The neuroendocrine control of clock-timed gonadotropin release in the female Syrian hamster: role of serotonin

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Abstract

We hypothesized that rhythms in hypothalamic serotonergic activity were permissive to daily and estrous cycle-related rhythms of LH, FSH and prolactin (PRL). In the Syrian hamster, proestrus (PRO) is characterized by a surge of LH, FSH and PRL; diestrus (DIE) by low LH and FSH and a small surge of PRL, while in photoperiod-induced anestrous (PIA) animals there is a surge of LH and FSH and low PRL. Turnover rates of serotonin (5HT) in four brain areas were determined for the three reproductive states at 2-h intervals. Turnover in the preoptic area and arcuate nuclei did not change, indicating that 5HT projections to these regions probably do not control LH, FSH or PRL release. Serotonin turnover in the median eminence (ME) was elevated at 0600 h in PIA females, at 0600 h, 0800 h, and 1400 h on DIE and at 0600 h and 2200 h on PRO. Since the pattern of 5HT turnover in the ME is different during each of the three reproductive states, 5HT in this area is likely not crucial to the control of LH, FSH and PRL. Turnover of 5HT also did not change in the suprachiasmatic nuclei (SCN) of PRO or PIA animals. However, 5HT turnover rates in the SCN were elevated at 1200 h, 2000 h, and 2400 h on DIE. The correlation of high 5HT turnover in the SCN of DIE but not PRO and PIA animals suggested that elevated serotonergic activity in the SCN is part of the mechanism by which the gonadotropin surge is prevented on DIE. To test this, PRO and DIE hamsters were injected with 5HT receptor ligands. Administration of a 5HT agonist attenuated the PRO surge of LH and blocked the surge of PRL. In contrast, administration of two 5HT antagonists failed to elicit a surge of LH in DIE and phenobarbital-blocked PRO females, an indication that other mechanisms also contribute to inhibition of gonadotropin and PRL surges.

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Introduction

The Syrian hamster is photoperiodic. Reproductive regression results from exposure to <12·5 h of light per 24-h day (Gaston & Menaker 1967). In females of this species, exposure to inhibitory photoperiods causes anestrus (photoperiod-induced anestrus; PIA). This physiological state is characterized by the lack of ovulable follicles, atrophy of the uterus (Reiter 1968), the absence of regular 4-day vaginal estrous cycles and the presence of a daily afternoon surge of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and progesterone (P_4) (Bridges & Goldman 1975). This daily surge may be timed by the circadian system in a manner similar to the timing of the proestrous (PRO) gonadotropin surge (Stetson 1978, Stetson & Anderson 1980, Turek *et al.* 1987).

There are several lines of evidence obtained in the rat which suggest that serotonin (5HT) is involved in the control of LH and prolactin (PRL) release. First, serotonergic neurons have been shown to terminate on LH-releasing hormone (LHRH) neurons in the preoptic area (POA) (Kiss & Halász 1985) and significant overlap of LHRH and serotonergic terminals has been described in the median eminence (ME) and organum vasculosum of the lamina terminalis (Jennes *et al.* 1982). Secondly, 5HT content changes in the hypothalamus (Walker 1984) and ME (Crowley *et al.* 1979) of PRO rats, while 5HT activity in several hypothalamic areas changes during the day in females characterized by LH surges: ovariectomized-estradiol implanted (OVX-E_2) rats (Johnson & Crowley 1986, Cohen & Wise 1988*a*) and PRO females (Vitale *et al.* 1984, Kerdelhué *et al.* 1989). Thirdly, surges of LH, PRL and/or ovulation are affected by treatments thought to alter endogenous 5HT availability. For instance, stimulation of the raphe nuclei, site of the 5HT cell bodies which project to much of the brain, inhibits the PRO surge of LH and ovulation (Coen *et al.* 1980, Morello *et al.* 1992) as well as LH surges in OVX-E_2-P_4-treated rats (Waloch *et al.* 1981). Furthermore, 5HT agonists attenuate the surge of gonadotropins evoked by electrochemical stimulation of the POA (Cramer & Barraclough 1978) and also diminish the preovulatory rise in LH (Morello *et al.* 1992, Taleisnik *et al.* 1993). However, 5HT can exert this

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latter effect by increasing basal levels of LH (Schneider & McCann 1970, Walker 1980). Paradoxically, administration of 5HT antagonists also blocks ovulation and the PRO surges of LH and PRL (Markó & Flückiger 1980, Walker 1980, Mistry & Voogt 1989, Dow *et al.* 1994), as does the administration of 5HT neurotoxins (Clemens 1978, Meyer 1978, Coen *et al.* 1980, Johnson & Crowley 1986, Arey & Freeman 1989). Furthermore, LH and PRL surges are also inhibited in OVX-E_2 females by similar treatments (Lawson & Gala 1976, Coen & MacKinnon 1979), suggesting a crucial role for 5HT in the elevation of LH and PRL levels.

Of interest as well is the possible role of 5HT in the function of the biological clock. 5HT content changes rhythmically in the whole hypothalamus of the rat (Quay 1968), mouse (Huie *et al.* 1989) and medial basal hypothalamus (MBH) of the Syrian hamster (Ferraro & Steger 1990). In the suprachiasmatic nuclei (SCN; site of the circadian clock (Stetson & Watson-Whitmyre 1976)) of *Phodopus sungorus* a diurnal rhythm of 5-hydroxyindole acetic acid (HIAA; the major 5HT metabolite) release, with maximum levels measured during the dark phase of the photocycle, suggests increased serotonergic activity during the dark period (Glass *et al.* 1992). Similarly, 5HT content in the SCN is elevated at 2400 h in the male rat (Poncet *et al.* 1993). Destruction of 5HT neurons shifts the phase angle of entrainment of Syrian hamsters such that activity onset occurs prior to lights off (Smale *et al.* 1990). Work with rat brain slices shows that application of 5HT (Medanic & Gillette 1992) or quipazine (Prosser *et al.* 1992) (a 5HT agonist) causes phase shifts in the rhythm of spontaneous electrical activity of SCN neurons. In addition, the effect of quipazine is independent of Na^+ and Ca^{2+} channels, suggesting that 'clock elements' are affected by quipazine directly (Prosser *et al.* 1992).

As well as its possible roles in the control of reproductive hormone surges, and in the entrainment of the circadian clock, there is also evidence that serotonergic activity changes in parallel with photoperiod-induced reproductive regression in male rodents. In the MBH, the ratio of HIAA to 5HT is increased on inhibitory photoperiods in the white-footed mouse (Glass *et al.* 1988) and Syrian hamster (Benson 1987) and by blinding in the Syrian hamster (Vriend 1989). Similarly, an increase in 5HT synthesis in the MBH has been reported in photoperiodically inhibited male Syrian hamsters (Steger *et al.* 1990). These studies suggest that photoperiod-induced increases in 5HT activity may inhibit the reproductive system and indeed, in the Siberian hamster, antagonism of 5HT receptors by daily administration of methysergide retards gonadal regression induced by daily injections of melatonin (Duncan *et al.* 1990). Interestingly, administration of 5HT over a period of 2 days decreases LHRH mRNA expression in male rats, suggesting that this neurotransmitter does suppress the reproductive axis (Li & Pelletier 1995).

Because the female Syrian and closely related hamsters are uniquely characterized by photoperiod-induced reproductive regression accompanied by daily afternoon surges of the gonadotropins and PRL, comparison with rhythms of LH, FSH and PRL secretion during the estrous cycle allows the opportunity to differentiate the role of 5HT in each of these physiological phenomena (i.e. reproductive hormone surges, circadian entrainment and photoperiod-induced reproductive quiescence). As a first step in addressing this question, we examined 5HT activity at 2-h intervals in the POA, SCN, ME, and arcuate nucleus (AN) of female hamsters on diestrus (DIE), PRO or when PIA. PRO hamsters are characterized by a surge of the gonadotropins and PRL, while DIE hamsters are characterized by basal levels of the gonadotropins and a small afternoon surge of PRL (Bast & Greenwald 1974). PIA hamsters, on the other hand, have an afternoon surge of the gonadotropins (Bridges & Goldman 1975), while PRL is low to undetectable throughout the day (Widmaier & Campbell 1981). We studied the POA and ME because they are the sites of LHRH nerve cell bodies and terminals respectively (Lehman & Silverman 1988), the SCN because they are the site of the biological clock (Stetson & Watson-Whitmyre 1976), and the AN because neonatal monosodium glutamate-induced lesions of this area in the female Syrian hamster prevent the transition from prepubertal daily gonadotropin surges to 4-day estrous cycles (Donham *et al.* 1990).

Based on the data obtained, we hypothesized that increased serotonergic activity in the SCN of DIE hamsters was part of the mechanism by which the surge of gonadotropins is prevented on this day of the estrous cycle. To test this hypothesis, we administered 5HT receptor ligands to DIE or PRO animals to determine whether manipulation of 5HT activity would evoke or prevent, respectively, surges of LH and PRL.

Materials and Methods

Animals

Female Syrian hamsters bred in our colony were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 86–23). All animals were kept under controlled lighting of 14 h light:10 h darkness (14L:10D); lights on 0600–2000 h) with food (Agway Prolab 3000, St Mary's, OH, USA) and tap water available continuously. PIA females were obtained by moving animals from 14L:10D to 12L:12D (lights on 0800–2000 h) at weaning (21 days of age). On the morning of estrus, Syrian hamsters are characterized by a vaginal discharge which is well correlated with the occurrence of ovulation the preceding night (Kent 1968). Therefore, beginning at 13 weeks of age, estrous cycles were monitored by examination for the postovulatory discharge until animals housed on the inhibitory

photoperiod (12L:12D) became anestrus at 16–20 weeks of age. For each experiment, females were observed for a period of at least 13 consecutive days just prior to the experiment to confirm both regular 4-day estrous cycles in long photoperiod (14L:10D)-housed animals and the continuous absence of postovulatory estrous discharges in those females housed on 12L:12D.

Experiment 1

This experiment was designed to confirm that pargyline (a monoamine oxidase inhibitor) would increase 5HT content in the brain areas of interest in female Syrian hamsters. DIE animals were injected with saline or pargyline (75 mg/kg body weight (BW), i.p.; 75 mg/ml) at 1630 h. A group of saline-injected animals was killed immediately to serve as an initial control. Equal numbers of animals in each treatment were killed at 10-min intervals for 30 min after injection. To obtain enough samples (n=11–13), all time-points for both treatments were collected on four consecutive calendar days. Brains were microdissected to isolate the POA, SCN, ME, and AN, and assayed for 5HT and protein content.

Experiment 2

Animals (n=7–10) were injected with saline or pargyline on DIE, PRO, or when PIA on 12L:12D at 2-h intervals beginning at 0600 h. All animals were killed 10 min after injection. Because of the number required, animals used in this experiment were born at four different times of year. To control for variation, animals were used at approximately the same age (4·5 months) and all groups were collected from each cohort. Trunk blood was processed for assay of LH, FSH and PRL, while brains were processed to determine 5HT concentrations in the POA, SCN, ME, and AN.

After confirming statistically that cohort did not affect 5HT content (P=0·1207), 5HT concentrations within a group were ranked and turnover rates were calculated prior to further statistical analysis.

Experiment 3

Based on the results from experiment 2, we hypothesized that increased serotonergic activity in the SCN of DIE hamsters was part of the mechanism by which the surge of gonadotropins is prevented on this day of the estrous cycle. Therefore, pharmacological elevation of serotonergic agonists should prevent an expected surge of LH. Decreases in 5HT availability (by administration of 5HT receptor antagonists), on the other hand, would not affect the expected surge and would perhaps evoke a surge in animals that had basal afternoon levels of LH.

To test this hypothesis, DIE and PRO hamsters (n=6/group) were injected with saline, quipazine (a 5HT agonist; 15 mg/kg BW, i.p.; Q1004; Sigma Chemical Co., St Louis, MO, USA), cyproheptadine (a 5HT antagonist; 7 mg/kg BW, i.p.; 27,907·2; Aldrich Chemical Co., Milwaukee, WI, USA), or methysergide (a 5HT antagonist with dopaminergic actions; 3 mg/kg BW, i.p.; Sandoz Pharmaceuticals, East Hanover, NJ, USA) at both 1100 and 1500 h. The dose of quipazine used here is the same as that employed to reduce LH levels in female rats (Lynch *et al.* 1984) and slightly higher than that used to reduce light-induced *fos* expression in the SCN of male Syrian hamsters (12·5 mg/kg; (Selim *et al.* 1993)). The doses of cyproheptadine and methysergide are the same as those used to inhibit ovulation (Markó & Flückiger 1980, Walker 1980) and LH secretion (Walker 1980) in proestrous rats. Injections were given twice so that levels of the receptor ligand would remain high throughout the afternoon. In addition, PRO hamsters were injected with saline or phenobarbital (100 mg/kg BW, s.c.; Eli Lilly, Indianapolis, IN, USA) at 1400 h, a treatment which blocks the surge of gonadotropins (Everett & Sawyer 1950, Stetson & Watson-Whitmyre 1977). These groups were included because it might be easier to elicit a surge from phenobarbital-blocked animals than from DIE animals. For instance, gonadotropin surges have been restored in barbiturate-blocked animals by administration of LHRH (Blake 1978, Siegal & Greenwald 1978) and P_4 (Turgeon & Greenwald 1972). To conserve animals, blood was collected from unanesthetized animals at 1330 h by cardiac puncture and at 1700 h by decapitation. This methodology has been used to obtain serial reproductive hormone measurements from Syrian hamsters in our laboratory (Stetson *et al.* 1981, Donham *et al.* 1984, 1990, 1994, Talamantes *et al.* 1984, Donham & Stetson 1985) as well as other laboratories (Siegel *et al.* 1976, Siegal & Greenwald 1978, Terranova & Greenwald 1979, Jorgenson & Schwartz 1987). We chose to collect blood from unanesthetized animals since commonly used anesthetics perturb hormone levels. Indeed, pentobarbital and ether can block the proestrous surge of gonadotropins (Kim *et al.* 1994) and exposure to ether has been documented to elevate levels of PRL (Reis *et al.* 1994) and adrenocorticotropin (Bruhn *et al.* 1984) within 5 min of exposure. Of particular concern for this experiment was the finding that ether-induced PRL release is mediated via serotonergic mechanisms (Jörgensen *et al.* 1992). Blood samples were processed and assayed for both LH and PRL.

Microdissection of specific brain areas

After animals were killed by decapitation, brains were removed, frozen on dry ice and subsequently stored at −65 °C.

Brain areas and nuclei (POA, SCN, ME, and AN) were dissected by the micropunch technique (Palkovits 1973, 1974, Palkovits & Brownstein 1988) modified for use

in the Syrian hamster brain. The procedure was as follows: 200 μm thick sections were cut by cryostat and thaw-mounted on glass slides kept on dry ice. Tissue was punched from these slices under a dissecting microscope using modified hypodermic needles according to the co-ordinates given by Knigge & Joseph (1968). Starting at the anterior commissure and working caudally, the POA was isolated from the 1st and 2nd 200 μm sections lateral to the third ventricle. A 500 μm diameter needle (21 gage) was used to remove this area. The SCN was isolated from the 5th and 6th sections, adjacent to the third ventricle and immediately dorsal to the optic chiasm. The SCN were removed using a 500 μm diameter needle, positioned medially. The AN and ME were isolated starting 300 μm caudal to the sections containing the SCN. The next two 200 μm sections were dissected to remove the ME (ventral to the third ventricle) with a 1000 μm diameter needle (17 gage) and the AN (lateral to the third ventricle) with a 300 μm diameter needle (24 gage). Tissue punches were stored at −65 °C in labeled plastic tubes until assayed for 5HT.

All micropunched tissue sections were preserved in 10% formalin and subsequently stained with cresyl violet and examined microscopically to confirm correct placement of the punch.

Measurement of 5HT turnover rates

Turnover rates of 5HT were determined by blocking the degradation of 5HT with pargyline (75 mg/kg BW dissolved in saline, i.p. (75 mg/ml); P8013; Sigma Chemical Co.). After injection of pargyline, 5HT is no longer degraded via the monoamine oxidase pathway, causing 5HT to accumulate over time (Costa & Neff 1970). This result was confirmed in experiment 1 and an interval of 10 min after pargyline injection was chosen to minimize the effects of increased amine concentrations on subsequent neuronal activity (Costa & Neff 1970, Weiner 1974, Barraclough & Wise 1982).

Measurement of 5HT by HPLC

Each microdissected sample was homogenized in 80 μl mobile phase (0·125 M monochloroacetic acid (24,060–5; Aldrich Chemical Co.); 1 mM EDTA (5–311); titrated to pH 3·00 with 5 M sodium hydroxide (SS256); followed by the addition of 7% acetonitrile (BP1170–4; all from Fisher Scientific Co., Fair Lawn, NJ, USA)) containing 15 ng/ml N-methyl-5HT (M1514; Sigma Chemical Co.) as an internal standard. After centrifugation, 50 μl supernatant were injected onto a 20 μl injection loop and subjected to HPLC (reverse phase column (MF6213; Bioanalytical Systems (BAS), Indianapolis, IN, USA)) with electrochemical detection (600 mV applied potential; LC17A and LC4B; BAS).

Protein measurement

Protein was measured by the method of Lowry *et al.* (1951) modified to permit measurement of protein in 30 μl fluid. BSA (A2153; Sigma Chemical Co.) diluted to known amounts in 0·15 M saline served as a standard. Samples were read at 630 nm on a Dynatech MR5000 scanner. Assay sensitivity was 0·3 μg protein/sample.

Calculation of 5HT turnover rate

Before 5HT turnover rate was calculated, levels of 5HT from individual animals were ranked within treatment group (saline or pargyline). Since 5HT accumulates over time after injection of pargyline, turnover rates were calculated using the formula: $(k=(C_t - C_o/C_o)\star 6)$; where k is the turnover rate, C_o is the concentration of 5HT in the saline-injected animal of the same rank and C_t is the concentration of 5HT in the animal injected with pargyline. Since animals were killed 10 min after injection, multiplication by 6 gives a rate/h. Turnover rates are expressed as pg/μg protein per h.

Measurement of LH, FSH, and PRL

Blood samples were collected by decapitation and allowed to clot at 5 °C. After centrifugation at 5 °C, serum was aspirated and stored at −20 °C until assayed for LH, FSH, and PRL.

Serum samples were assayed in duplicate by standard RIA using kits provided for rat LH and FSH by NIDDK (Baltimore, MD, USA) and for hamster PRL by A F Parlow (Pituitary Hormones and Antisera Center, Torrance, CA, USA). Reagents were LH (I6), FSH (I6) and PRL (AFP10302E) for iodination with Chloramine T; anti-rat LH (S10 or S11), anti-rat FSH (S11), and anti-hamster PRL (AFP7472988); goat anti-rabbit gamma globulin for the gonadotropins and rat anti-hamster gamma globulin for PRL (both from Antibodies, Inc., Davis, CA, USA); rat LH (RP2 or RP3 – noted on graphs), FSH (RP2), and hamster PRL (AFP10302E) were diluted to known amounts to serve as a standard curve. The sensitivities of the assays were 25 pg/tube for LH and PRL and 100 pg/tube for FSH. For any one experiment, serum samples were randomized and assayed within one assay. Intra-assay variation, determined with serum pools, never exceeded 20%.

Statistical analysis

Serum hormone data and brain neurotransmitter data from all experiments were log-transformed prior to statistical analysis. Therefore, the back-transformed means and standard errors generated by the GLM ANOVA procedure are graphed. Data were analyzed with SAS

(Version 6·06; SAS Institute, Inc., Cory, NC, USA). Where indicated, post-hoc analysis was performed using the predicted difference option of least-squares means. To avoid rejection of the null hypothesis during post-hoc comparisons due to random events, alpha (0·05) was divided by the number of groups in the experiment. Only *P* values below this value (see figure legends for each experiment) were accepted as statistically significant. In the case of serum hormone concentrations, surges were detected by comparison with 'basal levels' of hormone. For this purpose, basal levels were defined as levels measured at 0800 h and 1000 h during the same reproductive state. For experiment 1, a three-way GLM ANOVA with brain area, time after injection and drug treatment as the variables was used. In experiment 2, both serum hormone data and 5HT turnover rate data were analyzed with a three-way GLM ANOVA with time of injection, reproductive state and drug treatment as the variables for serum hormone measurements and time of injection, reproductive state and brain area as the variables for 5HT turnover rate measurements. For experiment 3, both LH and PRL data were analyzed by a four-way GLM ANOVA using reproductive state, time of day, drug treatment at 1100 and 1500 h and drug treatment at 1400 h as the variables.

Results

Experiment 1

Pargyline administration increased 5HT levels (drug treatment $F(1,228)=50·77$, $P<0·0001$; see Fig. 1), indicating that this monoamine oxidase inhibitor has the expected effect in female Syrian hamsters. There was no effect of time after injection ($F(3,228)=0·17$, $P=0·9172$); 5HT levels were maximally increased 10 min after administration of pargyline. There was an effect of brain area ($F(3,228)=6·14$, $P=0·0005$); levels of 5HT measured in the SCN were higher than 5HT levels in the other brain areas examined (post-hoc test). These data are similar to those reported by Cohen & Wise (1988*b*) for the rat. Although 5HT content did not appear to be elevated in the ME 10 min after this injection of pargyline, it certainly was in all other brain areas examined. Thus, to avoid underestimating turnover by choosing a longer period after injection, we chose 10 min post-injection for killing the animals in the subsequent experiment. As is obvious from the results of that experiment, this methodology is sensitive enough to measure increases in 5HT turnover in the ME.

Experiment 2

For LH, FSH, and PRL there were significant effects of time of injection (LH, $F(11,532)=28·44$, $P<0·0001$; FSH, $F(11,530)=6·18$, $P<0·0001$; PRL, $F(11,532)=11·09$, $P<0·0001$), reproductive state (LH, $F(2,532)=36·02$, $P<0·0001$; FSH, $F(2,530)=6·79$, $P=0·0012$; PRL, $F(2,532)=123·07$, $P<0·0001$), and the interaction between these two variables (LH, $F(22,532)=10·27$, $P<0·0001$; FSH, $F(22,530)=6·39$, $P<0·0001$; PRL, $F(22,532)=4·24$, $P<0·0001$), reflecting normal estrous cycle and PIA rhythms of hormone secretion. In other words, DIE animals were characterized by low levels of both LH and FSH and a small afternoon rise in PRL (Fig. 2), PRO animals had a preovulatory surge of all three hormones (Fig. 3), and PIA females had a gonadotropin surge and low levels of PRL (Fig. 4).

In addition, LH levels were affected by drug treatment ($F(1,532)=3·87$, $P=0·0498$) and the interaction of drug treatment with time of injection ($F(11,532)=2·55$,

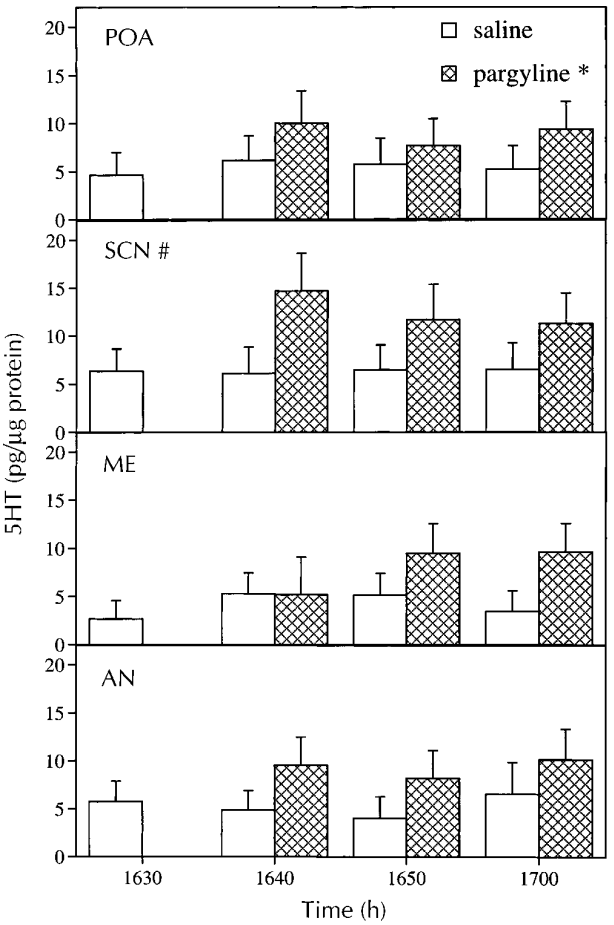

Figure 1 Pargyline administration at 1630 h increased 5HT content in the POA, SCN, ME, and AN. * Indicates significant accumulation of 5HT in pargyline-injected animals ($P<0·0001$); # indicates that 5HT levels in the SCN are higher than those in other brain areas ($P<0·0005$). There was no interaction between drug treatment and time ($P=0·9172$), indicating that maximal elevation of 5HT occurs by 10 min after injection of the drug.

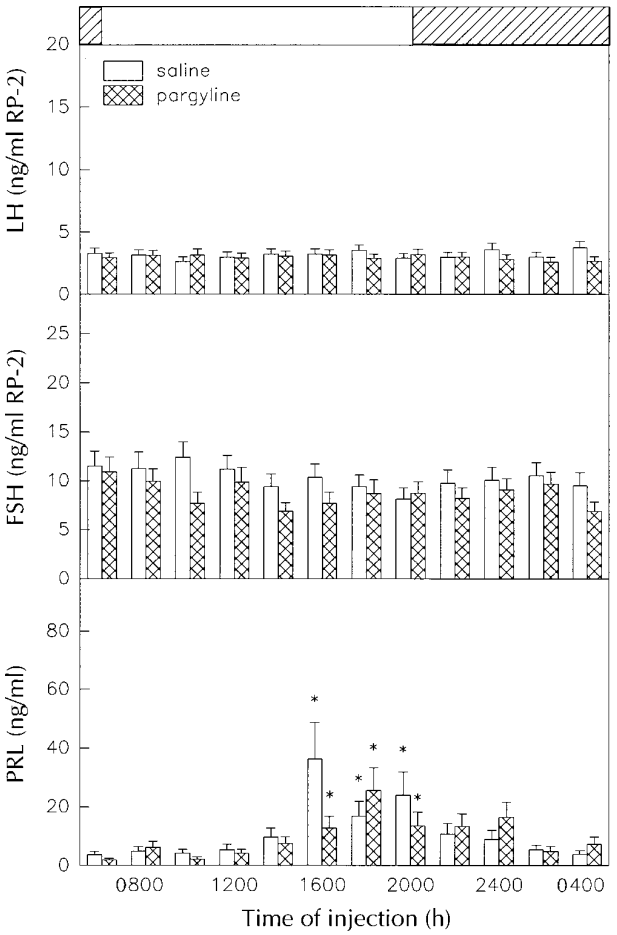

Figure 2 Lack of effect of pargyline on serum LH, FSH and PRL levels in DIE hamsters. Animals were killed by rapid decapitation 10 min after injection. * Indicates hormone levels that are significantly different from basal (0800–1000 h during the same reproductive state; $P<0{\cdot}0007$). The bar at the top of the figure indicates the photocycle.

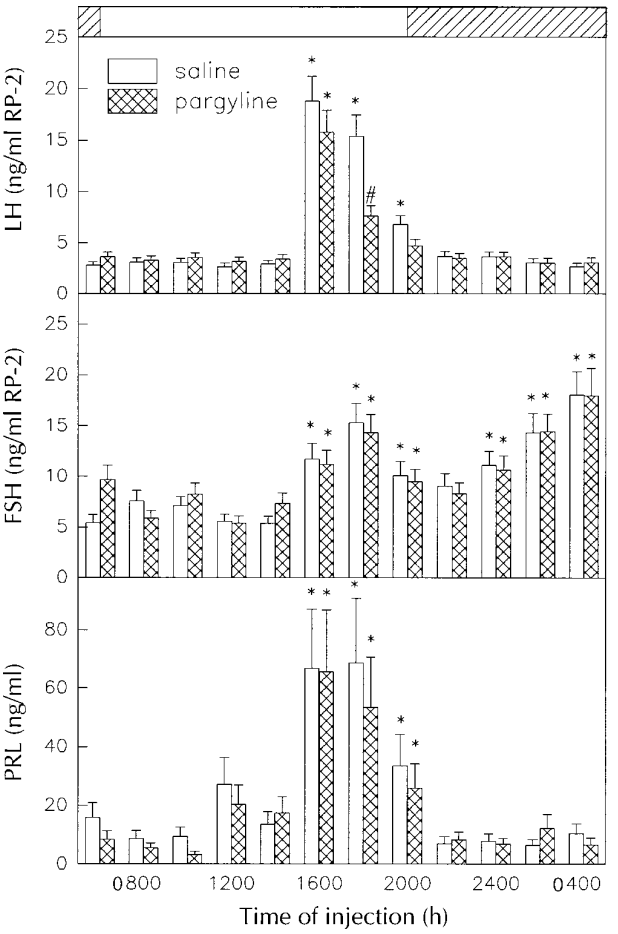

Figure 3 Effect of pargyline on serum LH, FSH and PRL levels in PRO hamsters. Animals were killed by rapid decapitation 10 min after injection. * Indicates hormone levels that are significantly different from basal, while # indicates LH levels in pargyline-injected animals that differ from LH levels measured in animals injected with saline at the same time ($P<0{\cdot}0007$ in both cases).

$P=0{\cdot}0039$). Post-hoc analysis showed that pargyline decreased LH levels (10 min after injection) at 1800 h in PRO (Fig. 3) and PIA (Fig. 4) hamsters. PRL and FSH levels were unaffected by pargyline in this experimental paradigm.

In regards to 5HT turnover rates, time of injection ($F(11,734)=2{\cdot}66$, $P=0{\cdot}0025$) and brain area ($F(3,734)=11{\cdot}93$, $P<0{\cdot}0001$) and all interactions significantly affected 5HT turnover rates (time of day*reproductive state, $F(22,734)=2{\cdot}36$, $P=0{\cdot}0005$; time of day*brain area, $F(33,734)=3{\cdot}14$, $P<0{\cdot}0001$; reproductive state*brain area, $F(6,734)=4{\cdot}38$, $P=0{\cdot}0002$; time of day*brain area*reproductive state, $F(66,734)=2{\cdot}16$, $P<0{\cdot}0001$). Post-hoc analysis revealed that on DIE, 5HT turnover rates were significantly increased at 1200 h, 2000 h and 2400 h in the SCN and at 0600 h, 0800 h and 1400 h in the ME (Fig. 5). On PRO, on the other hand, 5HT turnover was significantly increased only at 0600 h and 2200 h in the ME (Fig. 6). In contrast, in PIA females, 5HT turnover was only elevated at 0600 h in the ME (Fig. 7).

Experiment 3

Levels of LH and PRL were affected by reproductive state (LH, $F(2,273)=214{\cdot}95$, $P<0{\cdot}0001$; PRL, $F(2,272)=511{\cdot}01$, $P<0{\cdot}0001$), time of day (LH, $F(1,273)=144{\cdot}38$, $P<0{\cdot}0001$; PRL, $F(1,272)=301{\cdot}45$, $P<0{\cdot}0001$), treatment with 5HT receptor ligands (PRL, $F(3,272)=30{\cdot}85$, $P<0{\cdot}0001$), and treatment with phenobarbital (LH, $F(1,273)=224{\cdot}64$, $P<0{\cdot}0001$; PRL, $F(1,272)=14{\cdot}54$, $P=0{\cdot}0002$). The interactions of these factors also significantly affected levels of both LH and PRL, including the

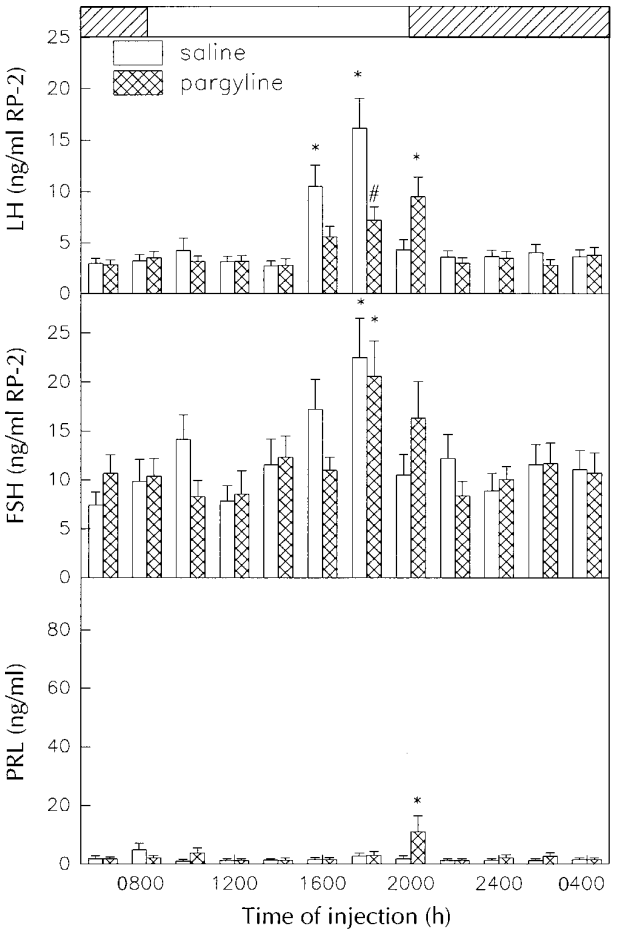

Figure 4 Effect of pargyline on serum LH, FSH and PRL levels in PIA hamsters. Animals were killed by rapid decapitation 10 min after injection. * Indicates hormone levels that are significantly different from basal, while # indicates LH levels in pargyline-injected animals that differ from LH levels measured in animals injected with saline at the same time ($P<0{\cdot}0007$ in both cases).

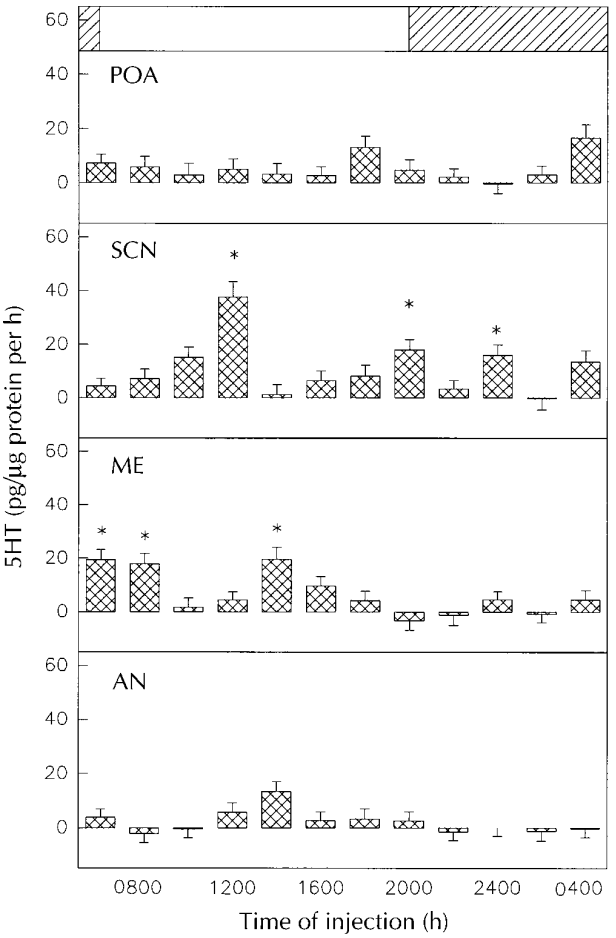

Figure 5 5HT turnover rates in microdissected brain areas on DIE. Animals were killed by rapid decapitation 10 min after injection and turnover rate was calculated as described in the Materials and Methods. * Indicates significantly elevated 5HT turnover rate ($P<0{\cdot}0003$ versus at least five other points).

interaction of all four factors (LH, $F(10,273)=25{\cdot}54$, $P<0{\cdot}0001$; PRL, $F(10,272)=3{\cdot}57$, $P=0{\cdot}0002$).

Post-hoc analysis of the LH data showed that the PRO surge was attenuated by administration of the 5HT agonist quipazine (Fig. 8). LH levels in DIE and in phenobarbital-blocked PRO animals were unaffected by any of the receptor ligands.

The pattern of the PRL response in these animals differed from the LH response (Fig. 9). First, the two 5HT antagonists did not affect PRL levels in the same way. Methysergide administration attenuated the PRL surge in both DIE and PRO animals that were not treated with phenobarbital. In contrast, cyproheptadine amplified the surge on DIE, while not affecting the amplitude of the PRO surge. Secondly, in PRO animals in which phenobarbital was administered at 1400 h, the surge of PRL was unaffected by phenobarbital or any of the 5HT receptor ligands. Thirdly, in regards to basal levels of PRL, both quipazine and cyproheptadine increased PRL levels measured at 1330 h in PRO, but not DIE, animals. However, quipazine administration blocked the PRL surge in both DIE and PRO females given saline at 1400 h.

Discussion

The data gathered in experiment 2 showed that at several times during the day high turnover rates of 5HT occur in the SCN of DIE hamsters (Fig. 5), but are not observed in the SCN of PRO and PIA hamsters (Figs 6 and 7). Thus on DIE, during which no gonadotropin surge occurs, high serotonergic activity was observed in the SCN (Fig. 2). Conversely, in PRO and PIA females, which are characterized by a surge in the late afternoon of

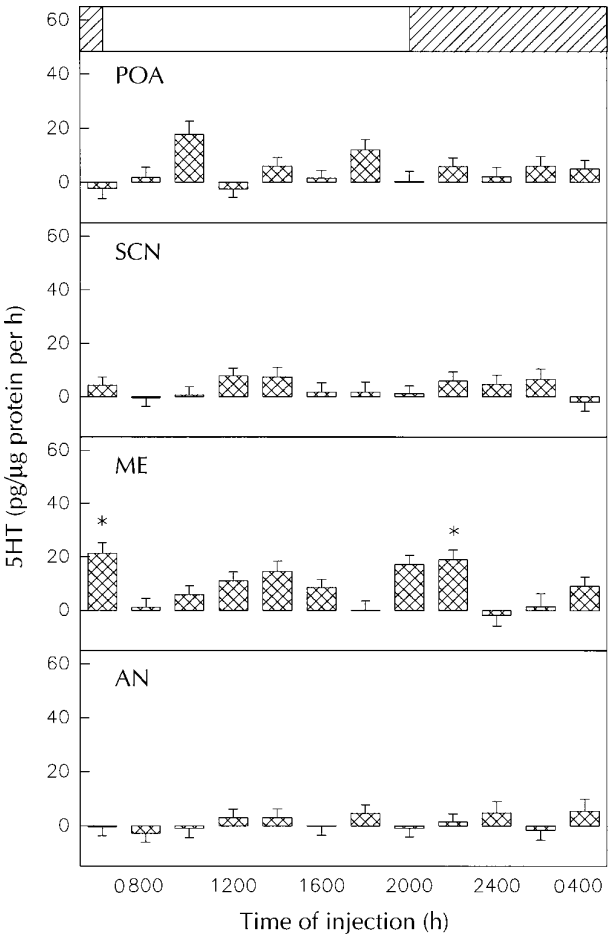

Figure 6 5HT turnover rates in microdissected brain areas of PRO hamsters. Animals were killed by rapid decapitation 10 min after injection and turnover rate was calculated as described in the Materials and Methods. * Indicates significantly elevated 5HT turnover rate ($P<0{\cdot}0003$ versus at least five other points).

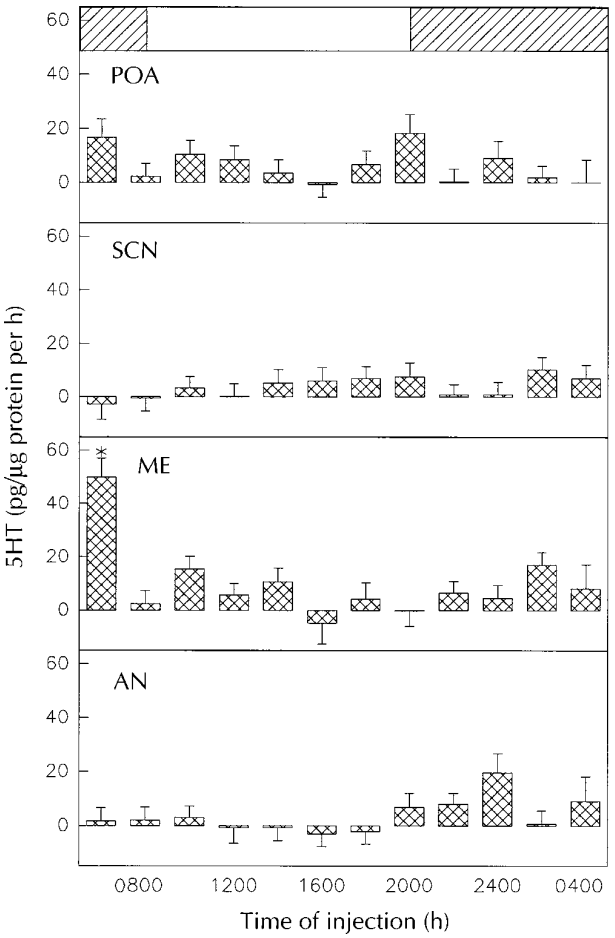

Figure 7 5HT turnover rates in microdissected brain areas of PIA hamsters. Animals were killed by rapid decapitation 10 min after injection and turnover rate was calculated as described in the Materials and Methods. * Indicates significantly elevated 5HT turnover rate ($P<0{\cdot}0003$ versus at least five other points).

LH and FSH (Figs 3 and 4), no significant elevations of 5HT turnover were measured in the SCN. This suggested the hypothesis that elevated serotonergic activity in the SCN is part of the mechanism by which the gonadotropin surge is prevented on DIE. Indeed, the serum hormone measurements from experiment 2 tend to support this idea in that administration of pargyline, which results in the accumulation of 5HT (Fig. 1), inhibits serum LH concentrations 10 min after injection during either the PRO or PIA surges (Figs 3 and 4).

Because 5HT turnover rates were low and unchanging throughout the day in the POA and AN of DIE, PRO and PIA females (see Figs 5, 6 and 7), serotonergic activity in these areas is probably not important in the generation of clock-timed gonadotropin surges. Other investigators (Johnson & Crowley 1986, Cohen & Wise 1988*a*) have also reported the absence of a diurnal rhythm of 5HT turnover in the medial POA and AN of OVX-E_2 rats.

In the ME, the data showed significant elevations of 5HT turnover rates occurring at different times of day in each of the three reproductive states examined. These differing diurnal rhythms are not easily correlated with gonadotropin or PRL release, nor are they correlated with the daily photocycle. Interestingly, in other studies which examined the ME specifically, 5HT turnover was found to increase during the afternoon in PRO rats (Vitale *et al.* 1984, Kerdelhué *et al.* 1989), to decrease during the afternoon in OVX-E_2 rats (Cohen & Wise 1988*a*), and to remain unchanged in OVX-E_2 and OVX-E_2-P_4-treated rats (Johnson & Crowley 1986). Evidently, since all of these models are characterized by clock-timed LH surges, serotonergic activity in the ME appears not to be critical to the generation of this hormonal rhythm.

We found no trend towards greater serotonergic activity in the MBH of inhibitory photoperiod-housed female

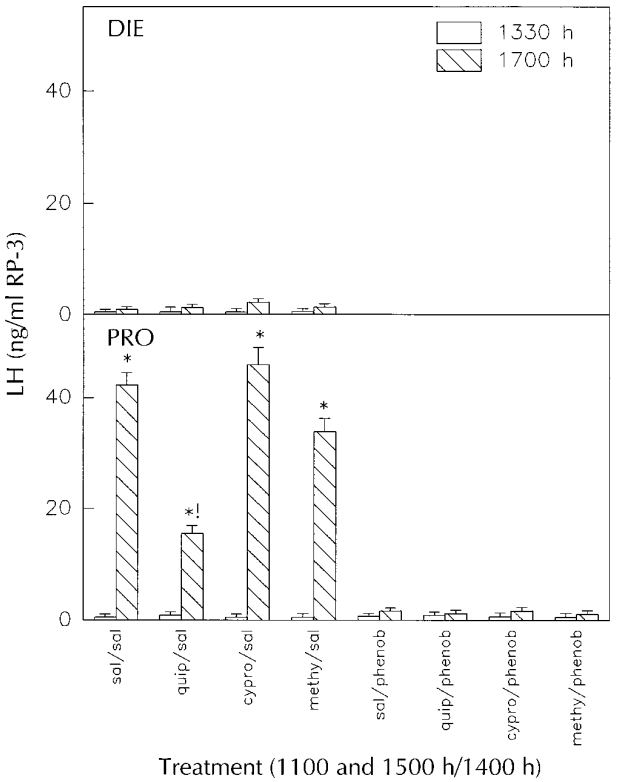

Figure 8 LH in DIE and PRO female hamsters from experiment 3. As indicated on the abscissa, animals were treated at 1100 and 1500 with an injection of saline (sal), quipazine (quip; a 5HT agonist), cyproheptadine (cypro; a 5HT antagonist), or methysergide (methy; a 5HT antagonist). At 1400 h, female hamsters were also treated with injections of saline or phenobarbital (phenob). * Indicates a hormone surge; ! indicates levels at 1700 h that differ from those in controls at the same time ($P<0{\cdot}0012$).

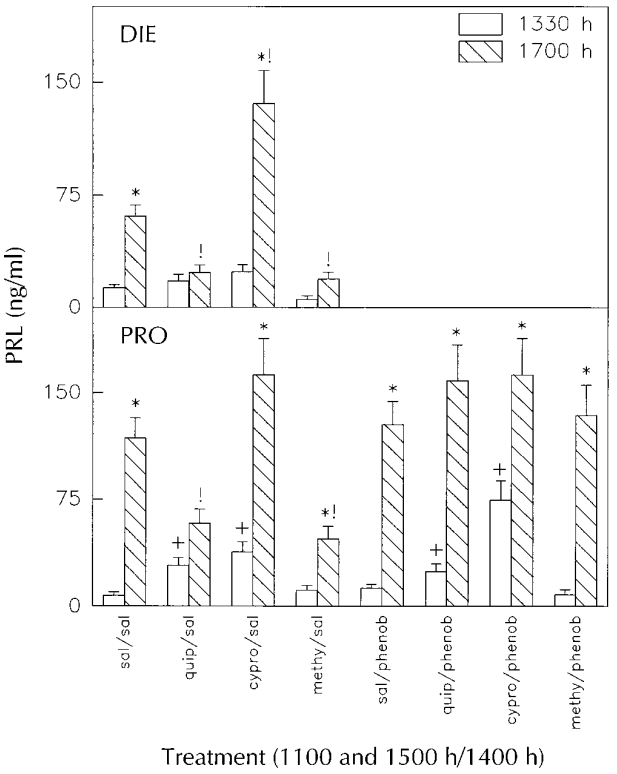

Figure 9 PRL in DIE and PRO female hamsters from experiment 3. As indicated on the abscissa, animals were treated at 1100 and 1500 with an injection of saline (sal), quipazine (quip; a 5HT agonist), cyproheptadine (cypro; a 5HT antagonist), or methysergide (methy; a 5HT antagonist). At 1400 h, female hamsters were also treated with injections of saline or phenobarbital (phenob). * Indicates a hormone surge; ! indicates levels at 1700 h that differ from those in controls at the same time ($P<0{\cdot}0012$); + indicates levels at 1330 h that differ from those in controls at the same time ($P<0{\cdot}0012$).

hamsters. In fact, 5HT turnover rates were increased only at one time in one brain area in PIA hamsters (0600 h, ME; Fig. 7). This is in contrast to the data collected from male rodents, in which 5HT activity is increased in reproductively regressed animals (Glass *et al.* 1988, Vriend 1989, Steger & Bartke 1991). This difference in results may be due to the anatomical specificity of the present study, precluding observation of an increase in parts of the MBH outside of the micropunches isolated in our study. Or, more likely, the discrepancies in our data may be a reflection of differences in the endocrine correlates of reproductive regression between male and female Syrian hamsters: males are characterized by a decrease in LH, FSH, and PRL (Steger & Bartke 1991), while females are characterized by a daily surge of LH and FSH (Bast & Greenwald 1974) and low levels of PRL (Widmaier & Campbell 1981). Thus, the neuroendocrine correlates of reproductive inhibition are also likely to differ between males and females.

Although substantial work has suggested a role for SCN 5HT in the generation of circadian rhythms (Levine *et al.* 1986, Smale *et al.* 1990, Medanic & Gillette 1992, Morin 1992, Possidente *et al.* 1992, Prosser *et al.* 1992, 1993, Selim *et al.* 1993), the data reported here do not support such a relationship. There was a diurnal rhythm of serotonergic activity in the SCN only in DIE females in which 5HT was elevated during the light. These data contrast with those of others who report high release of HIAA from the SCN during the dark period in the Djungarian hamster (Glass *et al.* 1992, 1993*a*,*b*), a high turnover of 5HT also during the dark period in young OVX-E_2 rats (Cohen & Wise 1988*a*), a high turnover rate of 5HT during the light period in young OVX rats (Cohen & Wise 1988*a*), and high content of 5HT during the light period in the SCN of male rats (Cagampang & Inouye 1994) and in the anterior hypothalamus (includes the SCN) of Syrian hamsters (Ferraro & Steger 1990). In hamsters, however, no rhythm of 5HT content in the

anterior hypothalamus (Ferraro & Steger 1990) or 5HT turnover in the SCN (Glass *et al.* 1993*a*) was evident under constant conditions, despite the maintenance of other circadian rhythms. Similarly, the rhythm in 5HT content in the SCN of male rats has a different pattern in animals exposed to either constant light or constant darkness (Cagampang & Inouye 1994). Also, Cohen & Wise (1988*a*) report the absence of a diurnal rhythm of 5HT turnover in middle-aged OVX and OVX-E_2 rats. It should be noted, however, that middle-aged rats still have normal estrous cycle rhythms (Cohen & Wise 1988*a*) and that the E_2-implanted animals would be characterized by a surge of LH. It is clear from the present data and the preceding reports that a diurnal rhythm of 5HT content or 5HT activity in the SCN is not necessary for the maintenance of overt circadian rhythms. However, these data do not refute a role for 5HT in modulating the entrainment or amplitude of rhythms timed by the SCN.

Experiment 3 was designed to test the hypothesis that elevated serotonergic activity in the SCN might be part of the mechanism by which the surge of gonadotropins is prevented on DIE. We employed pharmacological manipulation of 5HT systems in DIE and PRO hamsters and measured the effects on LH and PRL secretion. We reasoned that if elevated 5HT activity does prevent the surge of gonadotropins on DIE, mimicking this activity with the 5HT agonist quipazine should prevent the PRO surge. Similarly, prevention of 5HT receptor activation by administration of 5HT receptor antagonists (methysergide and cyproheptadine) would be expected not to affect the PRO surge (animals in which SCN 5HT activity is already low) and perhaps to elicit a surge in DIE and/or phenobarbital-blocked PRO animals. Although blood was sampled at 1330 h by cardiac puncture and at 1700 h by decapitation, published observations show that hormone concentrations are comparable in animals sampled via these two routes (Talamantes *et al.* 1984, Carlberg & Alvin 1992).

The results of this experiment showed that administration of either of the 5HT antagonists does not affect the PRO surge of LH (Fig. 8), in agreement with our prediction. Likewise, administration of the 5HT agonist quipazine does attenuate the PRO LH surge (Fig. 8). However, the PRO surge is not completely blocked, as the hypothesis had predicted. This indicates that another neuronal system(s?), one that is not inhibited by 5HT, is also involved in the generation of the LHRH and subsequent gonadotropin surges. In addition, antagonist administration does not evoke a surge of LH in either DIE or phenobarbital-blocked PRO females (Fig. 8). This indicates the existence of a non-serotonergic system which inhibits LHRH release on DIE. The failure to evoke a surge of LH in phenobarbital-blocked PRO hamsters may also be due to the same mechanism. A second possible explanation for this latter result is sensitivity of a stimulatory system (efferent to the 5HT-secreting neuron) to phenobarbital anesthesia. However, this stimulatory system must be afferent to the LHRH neurons themselves, since P_4 administration concurrent with administration of phenobarbital during the critical period reinstates the LH surge (Turgeon & Greenwald 1972).

The PRL data, although substantially different from the LH data, also support an inhibitory role for 5HT in the generation of PRL surges. On both DIE and PRO, administration of quipazine blocks the PRL surge (Fig. 9), a result similar to quipazine's effect on the PRO LH surge. However, methysergide and cyproheptadine, the two 5HT antagonists utilized, have opposite effects on PRL levels. Therefore, the actions of these drugs cannot be ascribed to antagonism of 5HT receptors. Rather, these data most likely reflect secondary actions at dopaminergic receptors, which methysergide may also activate (Douglas 1985).

In regards to basal levels of PRL, administration of both cyproheptadine and quipazine increased serum concentrations of this hormone in PRO hamsters (Fig. 9). The quipazine results, in particular, are interesting in that they may indicate two sites of action of 5HT, a stimulatory system that predominates during the early afternoon on PRO and an inhibitory system that predominates during the late afternoon on both PRO and DIE. Given that this drug acts as an agonist at more than one subtype of 5HT receptor (Van de Kar & Brownfield 1993), this hypothesis would be worthy of pursuit as more specific agonists become available.

Phenobarbital administration did not prevent the PRO surge of PRL. However, quipazine's action of suppressing the surge was abolished, indicating that the inhibitory effect of quipazine does not occur at the level of the anterior pituitary, but must occur afferent to a phenobarbital-sensitive neuron.

Like the 5HT turnover data, these pharmacological data support an inhibitory action of 5HT neuronal systems on the generation of reproductive hormone surges. These data also indicate that 5HT is involved in whether a surge will occur or not, rather than strictly inhibiting levels of hormone. This is evident when levels of PRL are measured, in that quipazine both increases basal levels and decreases surge levels in the same animals. Although differences in 5HT activity in the SCN are associated with the presence or absence of a gonadotropin surge, these differences are not easily related to plasma levels of gonadal steroids. Indeed, gonadotropin surges in the female Syrian hamster do not rely on ovarian steroids, since daily afternoon surges of LH and FSH persist in OVX animals which do not receive E_2 replacement (Bridges & Goldman 1975, Stetson *et al.* 1978). Not unexpectedly, our data also indicate that 5HT is not the only hypothalamic neurotransmitter involved in the regulation of LH, FSH, and PRL surges.

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