness (*9*). Two days later, each rat received 1 mg of progesterone subcutaneously to ensure that each rat was capable of displaying moderate to strong lordoses. The rats were tested for lordotic responsiveness (*10*) in two tests before being injected with progesterone and at 30-minute intervals after injection, for 4 hours. Rats displaying progesterone-induced increased lordosis reflex scores (at least 1.0 point on the four-point scale) were given this same estrogenic regimen about a week later. Instead of injecting progesterone, we microinfused peptides through the guide cannulas into the dorsal midbrain (*8*). Each rat was tested for lordotic responsiveness on three tests prior to infusion and at frequent intervals afterward.

Table 1. Effect of microinfusion of 400 ng of prolactin into the cerebral cortex of ovariectomized, estrogen-treated rats ($N = 6$) and into the central gray of adrenalectomized, ovariectomized, estrogen-treated rats ($N = 3$). Values are means (± standard error) of the lordosis reflex score (*10*).

Time	Infusion into	
	Cerebral cortex	Central gray
Before infusion*	1.3 ± 0.18	1.0 ± 0
0 minute	1.4 ± 0.36	1.0 ± 0
5 minutes	1.1 ± 0.26	1.0 ± 0
20 minutes	1.3 ± 0.21	1.8 ± 0.20
1 hour	1.3 ± 0.22	2.5 ± 0.24
2 hours	1.5 ± 0.19	2.5 ± 0.29
3 hours	1.4 ± 0.19	2.3 ± 0.33
4 hours	1.4 ± 0.30	2.4 ± 0.31
5 hours	1.2 ± 0.36	1.4 ± 0.31
6 hours	0.9 ± 0.31	1.0 ± 0
9 hours	0.5 ± 0.30	0.1 ± 0.07
24 hours	0.6 ± 0.33	0.7 ± 0.33

*This test was conducted 20 to 60 minutes before the prolactin infusion, which began immediately after time zero.

Microinfusion of prolactin (NIAMDD-rPRL-B-3) significantly increased lordosis reflex scores in a dose-related fashion, with a latency of approximately 40 minutes (Fig. 1A). Maximum responsiveness was obtained 90 minutes to 4 hours after infusion. Infusion of vehicle (0.025*M* phosphate-buffered saline, *p*H 7.2) had no effect. Microinfusion of 400 ng of prolactin into the cerebral cortex overlying the midbrain ($N = 6$) had no effect on lordosis (Table 1). The behavioral effect of prolactin did not require progesterone release from the adrenal gland, since infusion of 400 ng of prolactin into the midbrain of three adrenalectomized rats increased the lordosis scores, with a timing and magnitude similar to the effect in non-adrenalectomized rats (Table 1).

To determine whether the effect of prolactin was due to contaminants of the prolactin preparation (*11*), we infused three of these contaminants into the dorsal midbrain in great excess of the amounts calculated to be present in the prolactin preparation. Neither growth hormone (NIAMDD-rGH-B-6, 25 ng/μl; $N = 4$), vasopressin (Bachem, 1 ng/μl; $N = 4$), nor oxytocin (Bachem, 1 ng/μl; $N = 3$) significantly increased the lordosis reflex scores. Infusion of adrenocorticotropin [ACTH-(1–24), Bachem, 25 ng/μl, which is approximately equimolar to 200 ng of prolactin; $N = 3$] into the dorsal midbrain had no effect on lordosis.

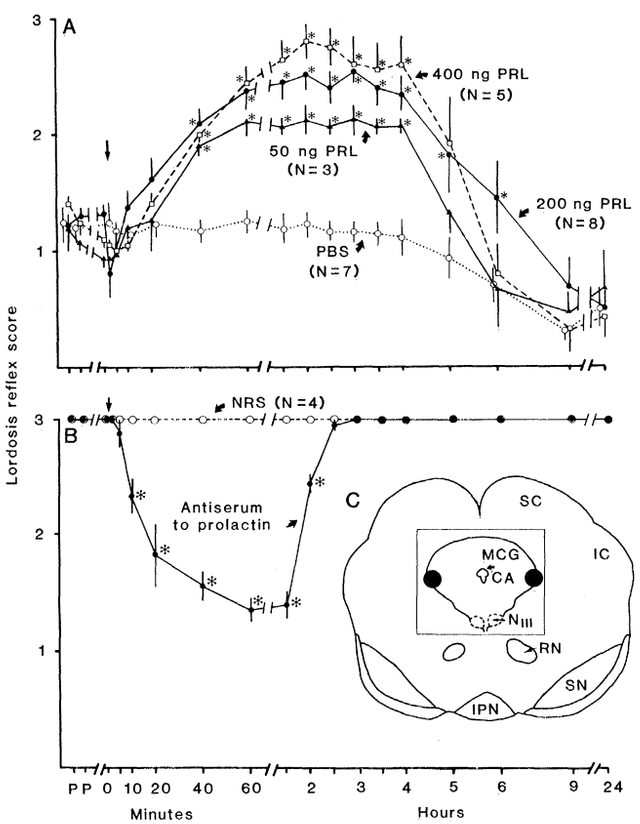
To investigate the physiological necessity of midbrain release of prolactin-like immunoreactive substances in the estrogenic maintenance of lordotic responsiveness, we infused undiluted antiserum to prolactin (NIAMDD-rPRL-IC-1) into the dorsal midbrain of rats displaying maximum lordoses because of prolonged estrogen administration. Infusion of the antiserum ($N = 5$) decreased lordosis reflex scores (Fig. 1B) with a latency of 10 minutes and for a duration of nearly 2 hours. Infusion of normal rabbit serum

(Pentex) into the midbrain of four of these same rats about a week later had no effect on lordosis (Fig. 1B).

To locate cell bodies containing prolactin-like immunoreactivity, we infused 50 μg of colchicine (Sigma) into the lateral ventricle of estrogen-treated, ovariectomized rats. One day after colchicine infusion the rats were killed and perfused, and the brains were prepared as described (*12*). Immunocytochemistry was performed on frozen sections with the same antiserum to prolactin as used for the behavioral studies but diluted 1:500 to 1:2000. To locate fibers containing prolactin-like immunoreactivity, we killed and perfused ovariectomized rats that had not been infused with colchicine. The brains were removed, postfixed, and cut on a Vibratome (75-μm sections). Free-floating sections were then processed for immunocytochemistry as above. Staining was completely eliminated by incubation of the tissue with normal rabbit serum or with antiserum absorbed with rat prolactin, but not when the antiserum was absorbed with β-endorphin or ACTH-(1–24). Cells in the mediobasal hypothalamus and fibers in the dorsal midbrain contained prolactin-like immunoreactivity. The cells were located in a band extending laterally from the arcuate nuclei to just ventral to the ventromedial nuclei, and from the rostral tip of these nuclei to premammillary levels (see Fig. 2A). Fibers containing prolactin-like immunoreactivity occurred throughout the midbrain central gray matter, especially ventral and lateral to the cerebral aqueduct, where there was a dense plexus of branching fibers (Fig. 2B).

These data suggest that a prolactin-like substance is produced by cells in the mediobasal hypothalamus, travels through fibers to the dorsal midbrain, and is released there in a way which facilitates lordosis. The data are consistent with our previous finding that infusion of colchicine into the mediobasal hypothalamus, which would block fast axoplasmic transport of substances from cell bodies to axon terminals, decreased lordotic responsiveness (*8*). The present behavioral results are also consistent with the time course of the loss of lordotic responsiveness following intrahypothalamic infusion of tetrodotoxin (*13*), which eliminates action potentials and thus should eliminate release of neuroactive substances from nerve terminals of hypothalamic cells.

The chemical nature of the prolactin-like immunoreactivity in the brain is unknown. Studies indicate that rat cells possess only one gene for prolactin (*14*) and that prolactin is not cleaved from a prohormone of larger molecular weight (*15*). These studies suggest that if the prolactin-like immunoreactivity in the brain shares significant sequence homology with pituitary prolactin, then the brain substance must be either prolactin per se or a post-transcriptionally modified product of prolactin. The available information on the structure and regulation of the prolactin gene (*14*) should facilitate the application of recent molec-



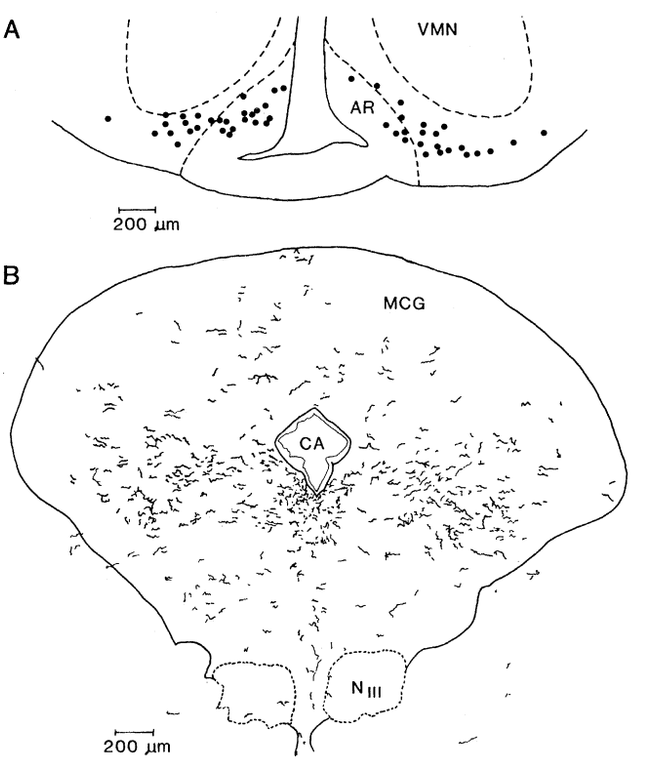


Fig. 1 (left). Effect of microinfusion of (A) prolactin or (B) antiserum to prolactin into the (C) dorsal midbrain on the lordosis reflex. Two pretests (*P*) were conducted 15 to 90 minutes before the infusion began immediately after time zero (at arrows). (A) Infusion of prolactin (*PRL*) increased the lordosis reflex; infusion of vehicle [phosphate-buffered saline (*PBS*)] had no effect. (B) Infusion of antiserum to prolactin decreased the lordosis reflex; infusion of normal rabbit serum had no effect. Each point represents the mean of the indicated number of rats. Vertical lines are standard errors. Asterisks represent significant differences (Mann-Whitney *U* test) from control infusions (PBS or normal rabbit serum). (C) Drawing of a coronal section through the midbrain (cortex not included) showing representative cannula tip placements [solid circles at lateral edge of midbrain central gray (*MCG*)]. The open square around midbrain central gray indicates extent of camera lucida drawing in Fig. 2B. Abbreviations: *CA*, cerebral aqueduct; *IC*, inferior colliculus; *IPN*, interpeduncular nucleus; N_{III}, oculomotor nucleus; *NRS*, normal rabbit serum; *RN*, red nucleus; *SC*, superior colliculus; and *SN*, substantia nigra. Fig. 2 (right). Camera lucida drawings of (A) hypothalamic cell bodies and (B) midbrain fibers containing prolactin-like immunoreactivity. Abbreviations: *AR*, arcuate nucleus; *CA*, cerebral aqueduct; *MCG*, midbrain central gray; N_{III}, oculomotor nucleus; and *VMN*, ventromedial nucleus.

ular biological techniques to this neuronal pathway. Brain cells that produce prolactin may concentrate estrogen from the circulation, since pituitary lactotrophs concentrate tritiated estradiol (*16*). Estrogen acts directly on lactotrophs to increase greatly the synthesis of prolactin (*17*) by increasing the synthesis of messenger RNA for prolactin (*18*). If such an estrogen-induced stimulation of prolactin synthesis occurs in the brain, this peptidergic neuronal system of cell bodies in the mediobasal hypothalamus and fibers in the dorsal midbrain could be a potent cell population mediating some of the behavioral effects of estrogen on the brain.

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10. Rats were tested by manual stimulation consisting of vigorous stroking of the flanks followed by grasping the flanks and perineum. The degree of dorsiflexion of the back was rated on a 4-point scale: 0, no lordosis; 1, slight; 2, moderate; and 3, maximum lordosis. Each rat was stimulated five times at intervals of a few seconds, and the average score of these five stimulations was used as the score for the rat at the indicated time-point. The inter-observer correlation (Spearman rank correlation coefficient) in lordosis reflex scores obtained by manual stimulation (performed by one author and scored by the same author and one other author) is 0.98, $N = 35$ tests. The intra-observer correlation (test-retest repeatability) is 0.96, $N = 23$ pairs of tests conducted 15 to 20 minutes apart on 23 different rats. For details see D. W. Pfaff, M. Montgomery, C. Lewis, *J. Comp. Physiol. Psychol.* **91**, 134 (1977); and (*8*).
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Tolerance Develops to the Disruptive Effects of Δ^9-Tetrahydrocannabinol on Primate Menstrual Cycle

Abstract. *Long-term exposure of sexually mature female rhesus monkeys* (Macaca mulata) *to thrice weekly injections of Δ^9-tetrahydrocannabinol resulted in a disruption of menstrual cycles that lasted for several months. This period was marked by an absence of ovulation and decreased basal concentrations of gonadotropin and sex steroids in the plasma. After this period, normal cycles and hormone concentrations were reestablished. These studies demonstrate that in rhesus monkeys subjected to long-term treatment with Δ^9-tetrahydrocannabinol tolerance develops to the disruptive effects of the drug on the menstrual cycle.*

The effects of marijuana and its principal psychoactive component, Δ^9-tetrahydrocannabinol (THC), on gonadal function in the female rhesus monkey have been studied in our laboratories. During the follicular phase of the menstrual cycle, monkeys treated with THC (2.5 mg/kg; day 1 to day 18) fail to ovulate and show decreased concentrations of estrogen and gonadotropins in the plasma. When exogenous gonadotropins are administered to THC-treated monkeys, ovulation is restored and normal luteal function follows (*1*). When monkeys are treated with THC (2.5 mg/kg) daily during the luteal phase of the menstrual cycle, the daily progesterone concentrations and the length of the luteal phases are no different from those in control groups. In addition, THC does not impair the normal pattern of response of the corpus luteum to increasing doses of human chorionic gonadotropin as measured by increases in serum progesterone concentrations (*2*). However, THC-treated monkeys exhibit abnormal menstrual cycles after THC treatment during the luteal phase (*3*). These results are consistent with studies from other laboratories that show that THC inhibits ovulation in rats (*4*) and rabbits (*5*) by a reversible effect on gonadotropin secretion.

We designed the present study to examine the effects of long-term THC treatment on the menstrual cycle. Five female rhesus monkeys (*Macaca mulata*) with normal menstrual cycles were used. Ovulation was detected by monitoring the concentrations of estrogen, luteinizing hormone (LH), and progesterone in the plasma and by laparoscopic examination (*2*). Daily swabs from the vagina were used to detect the onset of vaginal bleeding and duration of menses. Each monkey was followed for one control cycle and one cycle with vehicle treatment before being treated with THC. On day 1 of the third cycle the monkeys began thrice weekly injections of THC at doses of 2.5 or 1.25 mg/kg. The injections were continued for a total of 230 days or until two consecutive ovulatory cycles were observed. The injections were given on a Monday, Wednesday, and Friday schedule (at noon), and blood was sampled immediately before each THC injection.

The THC, which was obtained in solution in absolute ethanol (*6*), was prepared for injection by evaporating the ethanol under a constant stream of nitrogen gas. The residue was homogenized in Emulphor (polyethoxylated vegetable oil) in saline. The final concentration represented 10 percent Emulphor and 90 percent saline. The drug or vehicle was administered intramuscularly. A dose of 2.5 mg of THC per kilogram of body weight in monkeys is equivalent to mod-