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Further Evidence for Cholinergic Control of Gonadotropin and Prolactin Secretion[1] (38374)

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The existence of a cholinergic link between the brain and the pituitary was suggested in early experiments (1, 2), although the precise nature of this relationship has not been clarified. Recently, atropine sulfate, a cholinergic blocking agent, has been shown to suppress gonadotropin and prolactin secretion in male and female rats when injected subcutaneously (sc) or into the third ventricle (3). Furthermore, recent studies indicate that hypothalamic choline acetylase and acetylcholine esterase activities differ between sexes before and after puberty as well as within the stages of the estrous cycle (4). The present study was undertaken in order to further investigate the effect of cholinergic drugs on LH, FSH and prolactin secretion. Ovariectomized, estrogen-primed rats were used because of the high sensitivity of the pituitaries of such animals to LH-releasing factor (LRF) (5).

Material and Methods. Female (240–250 g) Sprague–Dawley rats obtained from Simonsen Laboratories (Gilroy, CA) were maintained in group cages in an air-conditioned room with controlled lighting (lights on 5 AM, off 7 PM). Food and water were provided *ad libitum*. Etherized rats were ovariectomized and were used for experimentation 3–6 weeks thereafter. Ten micrograms of estradiol benzoate (Sigma) in 0.2 ml corn oil were injected sc 48 hr before the start of the experiment.

Rats were lightly anesthetized with ether and an initial blood sample (1 ml) was withdrawn from the jugular vein with a heparinized syringe. Immediately thereafter, one or more of the cholinergic or anticholinergic drugs was injected sc dissolved in 0.9% NaCl in a volume of 0.1 ml/100 g body weight. Controls were injected with an equal volume of saline solution. The following drugs were used: pilocarpine HCl (Calbiochem), 5 or 50 mg/kg body weight; eserine SO_4 (Calbiochem), 0.5 mg/kg, atropine SO_4 (Pfaltz Bauer, NY), 20 mg/kg; atropine methyl nitrate (Sigma), 20 mg/kg.

When cholinergic blocking drugs were used, they were injected immediately after the initial blood sample was withdrawn and before the cholinergic drugs were injected, unless otherwise specified. Experiments were begun between 8:30 and 9:30 AM. Additional blood samples were withdrawn as before at ½, 1½, 6, and 24 hr after the injections. Throughout the experimental procedure, the rats were kept in individual cages.

All hormones were determined by radioimmunoassay. FSH and prolactin were measured using kits provided by NIAMD and results were expressed in terms of the RP-1 rat FSH and prolactin, respectively.[4] LH was measured by the procedure of Niswender *et al.* (6),[4] using a rat LH standard of known biological potency (ovarian ascorbic acid depletion assay) and ex-

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In rats treated with pilocarpine (50 mg/kg) or saline, 25 ng of synthetic LRF (sLRF)[4] were injected into the jugular vein at ½ and 6 hr after the cholinergic drugs were injected in order to establish the responsiveness of the adenohypophysis. Blood samples were removed as before immediately prior to and 10 min after injection of the decapeptide.

The significance of the changes in hormone levels in each group was determined by the paired *t* test.

Results. Effect of pilocarpine and eserine on plasma gonadotropin and prolactin titers. Plasma LH and prolactin titers in the saline-injected ovariectomized, estrogen-pretreated rats were unchanged throughout the experiment although LH and prolactin showed a tendency to increase during the afternoon (6 hr after injection) (Figs. 1 and 2). In the case of FSH, this increase reached statistical significance (Fig. 3).

Injection of either pilocarpine or eserine produced a biphasic effect on plasma LH and prolactin concentrations. Plasma LH was significantly decreased at both ½ and 1½ hr after injection of the 50 mg/kg body weight dose of pilocarpine (Fig. 1). By 6 hr plasma LH was elevated to values about three times the initial titers. The smaller dose of pilocarpine (5 mg/100 g body weight) gave similar results except that the increase in LH at 6 hr was not as pronounced. Eserine sulfate (0.5 mg/kg) also produced similar results in that there was a decrease in plasma LH at both ½ and 1½ hr followed by an increase at 6 hr.

Plasma prolactin titers followed the same pattern following the cholinergic drugs (Fig. 2). Both doses of pilocarpine produced a highly significant decrease in plasma prolactin at ½ and 1½ hr postinjection and by 6 hr there was a highly significant increase. Similar results were obtained in the eserine-injected animals.

In dramatic contrast to these findings was the failure of both cholinergic drugs to alter plasma FSH significantly (Fig. 3). There was a significant increase in plasma FSH in saline-injected controls at 6 hr, and this increase was not modified by either of the drugs used.

Effect of atropine SO_4 and atropine methyl nitrate (MN) in pilocarpine-injected rats. Atropine SO_4 (20 mg/kg) alone did not change plasma LH (Fig. 4, left panel). (Note that in this case the change in plasma LH rather than the absolute values was plotted). When the blocking drug was injected immediately prior to the

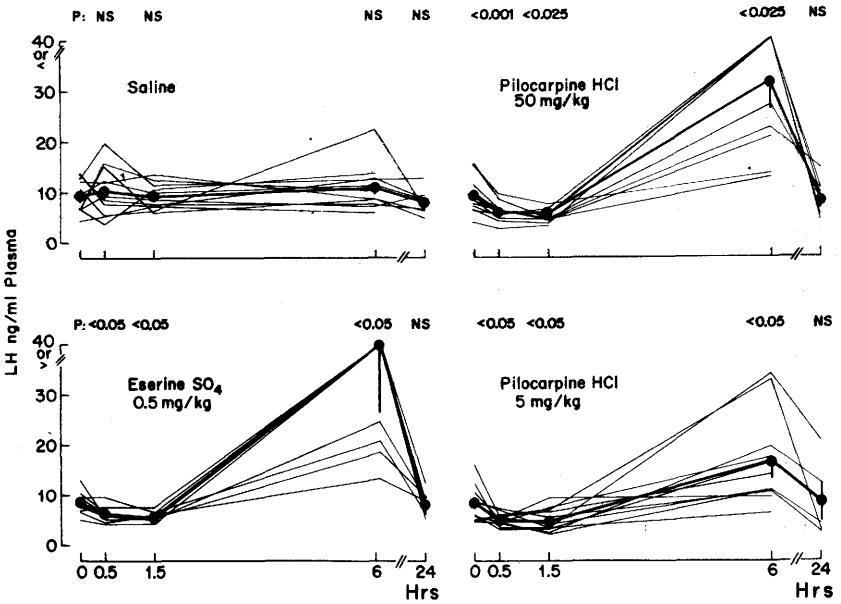

FIG. 1. Plasma LH prior to and after injection of saline, pilocarpine or eserine into ovariectomized estrogen-primed rats. *P* values indicate significant changes from the starting values at a particular time. Thin lines connect the values in individual rats. The heavy lines connect the mean values and the vertical bars give 1 SEM in this and subsequent figures.

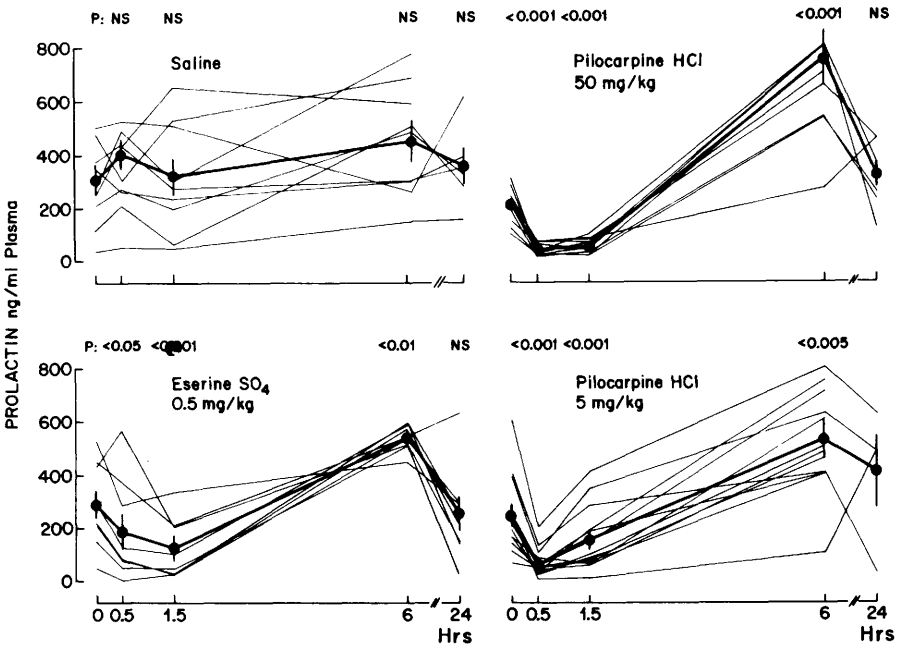

FIG. 2. Plasma prolactin in ovariectomized estrogen-primed animals injected with saline, pilocarpine or eserine.

higher dose of pilocarpine, it attenuated the initial decrease and later increase in plasma LH so that neither change was significant statistically. When atropine SO_4 was injected 4 hr after pilocarpine, the increase in LH at 6 hr was completely abolished, but the initial decline occurred as expected. The blocking effect of atropine was even clearer when the lower dose of pilocarpine was used (Fig. 4, right panel). On the other hand, when the quartenary ammonium derivative of the alkaloid, atropine (MN), was used, the biphasic effect of the cholinergic drug on plasma LH was unchanged.

Similarly, atropine SO_4 by itself did not alter plasma prolactin titers (Fig. 5, left panel). When injected immediately prior to the higher dose of pilocarpine, it only partially suppressed the biphasic effect of the drug. The decline in prolactin at 30 min was still significant statistically ($P < 0.05$), but the increase in prolactin at 6 hr was not significant. When the drug was injected at 4 hr, it blocked the later discharge that followed the initial decline of plasma prolactin in animals not receiving atropine. Atropine SO_4 also partially blocked the initial decline in prolactin produced by the lower dose of pilocarpine, but the peak at 6 hr was still significant (Fig. 5, right panel). Atropine MN was unable to modify the effects of the smaller dose of pilocarpine.

FSH values were not modified by the injection of atropine in the morning and, as before, there was no effect of the cholinergic drugs on FSH levels; however, when atropine SO_4 was injected at 4 hr, the increase in FSH at 6 hr failed to reach statistical significance (not shown).

Response to sLRF in the pilocarpine-injected rats. The pituitary sensitivity to sLRF was tested 0.5 and 6 hr after saline or pilocarpine (50 mg/kg) was injected (Table I). Values for plasma LH before injecting sLRF at 30 min and 6 hr after saline or pilocarpine were similar to those previously observed. The increment in plasma LH at 10 min after sLRF was significant in all groups, but the values obtained at 6 hr in the pilocarpine-treated animals were significantly greater than those obtained in the saline-injected animals at the same time. There was no increase in plasma FSH in any of the groups following sLRF (not shown).

Discussion. The results indicate that cholinergic drugs, such as pilocarpine and eserine, the first of which has primarily muscarinic action and the second both nicotinic and muscarinic action, can produce similar changes in plasma levels of LH and prolactin in the ovariectomized, estrogen-primed rat. There was an early decline

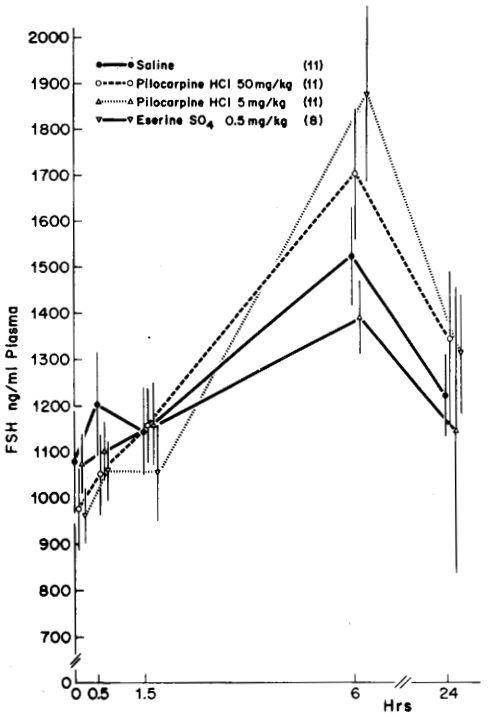

FIG. 3. Plasma FSH in ovariectomized estrogen-primed animals injected with saline, pilocarpine or eserine.

in the titers of both hormones which was coincident with the peripheral signs of action of the drugs. A few minutes after injection, there was profuse salivation, lacrimation, defecation, piloerection and tremor. The relative magnitude of these manifestations was dependent on the drug used. Salivation was most prominent with pilocarpine, and tremor predominated with eserine.

The initial decline in hormone levels was superseded by a rise in plasma concentrations of both LH and prolactin at 6 hr although there was greater variability in the responses from animal to animal. By this time the peripheral signs of action of the drugs had subsided. Both the initial decrease and the delayed increase in hormone release could be blocked by atropine SO_4, whereas atropine MN, a quartenary ammonium derivative which passes the blood brain barrier only with great difficulty, was totally ineffective. Both drugs blocked the peripheral muscarinic manifestations. Thus, it would appear that the effects on LH and prolactin release were due to a central action of the cholinergic drugs. The delayed increase in LH and prolactin observed at 6 hr postinjection was coincident with the timing of LH release in proestrus of intact rats (7) and in the afternoon of ovariectomized, estrogen-treated rats which have been injected with either a second injection of estrogen or progesterone (8). These elevations appear to be triggered by a circadian clock mechanism (9). It would appear then that the cholinergic drugs sensitize the animals so that the firing of the cyclic clock mechanism precipitates hormonal discharge. This thesis is supported by the finding that the discharge of LH at 6 hr after pilocarpine was attenuated if the animals were injected late in the day so that the clock would have fired

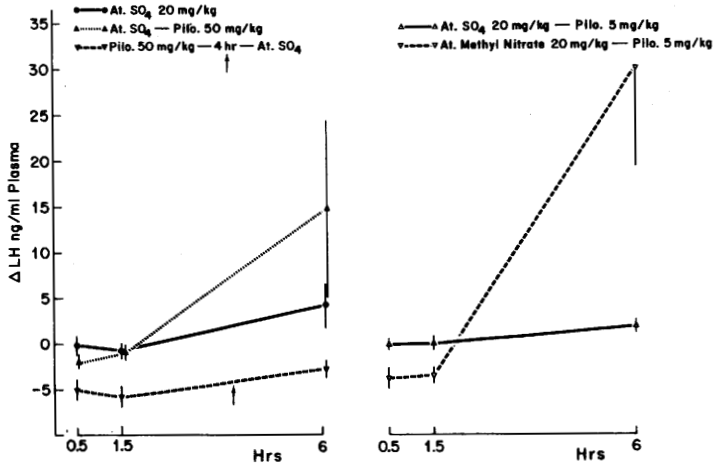

FIG. 4. The change in plasma LH at various times following administration of atropine SO_4, atropine SO_4 followed immediately by pilocarpine and pilocarpine followed at 4 hrs by atropine SO_4 (left panel), atropine SO_4 followed by the lower dose of pilocarpine and atropine MN followed by the lower dose of pilocarpine (right panel).

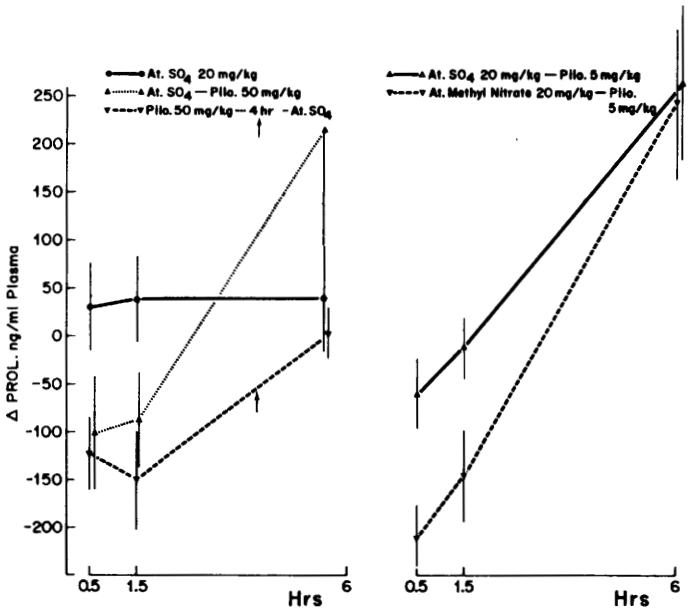

FIG. 5. Changes in plasma prolactin following administration of atropine SO_4, atropine SO_4 followed by pilocarpine, pilocarpine followed 4 hrs later by atropine SO_4 (left panel), atropine SO_4 followed by the lower dose of pilocarpine and atropine MN followed by the lower dose of pilocarpine (right panel).

sometime before 6 hr after drug injection.

Our results do not differentiate between the possibility that this delayed discharge of hormone was caused by the prior suppression of hormone release induced by the cholinergic drugs or the possibility that this was a delayed direct response to increased cholinergic tone in the afternoon. That cholinergic pathways may be involved in this delayed discharge is supported by the fact that atropine SO_4 could block the discharge when injected 4 hr after pilocarpine. The present results are reminiscent of those previously reported by Meyerson (10) who found that pilocarpine injected prior to the critical period could advance ovulation in 5-day cyclic rats, and that the effect was prevented by atropine.

No change in pituitary sensitivity to sLRF was found in pilocarpine-injected rats at ½ hr; however, at 6 hr after pilocarpine injection, there was an enhanced response to sLRF. Although this could be attributed to a direct effect of pilocarpine on the pituitary, a more likely explanation is that the elevated response was related to the high initial LH values at this time after pilocarpine. In every acute situation where plasma LH was elevated, we have observed an increased responsiveness to LRF, that is, on the afternoon of proestrus (11), during the LH discharge of estrogen–progesterone-treated rats (12) and in

TABLE I. The Effects of sLRF on Plasma LH (ng/ml) of Ovariectomized Estrogen-Primed Rats Injected with Either Saline or Pilocarpine.

		Plasma LH at varying times after pilocarpine		Increment in plasma LH at 10 min after sLRF	
Type of rat	No. of rats	30 min	6 hr	30 min	6 hr
Saline	15	7.7 ± 1.0[a]	10.7 ± 1.0	18.7 ± 4.2	16.1 ± 1.7
Pilocarpine	16	4.7 ± 0.7	21.7 ± 4.5[b]	20.0 ± 3.8	30.0 ± 5.4[c]

[a] Mean ± SEM.
[b] $P < 0.05$ versus saline control.
[c] $P < 0.02$ versus saline control.

the present circumstances. It is possible that the increased release of LRF in the pilocarpine-treated animals results in a sensitization of the gland. Aiyer *et al.* (13) have claimed that priming the gland with LRF enhances its sensitivity which would be in accord with this hypothesis.

In sharp contrast with the effects on LH and prolactin release was the lack of effect of the cholinergic drugs on plasma FSH levels. The only effect on FSH was the ability of atropine given 4 hr postpilocarpine to block the increase in FSH which occurred at 6 hr in all groups. The rise in FSH at 6 hr in the controls, at a time when LH was not increased could indicate that FSH is under control of a separate FSH-releasing factor (14). The rises in LH at 6 hr were not accompanied by increased FSH titers which may indicate that the increased decapeptide release was not sufficient to effect FSH since FSH secretion is much less sensitive to the decapeptide (15).

It is difficult to reconcile the present results with earlier experiments in which higher doses of atropine were able to block both gonadotropin and prolactin release in female and male rats (3). In the present experiments, cholinergic drugs, instead of producing the expected elevation in hormone titers, produced an immediate reduction and only at 6 hr was there an elevation. One could postulate that the higher doses of atropine used earlier produced a nonspecific suppression of hormone release. Alternatively there may be two sites of action of cholinergic drugs on prolactin and LH release. At one site, the drugs would stimulate the release of hormone and at the other, they would inhibit this release. Following the parenteral administration of the drugs used in the present study, access to the stimulatory site is delayed such that stimulation occurs only after a prolonged period. Further work will be required to resolve these discrepancies; however, the accumulated evidence suggests a role for acetyl choline as a transmitter in the complex circuitry which regulates gonadotropin and prolactin release.

Summary. Ovariectomized adult rats were injected with 10 μg of estradiol benzoate sc and 48 hr later with cholinergic drugs sc between 8:30 and 9:30 AM. Either pilocarpine HCl (5 or 50 mg/kg body weight) or eserine SO_4 (0.5 mg/kg) produced a drastic decrease in plasma LH and prolactin at ½ and 1½ hr postinjection which was followed at 6 hr by increased titers of both hormones in plasma. By contrast, plasma FSH remained unchanged in the drug-injected animals except for an increase above the initial value at 6 hr which was similar to that observed in saline-injected controls. Atropine SO_4 (20 mg/kg, sc) but not atropine methyl nitrate reduced the biphasic effect of pilocarpine on both LH and prolactin release. Since atropine methyl nitrate cannot pass the blood brain barrier, this indicates that the drugs were presumably acting on CNS muscarinic receptors to produce their effects on pituitary hormone release. Sensitivity of the pituitary to synthetic LH-releasing factor (sLRF) was evaluated in pilocarpine-treated animals. Sensitivity was unchanged during the period immediately after injection of the drug, at a time that plasma LH was lowered, but was increased in the afternoon at the time of the elevation in plasma LH. The results suggest that the immediate response to muscarinic drugs is a suppression in the release of both LH and prolactin and that this is superseded after a delay by an increased release, which is associated with an increased responsiveness of the pituitary to sLRF. The increased release occurring 6 hr after injection of the drug may be a secondary result of the earlier suppression of hormone release.

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