Atrazine Disrupts the Hypothalamic Control of Pituitary-Ovarian Function

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The chloro-S-triazine herbicides (i.e., atrazine, simazine, cyanazine) constitute the largest group of herbicides sold in the United States. Despite their extensive usage, relatively little is known about the possible human-health effects and mechanism(s) of action of these compounds. Previous studies in our laboratory have shown that the chlorotriazines disrupt the hormonal control of ovarian cycles. Results from these studies led us to hypothesize that these herbicides disrupt endocrine function primarily through their action on the central nervous system. To evaluate this hypothesis, we examined the estrogen-induced surges of luteinizing hormone (LH) and prolactin in ovariec-tomized Sprague-Dawley (SD) and Long-Evans hooded (LE) rats treated with atrazine (50–300 mg/kg/day, by gavage) for 1, 3, or 21 days. One dose of atrazine (300 mg/kg) suppressed the LH and prolactin surge in ovariec-tomized LE, but not SD female rats. Atrazine (300 mg/kg) administered to intact LE females on the day of vaginal proestrus was without effect on ovulation but did induce a pseudopregnancy in 7 of 9 females. Three daily doses of atrazine suppressed the estrogen-induced LH and prolactin surges in ovariec-tomized LE females in a dose-dependent manner, but this same treatment was without effect on serum LH and prolactin in SD females. The estrogen-induced surges of both pituitary hormones were suppressed by atrazine (75–300 mg/kg/day) in a dose-dependent manner in females of both strains evaluated after 21 days of treatment. Three experiments were then performed to determine whether the brain, pituitary, or both organs were the target sites for the chlo-rotiazines. These included examination of the ability of (1) the pituitary lactotrophs to secrete prolactin, using hypophysecto-mized females bearing pituitary autotransplants (ectopic pitui-taries); (2) the synthetic gonadotropin-releasing hormone (GnRH) to induce LH secretion in females treated with high concentrations of atrazine for 3 days; and (3) atrazine (administered in vivo or in vitro) to suppress LH and prolactin secretion from pituitaries, using a flow-through perfusion procedure. In conclusion, the results of these studies demonstrate that atrazine alters LH and prolactin serum levels in the LE and SD female rats by altering the hypothalamic control of these hormones. In this regard, the LE female appeared to be more sensitive to the hormone suppressive effects of atrazine, as indicated by the decreases observed on treatment-day 3. These experiments support the hypothesis that the effect of atrazine on LH and prolactin secretion is mediated via a hypothalamic site of action.

Key Words: atrazine; prolactin; luteinizing hormone; hypothalamus; reproduction.

Atrazine is a chloro-S-triazine herbicide that is employed extensively in agriculture worldwide (Gressel et al., 1984; Stevens and Sumner, 1991). In plants, the principal mode of action of chlorotriazines is to inhibit photosynthesis (Gysin and Knuesli, 1960). The adverse health effects of these compounds in mammalian species have not been well characterized. In studies using Sprague-Dawley (SD) rats, it was reported that dietary exposure to atrazine (400 ppm) or the related triazine, simazine, led to an earlier onset and greater incidence of mammary tumors (Stevens et al., 1994; Wetzel et al., 1994). Since neither atrazine nor simazine appears to be genotoxic, it has been proposed that chlorotriazine administration promotes the development of mammary gland tumors by inducing a premature reproductive senescence, thus creating an endocrine milieu conducive to tumor growth (Eldridge et al., 1998; Stevens et al., 1994). In a previous study, we reported that exposure to atrazine by gavage (75–300 mg/kg) led to a rapid disruption of ovarian cycling in SD and Long-Evans hooded (LE) females (Cooper et al., 1996b). At the highest dose tested, atrazine induced anestrus as indicated by prolonged vaginal diestrus, atrophied ovaries, and basal concentrations of serum gonadotropins and ovarian steroids (Cooper et al., 1996b). The hormonal changes observed following atrazine exposure suggested that this compound alters ovarian function through an action of the chlorotriazine on the brain and/or pituitary. In fact, we reported elsewhere that atrazine can...
inhibit the ovulatory surge of luteinizing hormone (LH) and prolactin and the pulsatile release of LH (Cooper et al., 1996a). Similarly, Simpkins et al. (1998) found decreased LH and prolactin secretion following atrazine exposure. In this study, we further characterized the effect of atrazine on pituitary hormone secretion in an effort to determine the possible target site through which this herbicide acts, whether or not these changes in pituitary hormone secretion are like those observed during normal aging in the female rats, and whether there is a difference in the way atrazine influences pituitary hormone secretion in LE versus SD female rats.

MATERIALS AND METHODS

Animals. Female LE and SD rats (60 days old) were purchased from Harlan Sprague-Dawley (Madison, WI) and housed in a room maintained at 22 ± 2°C with a 14:10 h light:dark schedule (lights on at 0500 h:lights out at 1900 h). Food and water were provided ad libitum. Beginning at 90 days-of-age, the ovarian cycle of each female was monitored for a period of 2 weeks by taking daily vaginal lavages as detailed elsewhere (Cooper et al., 1999). Only females displaying 4-day estrous cycles were included in the experimental groups.

Dosing. Technical grade atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine) of 97.1% purity was generously supplied by National Pesticides, Inc. (Greensboro, NC). The doses of atrazine employed in the present experiments are similar to those used by Eldridge et al. (1994) and Cooper et al. (1996b). Dosing solutions were prepared as suspensions in a vehicle of 1% methylcellulose (MC) in distilled water. The doses used for each experimental group are described below. Control animals were gavaged daily with 1% MC vehicle (0.5 ml/100 g body weight).

Ovariectomy and Estradiol Implants

In those studies evaluating the effect of atrazine on the estrogen-induced LH and prolactin surges, all females were anesthetized with a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg) administered ip and bilaterally ovariectomized on day 0. At the time of ovariectomy, an 8 mm, estrogen-filled silastic capsule (Scientific Products medical grade silicone tubing 1.57 mm ID × 3.18 mm OD) containing estradiol benzoate (4 mg/ml in sesame oil) was implanted subcutaneously in the right flank region. All surgeries were performed between 1200–1300 h. Previous work in our laboratory has demonstrated that these capsule dimensions and estradiol concentration maintained blood estradiol levels (80–100 pg/ml) comparable to those seen in the intact proestrus animal (Goldman and Cooper, 1993a). This implant also produces a daily, late-afternoon surge of LH and prolactin and thus provides a means to obtain a synchronized cohort of animals in which the timing and amplitude of each hormone surge can be evaluated under different doses of atrazine. Also, by using this model, any potential confounding effects of atrazine on ovarian hormone secretion or concentration is eliminated. In these experiments, we characterized the LH and prolactin surges on day 3 (72 h) after the capsule was implanted.

Experiment 1: Time and Dose-Response Effects of Atrazine on LH and Prolactin Secretion in Long-Evans Hooded and Sprague-Dawley Rats

In this study, we evaluated the effects of 4 different doses of atrazine on the estrogen-induced LH and prolactin surges after a single (1×) or multiple day (3 days = 3× or 21 days = 21×) exposure. 1× females. To evaluate the immediate effects of atrazine on pituitary hormone secretion, females in each strain were ovariectomized and received an estrogen implant on day 0. On day 3, groups of females were gavaged with 0, 50, 100, 200, or 300 mg/kg atrazine at 1200 h. Ten females receiving each dose of atrazine were then killed by decapitation at 1200, 1300, 1500, and 1800 h (e.g., 0, 1, 3, or 6 h post-gavage). Trunk blood was collected in 16-× 125-mm serum-separation tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged (1260 × g at 4°C for 30 min). The serum was subsequently stored at −70°C until assayed for LH, follicle-stimulating hormone (FSH), prolactin, thyroid stimulating hormone (TSH), and estradiol. The pituitaries were removed and the neural lobe was dissected free and discarded. The remaining anterior lobe was weighed, placed in 1 ml of HEPES-buffered (10 mM, pH 7.4) medium 199 containing 0.3% BSA (Sigma, fraction V), sonicated (Fisher Sonic Dismembrator, Model 300), and frozen immediately at −70°C until assayed for LH, FSH, prolactin, TSH, and estradiol.

3× females. Groups of LE and SD females were ovariectomized and estrogen-filled silastic capsules were implanted subcutaneously on day 0. Atrazine (0, 50, 100, 200, or 300 mg/kg) was administered by gavage at 1200 h on days 1, 2, and 3. Ten animals in each group were then killed at 1200, 1300, 1500, and 1800 h on day 3. The blood and pituitaries were processed as described above for the 1× females.

Experiment 2: Effect of Atrazine on Ovulation

We reported previously that continued dosing (up to 21 days) with atrazine interfered with regular ovarian cycles (Cooper et al., 1996b). To determine the effect of a single dose of atrazine on ovulation and ovarian cycling, intact LE females displaying regular 4-day estrous cycles were gavaged with 0, 75, 150, or 300 mg/kg atrazine at 1200 h on the day of vaginal proestrus (n = 20 each group). The effect of this treatment on ovarian function was determined in half of the animals in each dose group by examining the vaginal smear daily for 3 additional weeks after injection, using previously published methods (Cooper et al., 1999). In the remaining animals, the effect of the proestrous dose of atrazine on ovulation was determined. Oocytes were collected (1400–1500 h) on the day of vaginal estrus and quantified according to procedures described by Perreault and Mattson (1993). Briefly, the oviduct was dissected from the ovary and a cut made at its junction with the uterus. Under a dissecting microscope, the ampulla was flushed with 200 µl of Dulbecco’s PBS (Gibco, Gaithersburg, MD) containing 0.1% (w/v) BSA (fraction V, Sigma Chemical) using a 1-ml syringe with attached 30-gauge blunt needle inserted into the infundibulum. The cumulus mass was extruded from the cut end of the oviduct, and individual cumulus-encased oocytes were counted.

Experiment 3: Examining the Site of Action of Atrazine

Experiment 3a: Prolactin secretion from the ectopic pituitary. To determine whether atrazine alters pituitary prolactin secretion via a direct effect on the lactotrophs, the pituitary was removed from the sella turcica and implanted under the kidney capsule. For this procedure, LE female rats were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg) administered ip and placed in a David Kopf (model 1400) stereotaxic unit. The pituitary was aspirated from the sella turcica through a hypophysectomy ear bar (David Kopf Instruments, Tejunga CA). The aspirated anterior lobe fragments (approximately 1-mm3) were then placed in sterile saline and implanted under the kidney capsule as described by Nikitovitch-Winer and Everett (1958). The rats were then allowed 4 weeks to recover. On the test day, 2 baseline tail-blood samples (200 µl each) were obtained at 0 and 1 h prior to the administration of atrazine (300 mg/kg, by gavage) at 0 h (1300 h rat time). Six hourly post-dosing tail-blood samples (200 µl each) were then obtained from each female (n = 7) and the blood was processed for prolactin analysis as described above.
Experiment 3b: LH secretion following intravenous GnRH administration. LE rats were ovarectomized and implanted with an 8 mm silastic capsule containing estradiol benzoate at approximately 1330 h (see above). Three daily doses of atrazine (300 mg/kg) in 1% MC were administered by gavage, beginning 24 h after the surgery (i.e., 1330 h). Control females received 1% MC only. Immediately after the third dose, the females were fitted with indwelling cardiac catheters under halothane anesthesia as previously described (Goldman and Cooper, 1993a; Harms and Ojeda, 1974). Two h after placement of the catheters, a baseline bleed of 200 µl was taken and replaced with 100 µl of heparinized saline (ICN Biomedical, 10 IU heparin per ml saline). Ten min later another baseline bleed was obtained and immediately thereafter 50 ng/kg gonadotropin releasing hormone (GnRH) (Bachem, Torrance, CA), dissolved in saline, was administered through the catheter. Additional blood samples were collected at 5, 15, 30, 45, and 60 min following the bolus of GnRH. The blood was centrifuged and serum stored at −70°C until assayed for LH as described below.

Experiment 3c: Effect of atrazine on in vitro prolactin and LH release as determined by pituitary perfusion. To further determine any possible direct effects of atrazine on the pituitary hormone secretion, we examined LH and prolactin release using pituitary perfusion (Cooper et al., 1987; Goldman and Cooper, 1993b). After removing the pituitaries, the neural lobe was immediately dissected free and the anterior lobe placed on a sterile watch glass containing 1 ml ice cold Medium 199. The anterior pituitary piece was then hemisectioned along the rostral-caudal plane. One-half (randomly selected left or right portion) was weighed, sonicated in 500 µl Medium 199, and frozen for subsequent assays for LH and prolactin. The remaining half was promptly dissected free and the anterior lobe placed on a sterile watch glass containing oxygenated Medium 199. For collection of samples, both the perfusion system and procedure were consistent with that described in Goldman and Cooper (1993a,b). The buffer was Medium 199 (Earle’s salt with L-glutamine, Gibco formula #78–5051EA) with 0.2% BSA, 13 mM sodium bicarbonate, 10 mM HEPES, 50 µM Bacitracin (pH 7.4) prepared without phenol red as an indicator. After carefully transferring tissue fragments to the perfusion chambers, the tissue was perfused for an initial 2-h period to allow sufficient time for hormone secretion to reach baseline. Samples were then collected every 10-min, using a flow rate of 150 µl/min. After three 10-min baseline samples, the hemipituitaries were exposed to two 30-min challenges (spaced 60 min apart) with a combination of GnRH (85 nM), TRH (100 nM, Bachem, Torrance, CA). After each 10-min sample, tubes were checked for accuracy of flow rate and the collection tubes were sealed prior to being frozen at −70°C. After the second challenge, tissues were exposed once more (10 min) with a depolarizing stimulus of 60 mM KCl to assess tissue viability. Sample collections were then continued for an additional 40 min.

Radioimmunoassays

The concentration of LH, FSH, prolactin, and TSH in the serum and pituitary tissue and in perfusion media were determined with the following materials supplied by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases: Iodination preparations = I-6 (LH), I-6 (FSH), I-5 (prolactin) and I-9 (TSH); reference preparations = RP-2 (LH), RP-2 (FSH), RP-3 (prolactin) and RP-3 (TSH); Antisera = S-8 (LH), S-11 (FSH), S-9 (prolactin) and S-6 (TSH). The purified iodination preparations were radiolabeled with 125I (New England Nuclear) using the chloramine-T method of Greenwood et al. (1963). Each labeled hormone was separated from unreacted iodide on a BioGel P-60 (Bio-Rad Laboratories) column with potassium phosphate buffer (50 mM, pH 7.5). For the assay, goat anti-rabbit gamma globulin (Calbiochem) was used as the second antibody. The assay sensitivities of LH, FSH, and TSH were determined by a 24-h co-incubation of sample and first antibody prior to the addition of the labeled hormone. The respective sensitivities, inter-and intra-assay coefficients of variation for the 4 pituitary hormones were: 15 pg/tube, 7.7% and 5.9% (LH), 150 pg/tube, 8.3% and 12.4% (FSH), 32 pg/tube, 7.0% and 5.3% (prolactin) and 195 pg/tube, 7.2 and 6.2 % (TSH). Serum estradiol concentrations were determined using antibody-coated tube kits purchased from Diagnostic Products Corp. (Los Angeles, CA). The inter- and intra-assay coefficients of variation determined using controls provided by Diagnostic Products Corporation were 6.8% and 4.1%, respectively.

Statistical analysis. Serum and pituitary hormone levels and number of ova shed were analyzed for statistical significance by Analysis of Variance (ANOVA). Comparison of hormone levels among individual treatment conditions with the controls within each time period were further examined by Dunn’s t-test for multiple comparisons using INSTAT (San Diego, CA). In the 21-day-treated females, overall differences in body weight among control and treatment groups and the change in body-weight gain within a single dose group were tested using one-way, repeated measures analysis of variance (ANOVA) and Dunnett’s multiple comparison test.

RESULTS

Experiment 1: Time- and Dose-Response Effects of Atrazine on LH and Prolactin Secretion in Long-Evans Hooded and Sprague-Dawley Rats

The effect of a single or 3 daily doses of atrazine on the estrogen-induced LH and prolactin surges are shown in Figures 1 and 2. In SD rats, the prolactin surge was significantly reduced following 3 daily doses of 300-mg/kg atrazine. However, the secretion of these 2 hormones in SD females was not different from controls at any of the other time points or doses of atrazine examined. In contrast, atrazine exposure to LE females resulted in a marked attenuation of both the LH and prolactin surges. After a single dose of 300-mg/kg atrazine, the amplitude of the LH and prolactin surges were significantly reduced at 1, 3, and 6 h. Three daily doses of 100, 200, or 300 mg/kg atrazine completely suppressed the estrogen-induced surges of both hormones. The onset of the LH surge in control LE females occurred at 1 h with peak concentrations occurring in most animals at the 3-h time point. Although surges of both LH and prolactin were observed in the LE females receiving 3 doses of 50 mg/kg atrazine, the onset of the surge was delayed as evidenced by the presence of baseline values at 1 and 3 h and a peak appearing at 6 h (Fig. 2). In the 3×LE females, atrazine exposure inhibited the drop in pituitary prolactin concentration that was observed in control females during the observation period (Fig. 3).

The effect of atrazine on the LH and prolactin surge in females dosed for 21 days is shown in Fig. 4. In both strains, atrazine resulted in a significant dose-dependent suppression of serum LH and prolactin. In addition, in both strains, the concentration of LH and prolactin in the pituitary was altered following exposure to atrazine. In the 21-day-treated female, the amount of both LH and prolactin present in the pituitary was generally greater in both rat strains (Fig. 4).

Body weight was unaffected in the females receiving atrazine for 1 or 3 days. Similarly, atrazine was without effect on body weight in the 21×females at 75 and 150 mg/kg. However, the rate of body weight gain in those females receiving 300 mg/kg was significantly lower than control animals.
Mean pituitary weight was not affected in any of the groups evaluated. The concentration of serum or pituitary TSH and FSH was not different from controls at any of the doses or time points examined and serum estradiol concentrations were also similar in control and treated groups (data not shown).

**Experiment 2: Effect of Atrazine on Ovulation**

It is interesting that although the dose of 300 mg/kg did markedly attenuate the amplitude of both the estrogen-induced LH and prolactin surges in the LE females, this dose was without effect on ovulation (Table 1). Examination of the vaginal smears in a different group of rats treated with 300 mg/kg on vaginal proestrus revealed that 7 out of 9 females became pseudopregnant. The lower doses of atrazine either were without effect on the number of oocytes shed, as measured the morning after dosing, the day of estrus in the subsequent cycle, or on subsequent ovarian cycles as determined by vaginal smears. Importantly, continued dosing with 300-mg/kg atrazine for 3 days, beginning on vaginal diestrus day 1, did block the appearance of subsequent proestrus and ovulation (Table 1).

**Experiment 3: Examining the Site of Action of Atrazine**

3a: Prolactin secretion from the ectopic pituitary. In order to determine whether atrazine has the potential to alter prolactin by a direct action on the lactotroph, we examined the effect of this chlorotriazine on the prolactin levels in female rats bearing ectopic pituitary implants. Serum prolactin concentrations determined at hourly intervals, beginning one h before, at the time of injection, and for the next 6 h thereafter, remained unaltered at all the time points examined. This indicates that the secretion of this hormone by the pituitary is not altered by atrazine if the gland is removed from the influence of CNS factors (Fig. 5).

3b: LH secretion following intravenous GnRH administration. In order to determine whether or not the suppression of the estrogen-induced LH surge observed after three days of atrazine exposure could be reversed by intravenous GnRH
exposure, LH secretion was examined in atrazine-treated females bearing indwelling cardiac catheters. LH secretion remained at basal levels in atrazine saline-treated females (Fig. 6). However, an LH surge was observed in the atrazine GnRH females. In fact, serum LH concentrations in the atrazine GnRH females were comparable to the estrogen-induced LH surge observed in control females (e.g., see Figs. 1 and 2), indicating that the effect of atrazine on LH secretion is not the result of a direct impairment of LH release from the gonadotrophs.

3c: Effect of atrazine on in vitro prolactin and LH release as determined by pituitary perifusion. A pituitary perifusion system was used to further determine whether the observed effects of in vivo atrazine exposure on the circulating levels of LH and prolactin could be due to a direct pituitary response to toxicant exposure. No differences in either LH (see Fig. 7) or prolactin (data not shown) release were noted from the pituitaries of untreated females exposed to atrazine in vitro. Similarly, no changes in basal or GnRH-stimulated LH release or TRH-stimulated prolactin release were noted in the pituitaries obtained from females dosed with atrazine (0, 100, or 200 mg/kg) by gavage for 3 days (data not shown). In these same pituitaries, there was an initial increase in prolactin release from LE pituitaries that may be associated with a modest elevation in pituitary prolactin concentrations (see above, Experiment 1).

DISCUSSION

The data from these experiments demonstrate that atrazine has a dramatic effect on the neuroendocrine control of ovarian function in the rat. Although a clear strain difference was noted during the early treatment periods, continued dosing revealed that this herbicide inhibited the release of LH and prolactin in both LE and SD females in a dose-dependent fashion. In LE females, the amount of each hormone secreted in response to estrogen stimulation was markedly suppressed by a single dose of 300 mg/kg of atrazine, while neither hormone appeared to be altered by a single exposure to the lower doses. In SD females, a single dose of atrazine did not alter serum concen-
trations of LH or prolactin at any dose tested. In LE females, 3 daily doses of atrazine significantly altered the onset and the amplitude of both LH and prolactin surges, with the 2 higher doses leading to an almost complete inhibition of the hormone surges. At 50 mg/kg, 3 days of atrazine dosing caused a delay in the onset of the LH and prolactin surges, but did not appear to alter the peak concentration of either hormone once the surge did occur. The LH and prolactin surges in the females treated with atrazine for 3 weeks were suppressed in both strains and at each dose tested. We were not able to establish a NOAEL for the effect of atrazine on the serum hormones in the females of either strain treated for 21 days. Similarly, all 3 doses of atrazine altered the secretion of both hormones in 3× LE females. The 50-mg/kg dose, which delayed the onset of the LH and prolactin surges in the 3× LE females, is similar to that reported to induce mammary gland tumors in females exposed to atrazine for several months.

The fact that atrazine inhibits the estrogen-induced surge of LH would support the hypothesis that the chlorotriazines bring about changes in the neuroendocrine control of ovarian function similar to those known to occur prior to the loss of ovarian cycling in the aging rat. In most rat strains (LE and SD included), reproductive senescence develops by one year-of-age. It is characterized by the appearance of persistent or constant estrus, a condition in which the vaginal smear remains cornified and the ovaries are polyfollicular and without corpora lutea (Cooper et al., 1986; Everett, 1989). There is general agreement that the underlying neuroendocrine events responsible for the loss of ovarian cycling results from changes within the CNS that lead to a decrease in the amplitude and a delay in the onset of the proestrous LH surge (Cooper et al., 1980; van der Schoot, 1976). These alterations in the pre-ovulatory surge of LH are thought to result from an age-associated reduction in the frequency of the GnRH pulses (Scarbrough and Wise, 1990)). We reported elsewhere that atrazine can reduce GnRH pulse frequency (Tyrey et al., 1996). The age-dependent changes in pulsatile GnRH release are reported to result from the cumulative, lifetime exposure to endogenous estrogen (Brawer et al., 1980), as the regulation of GnRH in the older rat is affected only minimally if the female is ovariectomized at an early age (Scarbrough and Wise, 1991). Furthermore, age-related changes in ovarian cycles can be restored with centrally acting pharmacological agents (e.g., catecholamine precursors) known to enhance GnRH and LH activity in rats (Forman et al., 1980; Linnoila and Cooper, 1976; Watkins et al., 1975) and mice (Flurkey et al., 1987). Thus, the present studies indicate that this herbicide can bring about changes in LH secretion that are similar to those observed during reproductive aging in the female rat.

Although atrazine causes changes in LH secretion similar to those observed during aging, several questions remain concerning how this herbicide may contribute to premature reproductive senescence. At least two scenarios remain possible. First, if atrazine is able to suppress the LH surge, it would be expected that the effect of the
compound on LH would exacerbate any age-associated alterations that are present and subsequently lead to an earlier loss of cycling. To our knowledge, such effects in the middle-aged female have not been examined. Alternatively, the acceleration of reproductive aging reported by Stevens et al. (1994) and Eldridge et al., (1998) may have been the result of chronic, low-dose exposure to atrazine. In this case, atrazine may cause damage to hypothalamic-pituitary mechanisms that are either additive to, or synergistic with, the age-related changes in neuroendocrine function that normally occur in the female rat. Again, the precise mechanism(s) altered by atrazine remain to be determined. As noted above, normal aging in this neuroendocrine axis is believed to have resulted from a cumulative exposure to endogenous estradiol (Brawer et al., 1980), suggesting the possibility that atrazine acts in a fashion similar to that of estrogen in mediating premature senescence. However, atrazine does not bind to the estrogen receptor (Connor et al., 1996; Tennant et al., 1994). In fact, most of the current data on this compound indicate that it is anti-estrogenic in nature. For example, estrogens consistently lead to increased prolactin synthesis and release, whereas the current data clearly show that atrazine has an opposite effect on prolactin. Also, atrazine attenuates the estrogen-induced uptake of tritiated thymidine in the uterus and at higher doses reduces the estrogen-induced increase in uterine weight (Tennant et al., 1994). High doses of atrazine alone are without effect on these measures. Thus, if atrazine does cause a premature senescence.

![Image of Figure 4](https://example.com/figure4.png)

**FIG. 4.** Effect of 21 daily doses of atrazine (0, 75, 150, or 300 mg/kg) to ovariectomized females on peak serum and pituitary LH and prolactin concentrations in LE and SD females. On day 21, females received an estrogen-filled, silastic capsule. On day 24, groups of rats were killed at 1500 h. The data in this figure are different from that in Figures 1–3, because we did not characterize the full surge at each dose on day 21. Here, groups of animals were killed by decapitation at the time of the expected peak of LH and prolactin observed in our previous studies (i.e., 3 h). Bars represent the mean ± SEM; * = p < 0.05. (Note differences in the absolute y-axis values in this figure and Figures 1–3. This difference is the result of the difference in the time before estrogen replacement, which was immediate in the 1× and 3× females, but followed 21 days of atrazine treatment in the present experiment).
aging within the hypothalamus and pituitary, it does not appear to be due to any estrogenic properties inherent in this compound.

Prolactin secretion was decreased by atrazine, whereas no effect on FSH and TSH were noted under any of the conditions tested. Whether the lack of change in FSH and TSH secretion represents a differential effect of atrazine on the regulation of these two pituitary hormones, or an effect not identified under our test conditions, remains to be determined. The suppression of prolactin by atrazine was unexpected, as previous studies suggested that the chlorotriazines increase the serum concentration of this hormone (Cook et al., 1997; Stevens et al., 1994). The discrepancy between the current data and that of those previous reports may be attributed to several factors, such as differences in age (Stevens et al., 1994 examined old, noncycling females) and hormonal status (Cook et al., 1994 examined young, ovariectomized rats treated with atrazine without estrogen replacement). Serum prolactin levels in the females tested in our experiments were elevated because they all received estradiol implants. Under these conditions, prolactin release was clearly suppressed at doses greater than 100 mg/kg and the surge of this hormone was delayed at 50 mg/kg. Thus as with LH, we did not establish a NOAEL. Elsewhere, we noted that atrazine can inhibit suckling-induced prolactin release with doses as low as 12.5 mg/kg (gavage) administered twice daily (Stoker et al., 1998, 1999). The NOAEL for this effect was 6.25 mg/kg administered twice daily.

In addition to demonstrating a clear effect of atrazine on LH and prolactin secretion, the results of these experiments argue that the mode of action for this effect involves a disruption of the CNS signals regulating pituitary function and not the synthesis of the hormones. Consider the following: exposure to a single high dose of atrazine (300 mg/kg), which suppressed the LH and prolactin surge in both strains of rats, was without effect on prolactin release in those LE females bearing ectopic pituitaries. In this model, the influence of CNS factors on pituitary hormone secretion is removed when the graft is located under the kidney capsule. Thus, if atrazine affected the pituitary directly, prolactin release in the animals bearing ectopic pituitaries should have been suppressed (if the distribution of atrazine is equivalent in the kidney and the intact pituitaries).

In order to determine whether or not the pituitary of the

### TABLE 1

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<th>Dose (mg/kg)</th>
<th>Time of dose</th>
<th>Day of cycle</th>
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<td>Pro</td>
<td>13.8 ± 0.31</td>
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<td></td>
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<td>Pro</td>
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Note. Atr = atrazine, vehicle = 1.0% methyl cellulose, d I = diestrus day 1, d II = diestrus day 2, pro = proestrus, and E = estrus.

a On the day of the estrous cycle, the rat was injected with atrazine as determined by vaginal smear. b Mean ± SEM.

c Different groups of females were dosed with 300 mg/kg atrazine at different times on vaginal proestrus to optimize the possibility that sufficient atrazine would be in the blood at the time of the critical period for the neural trigger of the LH surge (usually 1400–1600 h in the afternoon).

d 7/9 of another group of females injected at this time become pseudopregnant (defined as displaying a diestrous vaginal smear for approximately 12 days or more and having elevated serum progesterone).

e Dosing was initiated on vaginal diestrus I and continued for 3 days. None of these females entered proestrus.
atrazine-exposed rat would secrete LH, we injected synthetic GnRH intravenously into atrazine-exposed females in which LH secretion was suppressed by high atrazine exposure. In every case, a substantial amount of LH was released, which indicated that the gonadotrophs of these animals are responsive to the releasing peptide and that the lack of LH secretion in the atrazine-treated rats with atrazine alone is most likely due to an attenuation in GnRH release.

The apparent lack of a direct effect of atrazine on pituitary function is also supported by the perifusion experiments in which the ability to release LH and prolactin was examined in vitro. In these experiments, direct exposure of the pituitary to atrazine was without effect on release of either hormone. However, we did note an enhanced release of prolactin from pituitaries obtained from rats exposed to atrazine in vivo for 3 days prior to perifusion. This observation would be expected, since we found that the initial concentration of prolactin present in the pituitaries of the 3-day-treated animal to be somewhat greater than that present in controls. Although a longer in vivo exposure may have been required for any effect of atrazine to become manifest, these data clearly do not support the suggestion that the pituitary is a target site for atrazine’s effect on LH and prolactin secretion.

The difference in the effect of atrazine on pituitary hormone release between the two strains of rats observed in the present studies is interesting. A similar difference between these 2 strains was noted in our earlier studies evaluating the effect of this chlorotriazine on ovarian cycles. The LE female appears to be more sensitive to the endocrine-disrupting effects of atrazine immediately following treatment. The reason for this strain difference could not be determined by the current data. However, one possible explanation may be a strain-related difference in the degradation and clearance of atrazine. The chlorotriazine sulfoxide metabolites bind to the sulfhydryl group on a cysteine residue of hemoglobin shortly after exposure (Hamboeck et al., 1981). This covalent binding may...

FIG. 7. Luteinizing hormone secretion (pg released/min/mg tissue) from perifused hemi-pituitary fragments taken from ovariectomized, estrogen-primed female SD rats and exposed to 100 μM atrazine in vitro. Control and atrazine group samples were collected every 10 min over the course of the 380-min perifusion. After an initial 120 min, 3 baseline collections were followed by two 30-min challenges with GnRH (85 nM)/TRH (100 nM) spaced 1 h apart (shaded regions). A final 10-min stimulation with KCl (60 mM, far right shaded area) was included to assess tissue viability. All data points for both control and atrazine samples indicate mean values for groups of 10 hemi-pituitaries. Standard error bars have been provided.
contribute to the fact that the reported half-life of $^{14}$C labeled atrazine is estimated to be between 8 and 24 h (Wu et al., 1998). It is interesting to note that, in the present study and elsewhere (Cooper et al., 1999), we found that before an effect on neuroendocrine function was noted, repeated (e.g., daily) exposure to atrazine was required (i.e., decreased surges in 3X LE females). We also noted that the maximum physiological response observed occurs within the first 6 h of exposure, when atrazine was administered either as a single high dose or as repeated low doses. One possible explanation for this type of physiological response may be that, upon initial exposure, the major proportion of the compound is bound to hemoglobin (Hamboeck et al., 1981), where it is sequestered and does not reach the target tissue. However, upon repeated dosing, the capacity for hemoglobin to sequester the chlorotriazine or its metabolites is saturated, thus allowing more of the free parent compound and/or its metabolites to reach the target tissues. Whether or not there is indeed a difference in the hemoglobin-binding affinity for atrazine between the 2 strains remains to be determined. It is also interesting that this binding to hemoglobin has only been reported in the rodent and the chicken, but not in other species, including humans (Hamboeck et al. 1981). Atrazine, as well as other chlorotriazines (i.e., simiazine and cyanazine) and chlorotriazine metabolites are found in the soil, ground and drinking water and foodstuffs (Ballantine et al., 1998). The observed concentrations of these compounds in the different media are in the parts per billion range. This would imply that the levels of atrazine and related chlorotriazines in humans and wildlife species are well below the concentrations used in the present study, however, detailed toxicokinetic and toxicodynamic studies for any species are lacking.

In summary, these experiments demonstrate a clear effect of atrazine on the estrogen-induced LH and prolactin surge. The atrazine-induced changes in LH secretion are consistent with the hypothesis that this compound can cause a premature reproductive senescence. Upon repeated dosing, the suppression of hormone release occurred more rapidly in the LE female compared to the SD female. Further research is needed to determine the factors responsible for this strain difference. Finally, the data presented in this study also indicate that the brain is the primary target site for the effect of atrazine on pituitary hormone secretion. Studies evaluating atrazine’s effects on those neuronal systems involved in the regulation of gonadotropin release are warranted.

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