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Endocrine-Disrupting Chemicals: Prepubertal Exposures and Effects on Sexual Maturation and Thyroid Activity in the Female Rat. A Focus on the EDSTAC Recommendations

Jerome M. Goldman,[1] Susan C. Laws,[1] Sharon K. Balchak,[2]** Ralph L. Cooper,[1] and Robert J. Kavlock[1]*

[1]Reproductive Toxicology Division, National Health & Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; [2]Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC 27599

* Correspondence to: J. M. Goldman, Ph.D., MD-72, Endocrinology Branch, RTD, NHEERL, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

** Current address: Notre Dame College of Ohio, South Euclid, OH 44121

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ABSTRACT: In 1996, the US Environmental Protection Agency was given a mandate by Congress to develop a screening program that would evaluate whether variously identified compounds could affect human health by mimicking or interfering with normal endocrine regulatory functions. Toward this end, the Agency chartered the Endocrine Disruptor Screening and Testing Advisory Committee in October of that year that would serve to recommend a series of *in vitro* and *in vivo* protocols designed to provide a comprehensive assessment of a chemical's potential endocrine-disrupting activity. A number of these protocols have undergone subsequent modification by EPA, and this review focuses specifically on the revised *in vivo* screening procedure recommended under the title Research Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats. Background literature has been provided that summarizes what is currently known about pubertal development in the female rat and the influence of various forms of pharmaceutical and toxicological insult on this process and on thyroid activity. Finally, a section is included that discusses technical issues that should be considered if the specified pubertal endpoints are to be measured and successfully evaluated.

KEY WORDS: endocrine-disrupting chemicals, prepubertal exposures, female rat, EDSTAC recommendations.

Abbreviations: γ HCH, Lindane; 1,2,3,4,5,6-hexaclorocyclohexane; **18-homo-estradiol,** 18-methylestra-1,3,5(10)-triene-3,17β-diol) estradiol; **3α-diol,** 5-α-androstane-3β,17β-diol; **AMPA,** DL-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid anandamide, *N*-arachidonylethanolamine; **AP-5,** DL-2-Amino-5-phosphonovaleric acid (a competitive NMDA receptor antagonist); **BPA,** Bisphenol A; **CB 47,** 2,2′4,4′-tetrachlorobiphenyl; **CB-154,** 2-Bromo-α-ergocryptine mesylate, a dopamine agonist; **CNS,** central nervous system; **CRF,** corticotrophin-releasing hormone; **DDE,** 2,2-*bis-p*-chlorophenyl-1,1-dichlorethylene; **DES,** diethylstilbestrol; **DNQX,** 6,7-dinitroquinoxaline-2,3-dione; **E1,** estrone; **E2,** 17β-estradiol; **E2B,** 17β-estradiol benzoate; **E785,** 3-(3,4-dihydro-6-methoxy-2-napthyl)2-dimethyl hexanoic acid; **E969,** 3-(3,4-di-hydro-6-methoxy-2-naphthy)2-dimethyl pentanoic acid; **EAA,** excitatory amino acid; **eCG,** equine chorionic gonadotropin; **EE,** ethynyl estradiol; **ER,** estrogen receptor; **EGF,** epdidermal growth factor; **FGF-2,** fibroblast growth factor; **FSH,** follicle-stimulating hormone; **GABA,** γ-aminobutyric acid; **GH,** growth hormone; **GHRH,** growth hormone-releasing hormone; **GnRH,** gonadotropin-releasing hormone; **hCG,** human chorionic gonadotropin; **HPTE,** 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane; **IGF-I,** insulin-like growth factor-I;

LH, luteinizing hormone; **MER-25,** ethamoxytriphetol; **MK-801,** methyl,10-11-dihydro-5H-dibenzo [*a,d*]-cyclohepten-5,10-imine maleate (a noncompetitive NMDA receptor antagonist); **MTD,** maximum tolerated dose; **NIADDKD,** National Institute for Arthritis, Diabetes, Digestive and Kidney Diseases; **NMDA,** *N*-methyl-D-aspartate; **NPY,** neuropeptide Y; ***ob*,** obese; **PCB,** polychlorinated biphenyl; **PGE2,** prostaglandin E2; **PMSG,** pregnant mare serum gonadotrophin; **POMC,** proopiomelanocortin; **QC,** quality control; **RU-486,** mifepristone; **T3,** 3,5,3′-triiodothyronine; **T4,** thyroxine or 3,5,3′,5′-tetraiodothyronine; **TCDD,** 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; **TGF-α,** transforming growth factor-alpha; **THC,** Δ-9-tetrahydrocannabinol; **TRH,** thyrotropin-releasing hormone; **TSH,** thyroid stimulating hormone; **VO,** vaginal opening.

I. INTRODUCTION

In 1996, in response to emerging concerns about the influence of environmental chemicals on human health, the U.S. Environmental Protection Agency was given a mandate by Congress under the Food Quality Protection Act and Safe Drinking Water Amendments. The Agency was to develop a screening program that would evaluate whether variously identified compounds could affect human health by an effect similar to one "produced by a naturally occurring estrogen, or such other endocrine effect as the Administrator may designate (U.S. EPA, 1998a)." Toward this end, in October 1996 the Agency chartered the Endocrine Distruptor Screening and Testing Advisory Committee (EDSTAC), which included government and non-government scientists, along with "stakeholder" representatives from various interest groups. The goal in assembling the series of screening and testing protocols was to provide sufficient information that could allow accurate classification of chemicals as endocrine disruptors,* described by EDSTAC as any exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny, and/or (sub)populations of organisms. In its ensuing deliberations, EDSTAC then decided to extend the scope of the screening and testing batteries to include both pure compounds and common mixtures that could enhance (mimic) or inhibit estrogenic-, androgenic-, and thyroid hormone-related processes.

The protocols recommended by EDSTAC consist of a number of *in vivo* and *in vitro* procedures grouped into a Tier 1 screen (T1S, Table 1). The expressed intention was to: (1) incorporate endpoints of sufficient diversity to permit decisions based on "weight-of-evidence" considerations, (2) allow for the detection of various modes of action associated with the endocrine measures of concern, (3) maximize the sensitivity of the evaluations and minimize the incidence of false negatives, and (4) expand the number of organisms used for such evaluations to allow for interspecies differences in metabolic activation/detoxification and receptor-associated mechanisms of endocrine activity (U.S. EPA, 1998b).

The proposed *in vitro* tests (i.e., receptor-binding assays, steroidogenic assessments, and placental aromatase determinations) are designed to complement the *in vivo* protocols by providing information specific to the ability of a chemical to interfere with steroid-mediated activities. Similarly, the rat uterotrophic, male and female pubertal, and Hershberger protocols, along with the frog metamorphosis and fish gonadal recrudescence determinations (Table 1), that comprise the *in vivo* components of the T1S recommendations also target these types of steroid- associated toxic insult. At the same time, the protocols allow for an evaluation of additional apical effects on reproductive function that may not necessarily be associated with an alteration in steroidogenesis or steroid-receptor mechanisms. This is particularly true for the pubertal screening procedures, that are less mechanistically targeted and allow for such a broadened casting of the endocrine disruptor net.

After recommendation, each of these protocols is subjected to a formalized process of validation before it can be brought into the laboratory for general use. However, it is first necessary to determine if there is sufficient information about the method to support a validation study. This involves both a comprehensive examination of the scientific literature relevant to the protocol and

* Use of the term "endocrine disruptor" has been the subject of some debate since it first entered the toxicological lexicon. More recently, the National Research Council in a critical review of the area (NRC, 1999) instead recommended "hormonally active agent" in its place. However, the appropriateness of such semantic adjustments will depend on individual perceptions concerning the scope of an insult to endocrine regulatory functions. The designation endocrine disrupting chemical has been used by the U.S. Environmental Protection Agency and will be applied within the present context.

TABLE 1
EDSTAC Recommended Endocrine Disruptor Tier 1 Screening Protocols[a]

Assay	Estrogen agonism	Estrogen antagonism	Androgen agonism	Androgen antagonism	Thyroid-related effects	Steroid synthesis	Aromatase inhibition	5-α-reductase inhibition	HPG4[b]
In vitro									
Estrogen receptor binding	x	x							
Androgen receptor binding			x	x					
Steroidogenesis						x			
Placental aromatase							x		
In vivo									
3-day uterotrophic	x		(x)[c]						
20-day pubertal female	x	x			x	x	x		x
Hershberger			x						LH[d]
Hershberger + T	(x)[e]			x				x	LH[d]
14-day intact male	x			x	x	x	(x)[f]	x	x
20-day pubertal male	x		x	x	x	x		x	x
Frog metamorphosis	x	?[g]	?[g]	?[g]	x	x	?[g]	?[g]	x
Fish gonadal recrudescence	x	x	x	x	?[g]	x	x	?[g]	x

[a] Table adapted from USEPA (1998b).
[b] HPG - indicates that the model has an intact hypothalamic-pituitary-gonadal axis (except for the Hershberger assay which does not), and that effects on hypothalamic-pituitary control of gonadal endocrine function would be evaluated.
[c] It is likely that aromatizable androgens would be detected in this assay; however, given that there are no examples of environmental androgens, this point cannot be empirically demonstrated.
[d] Agents that affect LH levels would be detected in the assay.
[e] Empirical demonstration that the assay detects estrogens is limited. The biology of the system suggests that they will be detected.
[f] Empirical demonstration that aromatase inhibitors are detected is limited. If sensitivity to aromatase inhibitors is lacking, a placental aromatase assay would be added to this option.
[g] The biology of these organisms suggests that these effects may be detectable. However, there are no empirical data to support the sensitivity of the assay for these endpoints.

the performance of a prevalidation assessment using a limited number of compounds selected to optimize and standardize conduct of the protocol. In support of this process, the following two companion papers focus on the pubertal protocols for both the female (whose performance in T1S is listed as required) and male rat (listed as an alternative assessment). They have been presented by EDSTAC under the names Research Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats and Research Protocol for Assessment of Pubertal Development and Thyroid Function in Immature (33 to 53-Day-Old) Male Rats (U.S. EPA, 1998c). The current versions of these protocols are modifications of the original recommendations that have been evaluated by an EPA intraagency committee comprised of scientific advisors from the Office of Research and Development and the Office of Pesticide Programs and Toxic Substances. Considerations of clarity, accuracy, and applicability for use under Good Laboratory Practice (GLP) procedures resulted in these amended versions that are the subjects of the present companion male and female reviews.

Both of these reviews focus on what is currently known about the mechanisms of mammalian pubertal development and, using the rat as a model species, provide a comprehensive presentation of those alterations in puberty that result from exposure to chemicals with a wide variety of modes of action. As the protocols are also concerned with evaluations of thyroid function, additional information is presented on developmental changes in the hypothalamic–pituitary–thyroid axis and the impact of chemical insult on thyroid function.

The material in the present review specifically addresses sexual maturation in the female rat and the influence of prepubertal exposures to endocrine disrupting chemicals on this process. The concern is primarily with those exposure parameters recommended in the current protocol (presented as Table 2), or ones that are chronologically similar. Effects of gestational exposures, those given to lactating pups during the first 2 weeks of postnatal life, or early neonatal treatments extending through puberty will not typically be included, because the impact of such types of insult may be quite distinct from treatments initiated in the 3rd and 4th week. Finally, a section is presented on technical issues that should be considered if the recommended endpoints (and those designated as optional) are to be measured and evaluated successfully.

II. PUBERTAL MATURATION IN THE FEMALE RAT

In the female rat, as in other altricial mammals, the postweaning period is a time during which a variety of interrelated neuroendocrine processes undergo a progressive integration that will culminate in the emergence of a physiologically mature reproductive system. As the toxicological database has grown, along with our understanding of the mechanisms involved in this transition, what has become increasingly clear is that the onset of puberty can be vulnerable to perturbations in one or more of those underlying processes that contribute to it. Moreover, the form of pubertal alteration, that is, a maturational delay or advancement, is dependent on the nature of the insult, and the sensitivity to these effects can vary with the species and strain of the animal model chosen. Although mammalian reproduction can show marked species variations in cycle length, seasonality and the importance of cervical stimulation as an ovulatory trigger, the underlying reproductive physiology generally exhibits a remarkable degree of homology among members of the class. While rats (and mice) have been used in a wide variety of biological research, the reproductive system of the females shares a number of addtional characteristics with that of the human female. Both are regularly cycling "spontaneous ovulators", having midcycle gonadotropin surges that trigger comparable follicular and oocytic maturational changes within the ovaries. Consequently, these animals have routinely been employed in laboratory studies designed to elucidate shared mammalian mechanisms of reproductive development and function.

The onset of puberty in the female is a transitional process and encompasses the period of vaginal opening (VO) and first ovulation. Vaginal opening (or vaginal patency) in the rat commonly takes place between postnatal days 30 and 37, although variations occur between strains and between different colonies (or commercial suppliers) of the same strain (see Rivest, 1991 for review). Such diversity is evident in representative VO summary data that have been reported by various labs for different strains (Table 3).

TABLE 2
Modified Research Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats

Purpose and Applicability

The purpose of this protocol is to outline procedures to quantify the effects of environmental compounds on pubertal development and thyroid function in the intact juvenile female rat. This assay detects agents that display antithyroid, estrogenic, antiestrogenic (estrogen receptor [ER] or steroid-enzyme-mediated) activity, or alter puberty via changes in luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL) and growth hormone (GH) secretion, or via alterations in hypothalamic function.

Required Endpoints:

Growth (body weight)
Age and weight at vaginal opening
Serum thyroxine (T4) and thyroid stimulating hormone (TSH)
Liver, kidney, pituitary, and adrenal weights
Thyroid histology
Uterine and ovarian weights and histology
Vaginal cytology

Optional Endpoints:

Serum tri-iodothyronine (T3), estradiol (E2), and prolactin
Thyroid weight
Liver, kidney, pituitary, adrenal and vaginal histology
Ex vivo ovarian and pituitary hormone production
Hypothalamic neurotransmitter concentrations
Estrous cycle length (requires extension of dosing)

General conditions

A. Rats are housed in clear plastic cages ($20 \times 25 \times 47$ cm) with heat-treated (to eliminate resins that induce liver enzymes) laboratory-grade pine shavings as bedding. Animals are maintained on a complete and balanced laboratory diet and tap water ad libitum, in a room with a 14:10 hour photoperiod (lights on at 0500 h, off at 1900 h), temperature of 20 to 24°C and a relative humidity of 40 to 50%. Reasonable variations of this portion of the protocol should be acceptable when documented and justified.

B. Animals: Juvenile Female Rats

The study will use Sprague-Dawley or Long-Evans hooded female rats weaned on day 21. The litters may be derived from individually housed pregnant females that were bred in-house or purchased from a supplier as "timed pregnant" on days 7 to 10 of gestation. Enough litters should be available to ensure a sufficient number of juvenile females to provide 15 pups per treatment group. To maximize uniformity in growth rates, the litters are culled to 8 to 10 pups per dam at postnatal day 3 or 4, and body weight is monitored on a weekly basis, with any unthrifty litters or runted pups excluded from the study. On day 21, the pups are weighed to the nearest 0.1 g and weight ranked. A population of rats that is as homogeneous as possible is selected for the study by eliminating the "outliers" (i.e., the largest and smallest of the pups with a range of 8 grams above or below the mean used as a guideline). Pups are then assigned so that treatment groups exhibit similar body-weight means and variances. In this regard, one nuisance variable, i.e., body weight at weaning, is experimentally controlled. After assignment to treatment groups, similarly treated females are housed 2 to 3 per cage.

C. Experimental Design

The treatment conditions are (1) vehicle and (2) xenobiotic treated. If necessary, the study can be conducted in blocks rather than at one time. In this case, the blocks should contain all treatment groups and balanced with respect to number of animals (i.e., two blocks with two treatment conditions, with 7 to 8 females/treatment/block). Varying dosage levels of the xenobiotic can be tested, although only one high dosage level at or just below the maximum tolerated (MTD) or limit dose is required.

D. Treatment

Treatments are administered daily by oral gavage beginning on day 22 and continuing through 42 days of age (see Figure 1). The dose should be administered between 0700 and 0900 h using an 18 gauge gavage needle (1 inch length with 2.25 mm ball) and a 1 cc glass tuberculin syringe in a volume of 2.5 to 5.0 ml corn oil/kg body weight. Doses should be administered on a mg/kg body weight basis and adjusted daily for weight changes

changes. Body weight (nearest 0.1 g) and the volume of the dose administered (nearest 0.1 ml) are recorded daily.

E. Vaginal Opening

Females are examined daily for vaginal opening. The appearance of a small "pin hole" or a vaginal thread, as well as complete vaginal opening should be recorded on the days they are observed. However, the day of complete vaginal opening is the endpoint used in the analysis for the age of vaginal opening. In addition, the weight at complete vaginal opening should be recorded. Following vaginal opening, daily vaginal smears are monitored until necropsy to determine the age of first estrus and/or the first vaginal cycle, thus providing a way to distinguish pseudo-precocious puberty from true precocious puberty.

F. Necropsy

The method of euthanasia will depend on the endpoints desired. If pituitary hormone analyses are not included, the females may be killed with CO2 on the last day of treatment. If pituitary hormone analyses are to be included, the females should be killed by decapitation, which is conducted in a room separate from the housing area and within 15 s of the animal's removal from the cage. Blood is collected, centrifuged and stored in siliconized microcentrifuge tubes at –20°C for subsequent thyroxine (T4) and TSH measurements. At necropsy, the ovaries, uterus, liver, pituitary and adrenals are removed and the weights recorded (to the nearest 0.1 mg). Care must be taken to remove mesenteric fat from the uterine horns and not to damage the uterus, so that the uterine fluid is retained. The uterus and cervix are separated from the vagina, and the weight of the uterus with fluid is recorded. The uterus is then placed on a paper towel, slit to allow the fluid contents to leak out, gently blotted dry and reweighed. The thyroid, ovaries, and uterus are prepared for histological evaluation by placing in Bouin's fixative for 24 h, after which they are rinsed and stored in 70% alcohol until embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E).

G. Statistical Analysis

All data (age at vaginal opening, weight at vaginal opening, body and organ weights at necropsy, and serum hormones) are analyzed using multivariate analysis of covariance, with the bodyweight at weaning as a covariate. If the treatment X bodyweight interaction is not significant, then differences among treatment means may be tested using a two-tailed test. Combining all endpoints into a MANCOVA will assure that issues involving multiple comparisons because of the number of endpoints examined will be properly addressed. If data display heterogeneity of variance, then appropriate data transformations or use of nonparametric analyses should be employed. Often log transformation of serum hormone data is required because the variance is proportional to the mean.

H. Data Summary

Table providing data from individual animals should be provided in conjunction with a summary table listing the mean, standard error of the mean (SEM), and sample size for each treatment group. The mean, SEM, and coefficient of variation (CV) values for the control data are examined to determine whether they meet acceptable QA criteria for consistency with normal values. Data presented should include age and weight at vaginal opening, thyroid, ovarian, uterine (with and without fluid), adrenal, liver and body weights at necropsy, body weight change from day 21 to necropsy, and serum T4 and TSH. Data may also be presented after covariance adjustment for body weight at weaning, but this should not replace presentation of the unadjusted data. A summary of any histological findings should be included.

Late follicular growth of the first ovulatory cohort is stimulated about 8 days prior to first ovulation (Meijs-Roelofs et al., 1982), an event that occurs at about the time of vaginal opening (Ojeda et al., 1976). However, there can be some dissociation between VO and this initial release of ooyctes (Firlit and Schwartz, 1977). The females then begin to display repetitive 4 to 5 day patterns of vaginal cytology and circulating hormones, but there tends to be a greater number of irregular cycles during the immediate post-pubertal period (Goldman et al., 1985).

A. Gonadotropin Secretion

The manifestation of the above pubertal events is a consequence of progressive functional shifts in signaling within the brain hypothalamic–pituitary–ovarian endocrine axis (Figure 2) and represents a culmination of processes that begin during infancy (for a general review see Ojeda and Urbanski, 1994). The occurrence of the first ovulatory episode is a response to gonadotropin stimulatory signals, which themselves are triggered by peripubertal pulses of gonadotropin-releasing hor-

TABLE 3
Reported Female Pubertal Parameters in Various Strains of Rats[ab]

Strain	Control VO (day)	Age- 1st E	Age- 1st Di	BW at VO	Ref.
Holtzman	36.8 ± 0.2	NR	NR	NR	Piacsek and Hautzinger, 1974
Holtzman	35.6 ± 0.7	NR	36.6 ± 0.7	129.7 ± 2.6	Advis et al., 1981
Holtzman	35.6 ± 0.8	NR	36.7 ± 0.8	114.9 ± 4.1	Advis et al., 1982
Holtzman	34.4 ± 1.2[c] 35.0 ± 0.3 35.6 ± 1.2	NR	NR	NR	Flaws et al., 1997
Sprague-Dawley	35.5 ± 1.0	35.9 ± 0.9	NR	115.9 ± 5.5	Pau and Milner, 1982
Sprague-Dawley	38.4 ± 0.4	NR	NR	180 ± 8[de]	Urbanski and Ojeda, 1987
Sprague-Dawley	35.2 ± 0.3	NR	36.6 ± 0.2	90 ± 2[e]	Dees and Skelly, 1990
Sprague-Dawley	34.0 ± 0.2	NR	35.0 ± 0.4	124.3 ± 4.4[d]	Junier et al., 1992
Sprague-Dawley	31.7 ± 0.4	NR	33.0 ± 0.4	NR	Nyberg et al., 1993
Sprague-Dawley	33.6 ± 0.5	NR	NR	125.9 ± 1.2	Smyth and Wilkinson, 1994
Sprague-Dawley	31.9 ± 0.4[c] 36.1 ± 0.4 37.9 ± 0.5 38.3 ± 0.5	NR	NR	126.1 ± 2.1[f]	Gruaz et al., 1994
Sprague-Dawley	34.2 ± 0.7[g] through 37.2 ± 1.5	NR	NR	129.1 ± 3.0[h]	Pierroz et al., 1995
Sprague-Dawley	33.4 ± 0.8[i]	NR	NR	NR	Clark, 1999
Wistar	41.7 ± 0.5	NR	NR	NR	Honma and Hiroshige, 1977
Wistar	37.4 ± 3.1	NR	NR	104.8 ± 20.5	Döcke et al., 1981b
Wistar	37.0 ± 0.8	NR	NR	123 ± 2.4	Gonzalez et al., 1984
Wistar	36.7 ± 0.3	NR	NR	NR	Kawagoe and Hiroi, 1989
Wistar	34.3 ± 0.8	35.1 ± 0.8	NR	101 ± 3	Van den Dungen et al., 1989
Wistar	35.4 ± 1.0	NR	NR	NR	Gallo et al., 1999
Wistar (R-Amsterdam substrain)	39.3 ± 0.5[c] 38.2 ± 0.8	39.5 ± 0.4 38.5 ± 0.9	NR	90.8 ± 2.0 90.6 ± 2.0	Kramer and Meijs-Roelofs, 1982
Wistar (R-Amsterdam substrain)	38.6 ± 0.4[g] through 42.1 ± 0.3	NR	NR	93.9 ± 2.8[gj] through 105.9 ± 1.8	Meijs-Roelofs et al., 1987
Fischer 344	36.5 ± 1.5	NR	NR	86.6 ± 3.2	Cooper et al., 1989
Long-Evans	39.0 ± 3.0	NR	NR	NR	Najam and Panksepp, 1989
Long-Evans	32–34[c]	33–34[c]	NR	97–116[c]	Gray et al., 1989
Long-Evans	35.0 ± 0.3	NR	NR	130 ± 2	Church et al., 1990
Long-Evans	36.2 ± 0.5	NR	NR	NR	Weinstein et al., 1992
Long-Evans	30.6 ± 0.2	NR	NR	114 ± 1.8	Gray et al., 1997

Note: Above data are taken from selected papers that are representative for each of the listed rat strains. VO, vaginal opening; E, vaginal estrus; Di, vaginal diestrus; NR, not reported.

[a] Values are group means ± standard errors of the mean.
[b] All cited studies employed light:dark photoperiods of 12 h:12 h or 14 h:10 h with the exception of Dees and Skelley and Nyberg et al., which reported 10 h:14 h.
[c] Numbers represent the range of control group means for different experimental blocks.
[d] body weight (BW) at 1st Di.
[e] Estimate from graphed data.
[f] BW at d35.

[g] Group range for a variety of control groups matched against different treatment conditions.
[h] BW at d37.
[i] Mean age ± standard deviation for control rats from studies conducted at Merck Pharmaceutical between 1989 and 1997.
[j] BW at 1st ovulation.

mone (GnRH) from nerve terminals in the hypothalamus.

Over the first 3 weeks of life, circulating concentrations of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) show modest elevations. There are sporadic bursts of secretory activity (MacKinnon et al., 1976) that are triggered by an induction of GnRH release in response to brief increases in hypothalamic noradrenergic activity. These elevations, particularly in the case of FSH, are considered important for ovarian maturation (e.g., Schwartz, 1974; Hage et al., 1978). At the beginning of week 4, the gonadotropin levels fall and there begins to emerge a pattern of small LH pulses spaced about 30 min apart. These pulses are more pronounced in the afternoon hours (Urbanski and Ojeda, 1985) and appear to be influenced by circulating steroid concentrations (Urbanski and Ojeda, 1987) that have begun to increase around this time. This activity then continues through puberty and presages the appearance of the cyclic patterns of gonadotropin secretion (the midcycle surges of LH and FSH) in the postpubertal female that function to stimulate follicular/oocytic maturation and ovulation. A general chronology of these changes and those to follow below are presented in Figure 3.

B. Hypothalamic Involvement in Sexual Maturation

1. Neurotransmitters and Hypothalamic Secretory Activity

As touched upon above, GnRH secreted in a pulsatile fashion from the hypothalamic median eminence region into the portal vessels that descend to the pituitary induces an episodic release of LH and FSH into the circulation (Figure 2). This secretion of GnRH from the network of hypothalamic GnRH neurons is associated with concurrent, synchronized increases in multiunit electrical activity in the mediobasal area. The mechanism that drives this phenomenon is referred to as the GnRH pulse generator, but exactly what constitutes this functional entity has still not been specified at an anatomical or cellular level.

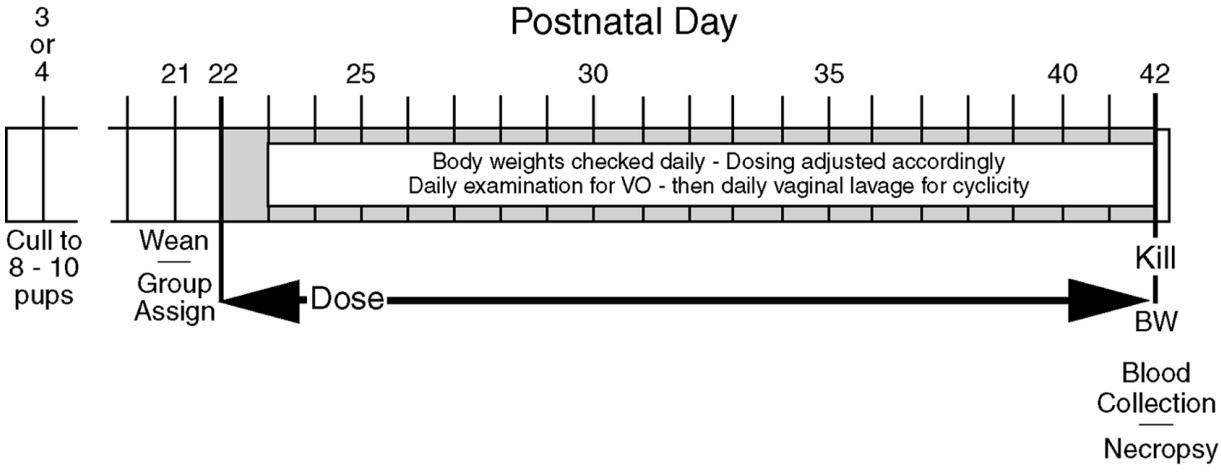

FIGURE 1. Timeline for the conduct of the female pubertal assay. Following group assignment on postnatal day 21, dosing is begun on day 22 and continued until necropsy on day 42. Body weights (BW) are taken daily, and the animals are checked each day for vaginal opening (VO). After VO is observed, vaginal lavages are taken daily for the remainder of the dosing period and evaluated for estrous cyclicity.

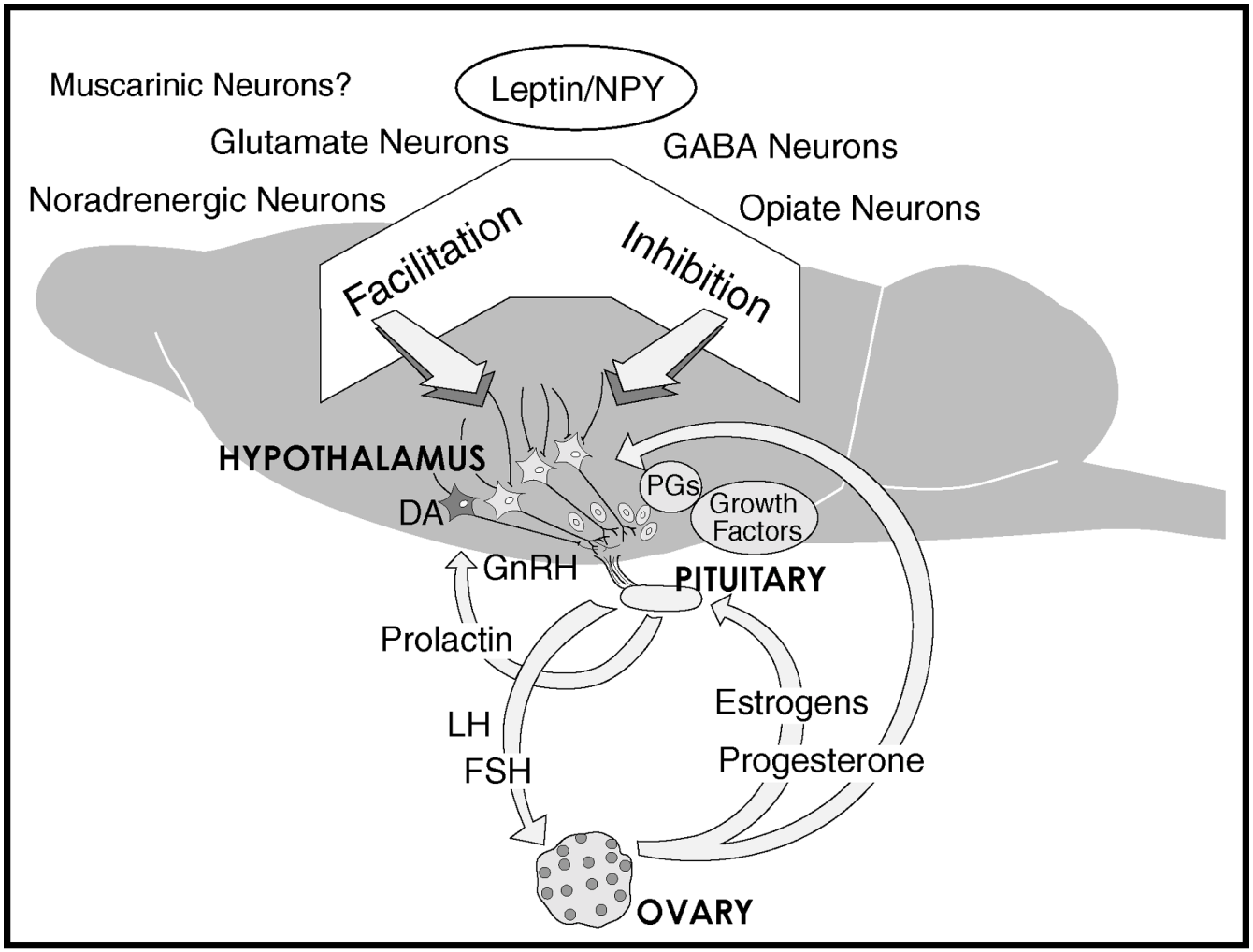

FIGURE 2. Generalized depiction of principal factors within the hypothalamic–pituitary–ovarian axis that participate in the onset of sexual maturation in the female rat. Both inhibitory and excitatory neuroendocrine input to the hypothalamic GnRH neurons underlie a pulsatile release of GnRH that in turn triggers the first ovulatory surge of LH. Moreover, the influence of the input during this period can show a dramatic change, as with GABA's shift from an exitatory signal in gonadotropin secretion to an inhibitory one (see text). Abbreviations: DA, dopamine; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; NPY, neuropeptide Y; PGs, prostaglandins.

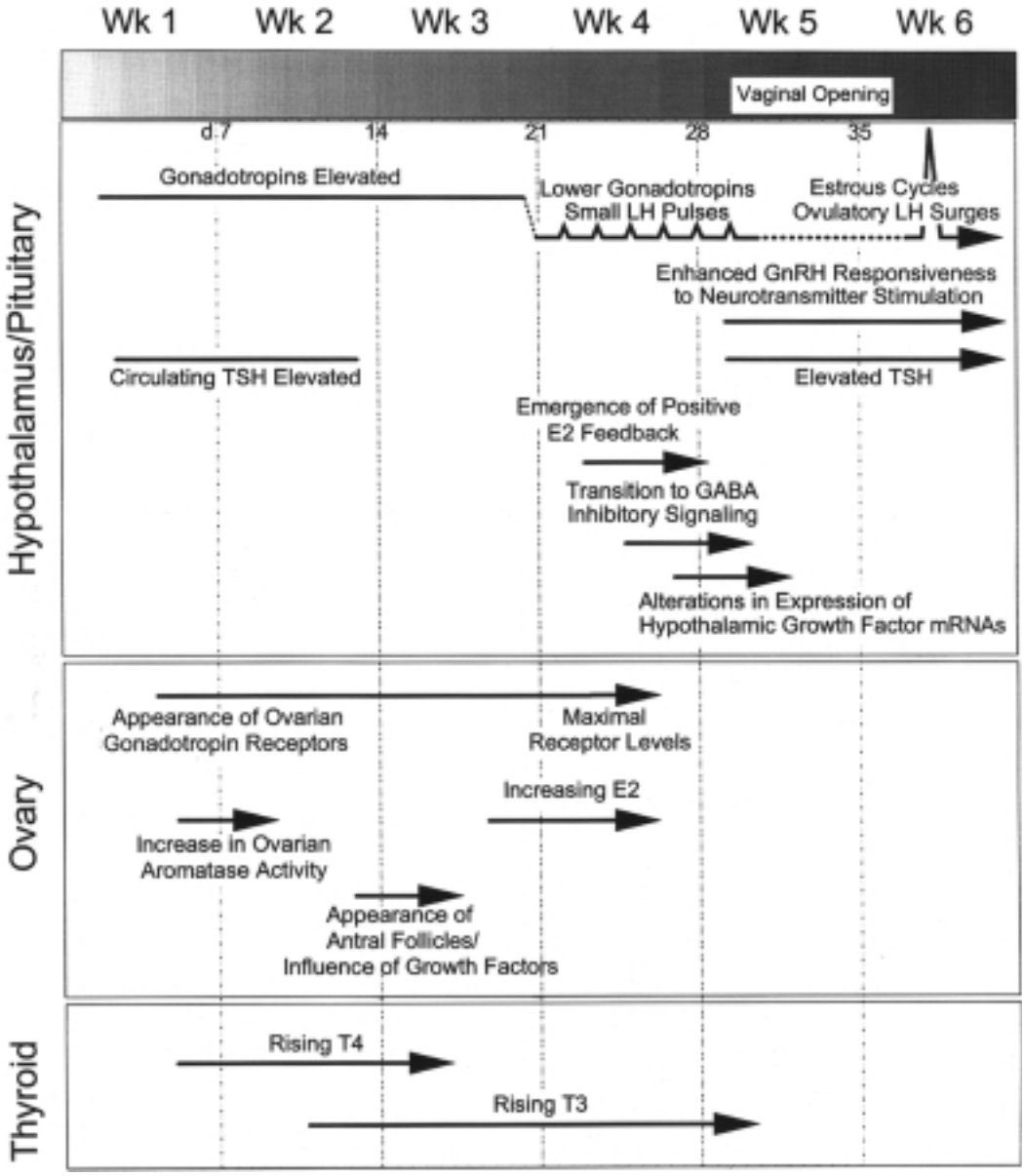

FIGURE 3. Chronology of maturational changes within the female rat hypothalamus, pituitary, ovaries, and thyroid over postnatal weeks 1 through 6. The arrows indicate alterations that have been reported to commence at the times indicated (see text for descriptions).

Nevertheless, its development is critical to the process of puberty.

The network of GnRH neurons actually arises outside of the brain from progenitor cells in the olfactory placode (Wray et al., 1989). By gestation day 12, GnRH can be detected in the rat hypothalamus (Aubert et al., 1985), and the levels gradually increase until a few days after parturition (Chiappa and Fink, 1977). A steep rise then takes place through the second postnatal week, followed by a further increase in the female that continues until puberty (Chiappa and Fink, 1977). At puberty, the responsiveness of the GnRH neurons to neurotransmitter stimulation becomes enhanced (e.g., Ojeda et al., 1986), as a previously inhibitory influence of estradiol declines (e.g., Docke et al., 1981a). There is no change over this time in the total number of GnRH neurons, although they do seem to exhibit a steroid-

related morphological increase in spiny projections (Wray and Hoffman, 1986; Becu-Villalobos and Libertun, 1995).

A number of neurotransmitter systems participate in GnRH release and as a consequence are involved in the progression through puberty in the rat. Catecholamines, γ-aminobutyric acid (GABA), the excitatory amino acid glutamate, and possibly acetylcholine all have been implicated in the process, which now appears to involve shifts in the interrelationships among the regulatory input by these systems (Figure 2). Moreover, for an individual transmitter system, puberty can represent a period of functional transformation, where a previously excitatory stimulus transitions to an inhibitory one. Such has been reported to be the case for GABA, which in prepubertal (16 d) female rats is able to stimulate gonadotropin secretion (through a likely effect on GnRH secretion), whereas in the peripubertal (30 d) animal, the signal becomes an inhibitory one. During both of these times, the action appears to involve the GABA-A receptor subtype (Moguilevsky et al., 1991). Subsequent evidence has implicated an activation of excitatory amino acid neurotransmission that mediates this early prepubertal effect (Scacchi et al., 1998).

The shift in GABAergic input from excitation to inhibition seems to take place around the week preceding the onset of puberty (Figure 3). Between day 20 and the emergence of the pubertal indices, LH levels in the rat are very low, and at that time the hypothalamic mechanism for generating GnRH pulses appears to be suppressed (Roth et al., 1997). At 30 days, injections of the GABA-A receptor blocker bicuculline were able to increase serum LH (Roth et al., 1997), suggesting that during this prepubertal period GABAergic transmission imposes a tonic inhibition on the pulsatile GnRH release mechanism.

The excitatory amino acid (EAA) receptors are now believed to be the primary receptors mediating excitatory neurotransmission in the brain (for reviews see Headley and Grillner, 1990; Brann, 1995). They appear to principally bind glutamate and aspartate, as these are the brain's most abundant EAAs, although others such as L-homocysteic acid and the tryptophan metabolite quinolinic acid are present and may have functional roles. While EAA receptors can be broadly classified as either ionotropic (linked to cation specific channels) or metabotrophic (coupled to G-protein-associated second messenger pathways), it is the ionotrophic class that has been most closely associated with the process of pubertal development. Three types of these ionotrophic receptors have been identified (see Brann, 1995), each with multiple subforms: NMDA (*N*-methyl-D-aspartate) receptors, AMPA (DL-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid) receptors and kainate receptors.

Most of the available data have implicated activation of the NMDA receptors as excitatory participants in the female pubertal process (e.g., Veneroni et al., 1990; Meijs-Roelofs et al., 1991; Smyth and Wilkinson, 1994). A role for the other two types of receptors is still uncertain. AMPA receptor binding, unlike that for the other two classes, does show an increase in the hypothalamus at the time of puberty (Zamorano et al., 1998), although GnRH neurons apparently do not express the message for that receptor subtype (Eyigor and Jennes, 1997). On the other hand, these neurons do contain the mRNA for certain subtypes of both the NMDA and kainate receptors (Eyigor and Jennes, 1997), and there is some evidence that kainate receptors regulate gonadotropin release in the prepubertal male (Pinilla et al., 1998). In females, the maturational shift from negative-to-positive feedback in the influence of estradiol/progesterone on gonadotropin secretion has been reported to be linked to a similar influence of this combination of steroids on EAA release (Carbone et al., 1995). Previous work on the stimulation of NMDA receptors by EAAs have implicated progesterone as primary potentiating factor in this effect (Brann and Mahesh, 1991).

Norepinephrine is a catecholamine neurotransmitter that has long been known to participate in the release of GnRH. In immature rats, the administration of pregnant mare serum gonadotropin will cause a drop in the numbers of α-noradrenergic receptors (Wilkinson et al., 1979) and induce the appearance of a GnRH-triggered LH surge, something that is prevented by the destruction of dorsal hypothalamic noradrenergic input (Sarkar et al., 1981). Moreover, during puberty, norepinephrine turnover in the region is enhanced (Advis et al., 1978; Raum et al., 1980), which may be a response to increases in estradiol concentrations (e.g., Crowley, 1982). For the secretion of GnRH, the action of norepinephrine is Ca^{2+}-dependent

and probably uses prostaglandin E2 (PGE2) as an intermediate (Ojeda et al., 1988), because a suppression of PGE2 synthesis by cyclo-oxygenase inhibition will prevent a norepinephrine-induced release of GnRH (Ojeda et al., 1979).

A blockade of the receptors for the catecholamine neurotransmitter dopamine beginning on day 28 was reported to advance both VO (day 38 → day 35) and first ovulation (Docke et al., 1987), while administration of a specific receptor agonist from day 22 onward was observed to delay VO and ovulation (Advis et al., 1981a). It has been well established that dopamine released from hypothalamic terminals of tuberoinfundibular neurons into hypophyseal portal capillaries acts to tonically inhibit pituitary prolactin release. During the prepubertal period in the female rat, there is a gradual increase in serum levels of prolactin that peaks around the time of vaginal opening (Dohler and Wuttke, 1974; Ramaley, 1981). In the 1960s, it had been observed that prolactin introduced into the hypothalamic median eminence was able to advance puberty in immature females (Clemens et al., 1969), while somewhat later it was found that a chronic suppression of prolactin from day 22 onward caused a marked delay (Advis et al., 1981a). Moreover, 21-day-old rats made hyperprolactinemic by grafting a pituitary from a littermate donor under the kidney capsule (thus dissociating the grafted tissue from the inhibitory influence of hypothalamic dopamine) also showed a significant advancement in vaginal opening (Gonzalez et al., 1984), reinforcing a role for prolactin in the transition to sexual maturity. As with the hypothalamic implants, this effect did not appear to be dependent on ovarian estradiol feedback (Lung and Docke, 1981).

A direct participation of cholinergic mechanisms in puberty is still unresolved. In the female, a cholinergic mechanism may contribute to the peripubertal appearance of a diurnal rhythm in tuberoinfundibular dopaminergic (TIDA) neuronal activity (Shieh and Pan, 1998). This rhythm is important in maintaining the afternoon prolactin surge that occurs in the postpubertal female on the days of proestrus and estrus. Even so, a cholinergic role in sexual maturation may be minor. An advancement of ovulation by the administration of estradiol to sexually immature rats has shown that there is an accompanying decrease in the hypothalamic concentration of acetylcholine (Modak et al., 1979). At the same time, a prepubertal blockade of muscarinic receptor subtype was reported to delay VO and first estrus (Trkulja and Lackovic, 1996), suggesting some type of involvement.

The pubertal maturation of the TIDA rhythm in the rat also appears to involve input from the opioid neurons in the area (Shieh and Pan, 1998). These neurons, together with the TIDA system, comprise the principal inhibitory neuronal systems in the prepubertal female (Becu-Villalobos and Libertun, 1995). The regulatory role of endogenous opioid peptides in gonadotropin secretion has been well established in the adult (e.g., Ieiri et al., 1980; Kubo et al., 1983; Adler and Crowley, 1984) and is functional in the immature female (e.g., Ieiri et al., 1979; Cicero et al., 1986). During puberty, an attenuating effect of the endogenous opioid β-endorphin on LH secretion is diminished. The effect has been attributed to an increase in the metabolic inactivation of this peptide in the hypothalamus (Martensz, 1985) and suggests that one factor in the emergence of a pattern of LH pulsatility is a lessening of an inhibitory restraint.

2. Growth Factors

The actions of a variety of growth factors have been implicated in the process of puberty in the female rat. Transforming growth factor-alpha (TGF-α), epidermal growth factor, insulin-like growth factor-I (IGF-I), and fibroblast growth factor (FGF-2) all appear to undergo changes at the time of puberty (Figure 3). There is evidence for changes in concentration or shifts in the expression of relevant mRNAs for these factors or their receptors (Hiney et al., 1991; Ma et al., 1992, 1994; Smyth et al., 1997). As glial cell-secreted neurotropic factors, they may be involved in any maturational processes that occur. While the data right now are generally correlational, it has been observed that a suppression in the action of TGF-α in the median eminence region can delay puberty (Ma et al., 1992). Moreover, in immature females an activation of TGF-α expression in the hypothalamus using grafted fibroblasts retrovirally transfected with a human TGF-α gene under the control of a metallothionein promotor was able to advance first ovulation (Rage et al., 1997a). Similar grafts not expressing hTGF-α had no such effect.

Prepubertal rats with lesions of the anterior hypothalamus have frequently been reported to exhibit a premature VO accompanied by ovulation (Donovan and van der Werff Ten Bosch, 1956). These lesioned females have elevated concentrations of circulating gonadotropins in the afternoon, and a commonly accepted hypothesis was that the lesions removed a central restraint on gonadotropin secretion (Donovan and van der Werff Ten Bosch, 1956). More recent data have indicated that the damage caused increased TGF-α gene expression in proliferating astrocytes around the lesion (Junier et al., 1991), which could underlie structural and activational remodeling in the area (Junier et al., 1992) and stimulate GnRH secretion via an enhancement of glial PGE2 production (Ma and Ojeda, 1997). This process has been determined to involve the activity of a particular class of transcription factors in what is known as the POU-domain gene family, which has been shown to play critical roles in nervous system development (Latchman, 1999). These factors are products of phylogenetically conserved developmental control genes that serve to regulate the transcription of other genes. POU proteins have been found to act as transcriptional regulators of neuropeptide or neutrotransmitter genes expressed in the hypothalamus and pituitary (e.g., Simmons et al., 1990; Rosenfeld et al., 1996). One of these regulatory factors, termed Oct-2, now appears to be a component of a normal process by which the glial cells up-regulate the neuronal secretion of GnRH in the pubertal female rat by activating the TGF-α gene (Ojeda et al., 1999).

C. Ovarian Maturation and Steroid Production

1. Ovarian and Follicular Maturation

In the fetal rodent ovary, oogonia that are formed from proliferating germ cells undergo a further meiotic division to reach an arrested diplotene stage of development between gestational day 17 and postnatal day 5 (Byskov, 1974). At the same time, each becomes surrounded by a structure of supporting cells, which together comprise a primordial follicle. Under hormonal influence, both the arrested oocyte and this immature follicle must progress through a number of further maturational stages before ovulation takes place.

Until about postnatal day 12, maturing follicles containing an antral cavity are completely absent from the immature ovary (Carson and Smith, 1986). The progression in development from a primordial follicle to a mature preovulatory one requires the proliferation and differentiation of granulosa cells surrounding the oocytes. Few follicles actually progress this far, and it is estimated that upward of 95% undergo atresia during their antral stage (see Greenwald and Terranova, 1988 for review). The impetus for the proliferative process appears to be due to a coordinated regulation by growth factors and FSH. While FSH has been implicated in these changes, it may actually be that the early follicular growth and steroid production are equally dependent on the presence of other bioactive compounds. Nerve growth factor, transforming growth factor-β, epidermal growth factor, insulin-like growth factor I, and vasoactive intestinal polypeptide (VIP) have all been reported to be important contributors to follicular cell maturation in the immature ovary (e.g., Funkenstein et al., 1980; George and Ojeda, 1987; Tornell et al., 1988; Ojeda et al., 1992; Dunkel et al., 1994; Roy and Hughes, 1994; Mayerhofer et al., 1997; Yoshimura, 1998). Moreover, it now appears that a number of these factors may facilitate follicular maturation by enhancing FSH receptor number and affinity (e.g., Dunkel et al., 1994; Mayerhofer et al., 1997).

2. Steroid Production and Feedback Signaling

Ovarian FSH receptors begin to increase over the first few weeks of life and show maximal levels by the 4th week (Smith-White and Ojeda, 1981) (Figure 3). The earliest FSH binding can be detected sometime over the latter half of the first or beginning of the second week (Uilenbroek and van der Linden, 1983; Sokka et al., 1992). Similar binding data (using human chorionic gonadotropin, hCG) were reported for the LH receptor (Sokka and Huhtaniemi, 1990), although a functional response (cAMP production) to this binding occurs somewhat later (d7) than for FSH (d4). At this time, FSH becomes able to stimulate the aroma-

tase activity necessary for the conversion of testosterone to estradiol (George and Ojeda, 1987). By day 7, aromatase activity has significantly increased, but low levels of progesterone and testosterone limit estradiol production (Carson and Smith, 1986). The rise in estradiol is not until the latter half of week two, when a follicular theca cell layer emerges to surround those granulosa cells that had already begun to proliferate.

During the first 2 weeks, the rising levels of estradiol are relatively ineffective in depressing gonadotropin levels (e.g., Frawley and Henricks, 1979), which may account for the modest elevations in LH and FSH during that time (Figure 3). This is primarily attributable to the high circulating concentrations of α-fetoprotein at this time, which bind up the available estrogens (e.g., Meijs-Roelofs and Kramer, 1979). As the postnatal levels of α-fetoprotein undergo a decline (Esumi et al., 1982), the augmentation in the effectiveness of estradiol negative feedback causes LH and FSH to decline around the beginning of the 3rd week (Andrews and Ojeda, 1981; Andrews et al., 1981).

In the 4th week, there occurs a transition in the feedback effects of estradiol, and the rat hypothalamus and pituitary begin to respond positively, resulting in a stimulatory release of LH (Andrews et al., 1981; Kawagoe and Hiroi, 1983). As the rat continues to mature, the hypothalamus and pituitary become more sensitive to estrogen, an effect that may be linked to an enhancement by estradiol of PGE2 production (Ojeda et al., 1986; Rage et al., 1997b). This increased synthesis of PGE2 would then augment the stimulation of GnRH secretion by noradrenergic neuronal activity. In turn, the magnitude of LH release increases that, along with the occurrence of vaginal opening, culminates in the appearance of the first preovulatory surge of LH (Ojeda and Urbanski, 1994) and the initiation of adult-like cyclic changes in the circulating endocrine profile.

During the 3rd week, a cohort of immature, primordial follicles is stimulated by elevated levels of circulating FSH to undergo a series of maturational changes that will prepare surviving members of the cohort to respond to the first ovulatory surge of LH. The progression in follicular development from a primordial follicle to a preovulatory follicle involves growth in two major cellular regions, the outer theca cell layer and the inner granulosa cell layer. In the rat, the theca cells emerge somewhat later in development than the initial granulosa cell layer, appearing after two to three layers of granulosa cells are formed (Hirshfield, 1991). The theca layer in the maturing follicle becomes well vascularized (Bassett, 1943) and provides the blood supply necessary for conveying nutrients and hormonal factors to the layers of nonvascularized granulosa cells.

The proliferation and differentiation of the granulosa cells surrounding the oocytes is a process that not only involves the stimulatory action of FSH (McNatty et al., 1979), but previously mentioned contributions from a number of growth factors as well. TGF-β, in combination with FSH, has been shown to promote DNA synthesis in immature rat granulosa cells (Skinner et al., 1987; Dorrington et al., 1988; Adashi et al., 1989), and regulatory roles have also been found for IGF-I and epidermal growth factor (EGF) (e.g., Adashi et al., 1985, 1991; Feng et al., 1986; Bendell and Dorrington, 1990).

Granulosa cells stimulated by FSH produce the glycoprotein hormone inhibin, which in adults exerts a specific negative feedback effect on pituitary FSH secretion. In juvenile female rats, this feedback action does not appear until postnatal week 4 (Rivier and Vale, 1987; Culler and Negro-Vilar, 1988). The importance of inhibin in sexual maturation is still unclear, although there are some data to suggest that stress- or exercise-induced increases in prepubertal circulating concentrations may contribute to a pubertal delay (Pellerin-Massicotte et al., 1987).

As the growing follicle matures, an antrum or cavity forms within the granulosa cells. This process is gonadotropin dependent (Eppig, 1991) and is augmented by estrogen synthesized within the follicle (Goldenberg et al., 1972). At this time, the granulosa cells immediately adjacent to the oocyte (corona radiata cells) develop intimate contact with the oocyte until the preovulatory stage of development. They are part of the cellular mass that surrounds the oocyte (*cumulus oophorus*) and attaches it to the follicle wall, providing both physical and nutritional support. The majority of the granulosa cells form the mural, or parietal granulosa cells lining the follicular cavity.

Under LH stimulation, cells in the thecal layer synthesize and secrete androgens, which are then

transported to the granulosa cells for conversion to estrogens by the action of cytochrome P450 aromatase (P450 arom) (e.g., Fortune and Armstrong, 1977; Liu and Hsueh, 1986; Roberts and Skinner, 1990). The steroids produced stimulate further follicular growth and enhance LH receptor formation that augments the granulosa cell responsiveness to the LH surge in mature antral follicles (Kessel et al., 1985).

D. Pubertal Development, Food Intake, and Body Weight

In the female rat, it has long been known that reductions in body weight during development are able to cause either delays in the onset of puberty, or a loss of fertility in the adult. Amenorrhea and infertility are also seen in women who have maintained a reduced body weight by caloric restriction or vigorous exercise (e.g., Frisch et al., 1980; Bates et al., 1982; Warren et al., 1999). For rats in which delays were induced by dietary restriction, pubertal changes were observed to appear at the same weight as in fully fed female controls (Wilen and Naftolin, 1978; Holehan and Merry, 1985), although this relationship between body weight and puberty has not always held true (Bronson, 1987; Ikeda et al., 1994).

Overweight mice with mutations in what has been termed the *ob* (obese) gene were found to be infertile, with an immature reproductive axis (Swerdloff et al., 1976). In 1994, the structure of this gene and its human homolog were described (Zhang et al., 1994). Its product is a 167 amino acid protein secreted from adipocytes that has been termed leptin (from the Greek *leptos,* meaning thin). Over the last several years, a spate of published studies have implicated leptin as a regulatory factor in food intake and metabolism (e.g., Ahima et al., 1998; Buchanan et al., 1998; Rosenbaum and Leibel, 1998) and one that plays a critical role in the timing of puberty (e.g., Barash et al., 1996; Sahu, 1998; Cunningham et al., 1999). Although the weight of evidence indicates that adequate levels of leptin in the circulation are important (but not sufficient) for pubertal progression, it has been reported that immature mice injected with leptin exhibited an advancement in puberty of up to 9 days (Chehab et al., 1997). In the genetically obese *ob/ob* mice (Yu et al., 1997) or food-restricted, ovariectomized/steroid-primed rats (Kohsaka et al., 1999), leptin administration elevated gonadotropin secretion and stimulated increases in gonadal weights and ovarian tissue (Barash et al., 1996) that were consistent with a gonadotropin activation. The brain has been established as a target site, with the mRNA for the *ob* receptors being found in the hypothalamus and various other regions of the central nervous system (Elmquist et al., 1998). Its receptors are colocalized on hypothalamic estrogen-containing neurons (Diano et al., 1998), and an estrogen-induced elevation in leptin production *in vivo* (Shimizu et al., 1997; Brann et al., 1999) suggests that its role during puberty is tied to the increases in estradiol production at this time. Receptors are also found in other organs, including the ovaries, and leptin administered *in vitro* is able to attenuate ovarian steroid synthesis (Spicer and Francisco, 1998; Barkan et al., 1999). Such direct action implies that leptin functions as an additional regulatory factor in the feedback control of steroid production.

Another factor that has been implicated in the relationship between body weight and sexual maturation is Neuropeptide Y (NPY). NPY is a biologically active 36 amino acid peptide that is now known to function as a peptidergic co-transmitter, interacting with the classic neurotransmitters. It is synthesized in adrenergic neurons and for the regulation of GnRH likely acts in the adult to amplify the catecholaminergic signals (see Kalra and Crowley, 1992 for review). There does appear to be an increase in immunoreactive NPY within the hypophyseal-portal circulation around the time of VO (Sutton et al., 1988), and its immunoneutralization on the day of first proestrus was able to attenuate the subsequent LH surge (Minami et al., 1990). However, evidence is also emerging that leptin is able to regulate NPY production (Dube et al., 1999) and that NPY levels increase during periods of food restriction or deprivation. This response may be due to activation of a subset of NPY neurons in the arcuate nucleus (Baskin et al., 1999) and could represent an inhibitory maturational signal during a time of adverse metabolic conditions. In this regard, centrally administered NPY has been observed to completely block sexual maturation in previously food-restricted rats re-

turned to a normal diet (Gruaz et al., 1993). However, a mediation by NPY is likely not the sole mechanism by which leptin acts on the hypothalamus. Mouse mutants devoid of NPY appear normal (Palmiter et al., 1998), and a cross of NPY-deficient mice with *ob/ob* mice results in offspring that still exhibit some degree of obesity (Erickson et al., 1996). In fact, studies have shown that a variety of hypothalamic factors, including proopiomelanocortin (POMC) neurons and the bioactive peptides galanin and neurotensin, are also affected by leptin (Cheung et al., 1997a; Sahu, 1998).

Some of the recent data have underscored a melanocortin role in leptin signaling (e.g., Schwartz et al., 1997; Zemel, 1998; Watanobe et al., 1999). Melanocortins are a group of peptides that includes adrenocorticotrophic hormone (ACTH) and the α, β, and γ-melanocyte-stimulating hormones. They are cleaved from the POMC precursor, and the administration of specific melanocortin receptor agonists has been found to reduce food intake (e.g., Fan et al., 1997; Murphy et al., 1998). Five melanocortin receptors have thus far been identified, and this hypophagic effect appears to be associated most prominently with binding to the MC4-R subtype (Giraudo et al., 1998; Harrold et al., 1999).

E. The Thyroid

The influence of thyroid hormones is virtually ubiquitous throughout the body. They regulate growth and development, cellular metabolism, and oxygen use and basal metabolic rate. A variety of hormones, including testosterone, growth hormone, and norepinephrine, have some effect on metabolic rate, but the thyroid hormones, thyroxine (3,5,3′,5′-tetraiodothyronine or T4) and 3,5,3′-triiodothyronine (T3), are by far the most important in this regard. The secretion of these thyroid hormones is part of a functional axis that is structurally comprised of the hypothalamus, pituitary, and thyroid. Thyrotropin releasing hormone, secreted from the hypothalamus, reaches the pituitary via the portal vessels and prompts the release of thyroid stimulating hormone (TSH). TSH then enters the general circulation and serves as the trigger for T3 and T4 synthesis and release. These thyroid hormones then feed back on the hypothalamus and provide a modulatory signal to the axis, attenuating hypothalamic-pituitary secretory activity during times when thyroid hormone concentrations are elevated.

1. Thyroid Influences on Pubertal Development

In the developing female rat, circulating concentrations of TSH show a bimodal pattern (Figure 3). There is an early peak during the first 2 weeks of life that is followed by a second elevation after day 30 (Fukuda and Greer, 1978), an increase that is paralleled by concentrations within the pituitary. At birth, T4 and T3 are very low. T4 then undergoes a marked rise between days 4 and 16, while T3 levels remain low until about day 10 before reaching a maximal concentration by day 30 (Dussault and Labrie, 1975; Fukuda and Greer, 1978).

The presence of increased concentrations of TSH in the anterior pituitary and serum at the onset of puberty suggest that the thyroid may also mediate the process of sexual maturation in the female rat (Simpkins et al., 1976). As mentioned above, TSH regulates the synthesis of T4 and T3 and stimulates their release into the circulatory system, where they bind to transporting proteins (albumin and prealbumin (transthyretin)) that distribute the hormones to peripheral tissues (Larsson et al., 1985). There, T4 is converted to the active T3 form, which binds to its intracellular nuclear receptor and stimulates gene transcription (Apriletti et al., 1998). Together, these hormones are critical for regulating normal growth and development in the young (Griffin, 1988).

To assess the role of thyroid hormones during the onset of puberty, Tamura et al. (1998a) measured plasma serum estradiol, progesterone, and inhibin concentrations during gonadotropin-induced ovarian development in 26-day-old thyroid-deficient female rats. Elevated serum concentrations of estradiol and inhibin, but not progesterone, were observed 24 and 48 h after equine chorionic gonadotropin (eCG) treatment in the thyroidectomized animals. The number of large healthy follicles and ovarian weight were also increased in this group when compared with controls. Daily administration of T4 following thyroidectomy prevented these

eCG-induced changes in ovarian hormone concentrations and follicular development. Thyroidectomy without eCG treatment did not increase either of the hormones. These results suggest that an interaction may exist between thyroid hormone and gonadotropin, which influences the fate of developing ovarian follicles in the immature rat. Subsequent studies have shown that hypothyroidism can block gonadotropin-induced first ovulation in immature rats by decreasing FSH and LH serum concentrations (Tamura et al., 1998b). Ovulation and serum LH concentrations in the thyroidectomized animals were restored to control levels with co-administration of GnRH.

F. Additional Influences on Sexual Maturation

Two additional factors whose role in female pubertal development has received attention are growth hormone (GH) and the pineal secretory product melatonin. GH implants placed in the hypothalamic median eminence will result in a negative feedback inhibition of GH secretion and retard sexual maturation for a few days, but not prevent it (Advis et al., 1981b). Also, daily injections of large doses of GH (13.6 or 68 mg/kg body weight [bw]) beginning on day 26 lowered pituitary GH by up to 58% and caused similar delays (Groesbeck et al., 1987). However, a passive immunization of immature female rats from day 15 onward with an antibody against rat growth hormone-releasing hormone (GHRH) resulted in significant depletions of circulating GH and IGF-I, but did not affect VO (Gruaz et al., 1994). This work also showed that the maturational recovery that occurs when food-restricted rats are returned to normal diets was not prevented by GH deprivation, implying that GH is not an essential participant in the recovery process. Moreover, it could be that the above influence of GH on the sexual maturation may well be mediated by reported roles of NMDA receptor activation (Veneroni et al., 1990; Cocilovo et al., 1992) or leptin (Aubert et al., 1998; Carro et al., 1999) as regulators of GH release via an effect on GHRH. It is also possible that other GH regulatory factors play a maturational role. For example, a newly discovered orphan G-protein-coupled receptor triggers an episodic release of GH when activated by synthetic ligands. It apparently functions independently of GHRH or the GH inhibitory factor somatostatin (Smith et al., 1997, 1999)

The indoleamine melatonin is a secretory product of the pineal gland. Attempts to determine its involvement in female pubertal development have generated contradictory results. A role for melatonin is more strongly supported in those species that display a seasonal reproductive cycle. In the rat, even though it is a photosensitive species, seasonal cycles are at best a minor component of reproductive activity, and no relationship appears to exist in humans. Experiments in laboratory rodents have demonstrated that melatonin can both suppress sexual activity in adult males (Kinson, 1976) and delay pubertal development in immature ones (Lang et al., 1985; Kennaway and Rowe, 1997). Data in the immature female are inconsistent (e.g., Sizonenko et al., 1985; Badawi and Wilkinson, 1988), and the results of exogenously administered melatonin may depend on the stage of postnatal development (Batmanabane and Ramesh, 1996) and the point in the photoperiod (Sizonenko et al., 1985) when the exposures take place. Nevertheless, endogenous melatonin may have some role in shaping the final pattern of LH secretion that emerges during puberty (Rivest, 1987).

III. EXOGENOUS INFLUENCES ON THE ONSET OF PUBERTY

In keeping with the present focus on the pubertal assessment of toxicant exposure, the following section considers those forms of chemical insult, both environmental and pharmacological, to the maturing female reproductive system that take place during the 7- to 10-day prepubertal interval. Perinatal and early postnatal exposures, while no less important within the context of reproductive impact, broaden the scope of such insult to include earlier developmental processes that precede those events critical to the postweaning emergence of sexual maturity. In this section, the material is partitioned into (1) sex steroid-related and (2) non-steroid-related toxicant-induced alterations, and (3) induced alterations in thyroid endpoints. However, it should be understood at the

outset that because puberty is the result of a series of interrelated processes, such partitioning should be viewed simply as a matter of convenience.

A. Steroids: Agonistic, Antagonistic, or Direct Effects on Steroid-Related Processes

1. Exogenous Estrogen Exposure

While all gonadal and adrenal steroids are important for the development and function of the female reproductive system throughout her life span, it is estrogen that plays the key role in the initiation of sexual maturation. Ovariectomy prior to the onset of puberty prevents further maturation of the reproductive system (Critchlow and Bar-Sela, 1967) and exogenous estrogens when administered around the time of weaning can advance the onset of puberty (see Table 4 for summary). The first experiments that demonstrated such an advancement were reported by Allen and Doisy (1924) describing an induction of VO following the injection of follicular extracts from adult ovarian homogenates into immature rats. Subsequent studies demonstrated that the active hormone in the follicular extracts was 17β-estradiol (E2; for review see Goldzieher, 1994), and that daily injections of 17β-estradiol benzoate (E2B) beginning on postnatal days 5 or 26 caused an advancement in VO when compared with controls (Ramirez and Sawyer et al., 1965). In addition, increased plasma LH concentrations, along with an initiation of estrous cycles, indicated the induction of true precocious puberty in the E2B-treated animals. A general summary of these effects is presented in Table 5.

Edgren et al. (1966) and Odum et al. (1997) have evaluated the effects of a variety of estrogens on VO in rodents. When initiated on day 25, the effective doses of E2 and 18-homoestradiol (18-methylestra-1,3,5(10)-triene-3, 17β-diol) that advanced VO in 100% of rats by 30 days of age were 0.03 μg/rat and 0.3 μg/rat, respectively (Edgren et al., 1966). Odum et al. (1997) compared the ability of E2, E2B, and ethynyl estradiol (EE) to induce VO in immature mice following 3 days of exposure by oral gavage or subcutaneous injection. Of these estrogens, lower doses of EE (2 μg/kg, s.c.) induced VO in 100% of the animals when compared with higher concentrations of E2 (E2; 20 μg/kg, s.c.; 66% VO) or E2B (10 μg/kg, s.c.; 35% VO). In addition, exposure by oral gavage was less effective when compared with a subcutaneous route for all three estrogens.

2. Steroid Receptors

Data describing reproductive effects in the female following prepubertal exposures to environmental toxicants have primarily focused on those compounds that have been demonstrated to affect steroid receptor binding or steroidogenesis. Many of the physiological processes regulated by estrogen, progesterone, and testosterone during development and reproductive function are mediated through intracellular receptors. These receptors, after binding to their respective ligands, then undergo structural modifications that permit an interaction with the steroid binding element of specific steroid-regulated genes (see Jensen, 1996 for review). It is the interaction of the ligand-receptor complex, along with the association of additional coactivators, which stabilize the DNA and allows gene transcription to occur resulting in the synthesis of new proteins within several hours (Shibata et al., 1997).

In vitro tests have demonstrated that many chemicals can bind to intracellular steroid receptors (Kelce et al., 1995; Shelby et al., 1996; Danzo, 1997; Bolger et al., 1998; Kuiper et al., 1998). They can mimic steroid activity *in vivo* and induce agonistic or antagonistic cellular responses by binding to the steroid receptor (Shelby et al., 1996; Nimrod and Benson, 1996; Kelce et al., 1997; Ostby et al., 1999), or by activating/inhibiting steroid receptor function via phosphorylation or modifications in the action of receptor coactivators (Aronica and Katzenellenbogen, 1993; Jenster 1998; Macgregor and Jordan, 1998). Similarly, *in vitro* studies using mammalian or yeast cell systems transfected with steroid receptor expression vectors and steroid receptor-linked reporter genes have demonstrated that environmental chemicals can induce steroid-regulated gene transcription (Tran et al., 1996; Gaido et al., 1997; Kelce and Wilson, 1997).

Multiple isoforms of both estrogen and progesterone receptors have been identified (e.g., Kato

TABLE 4
Chemicals That Alter The Onset of Puberty by Disrupting Steroid Function

Treatment	Dose-route (duration)	Effect	Putative/suggested mechanism(s)	Ref.
Estrogens, agonists				
Ovarian follicular hormone	0.5–1 cc/rat/d–injection (d21–VO)	Advanced VO	Feedback to hypothalamus	Allen and Doisy, 1924
Estradiol	0.4 mg/kg/d–oral (d21–23)	Advanced VO	Feedback to hypothalamus	Ashby et al., 1997
17β-Estradiol	0.03 μg/rat/d–sc (d25–42)	Advanced VO	Feedback to hypothalamus	Edgren et al., 1966
17β-Estradiol	0.5–400 μg/kg/d–sc 10–300 μg/kg/d-oral, (d21–23)	Advanced VO, 20–400 μg/kg/d–sc	Feedback to hypothalamus	Odum et al., 1997
17β-Estradiol	Silastic implant 25–100% E2	Advanced VO followed by 12–17 d persistent diestrus before regular estrous cycles	Feedback to hypothalamus	Nass et al., 1984
17β-Estradiol benzoate	0.5 μg/100 g (d5–VO; d26–VO)	Advanced VO; ↑ LH	Feedback to hypothalamus	Ramirez and Sawyer, 1965
17β-Estradiol benzoate	0.1–200 μg/kg/d–sc, 10–400 μg/kg/d–oral,	Advanced VO, 10–200 μg/kg/d–sc	Feedback to hypothalamus	Odum et al., 1997
17β-Estradiol benzoate ± Progesterone	1 μg/rat–sc ± 2 mg progesterone, sc (d22)	E ↑ ovarian wt. after hCG augmentation, P inhibited	E increased output of pituitary FSH; P inhibited response	Naqvi and Johnson, 1970
18-homoestradiol (18-methylestra-1,3,5(10)-triene-3,17β-diol)	0.3 μg/rat/d–sc (d25-42)	Advanced VO	Feedback to hypothalamus	Edgren et al., 1966
Ethynyl estradiol	0.05–400 μg/kg/d–sc, 0.05–400 μg/kg/d–oral,	Advanced VO, 2–400 μg/kg/d–sc	Feedback to hypothalamus	Odum et al., 1997
Ethynyl estradiol	1–5 μg/kg/d–oral (5 d)	Advanced VO, cornified vaginal cells 5 μg/kg/d	Feedback to hypothalamus effect on vaginal epithelium	Singh and Kamboj, 1980

Diethylstilbestrol (DES)	Silastic implant 50% DES (d12-VO)	Advanced VO followed by 12–17 d of persistent diestrus before regular estrous cycles	Feedback to hypothalamus	Nass et al., 1984
E696, E785	1–5 μg/kg/d–oral (5 days)	Advanced VO, cornified vaginal cells 5 μg/kg/d	Feedback to hypothalamus, effect on vaginal epithelium	Singh and Kamboj, 1980
Estrogen-antagonists, partial agonists and selective ER modulators				
Tamoxifen	1.0 mg/kg–sc (d21–23)	Incr. uterine wt.	Partial agonist effect on estrogen receptors	Wakeling and Bowler, 1988
MRI-41 (Clomiphene citrate)	0.5 mg/kg/d–sc (d18–21)	Advanced VO	Possible inhibition of E negative feedback of LH; most likely agonist effect on estrogen receptor (ER)/hypothalamic feedback	Coppola and Perrine, 1965
MRI-41 (Clomiphene citrate)	0.25–3.0 mg/kg/d–oral (d28–32)	0.5 mg/kg/d ↑ ova released during PMSG induced ovulation; 1.5–3.0 mg/kg/d ↓ ova released	Possible inhibition of E negative feedback of LH; most likely agonist effect on ER/CNS, pituitary and ovary	Coppola and Perrine, 1965
Raloxifene	0.01–1.0 mg/kg/d–oral (d21–23)	No effect on VO	—	Ashby et al., 1997
MER-25 (ethamoxytriphetol)	5 mg MER–25/kg feed; (weaning to VO)	Advanced VO	Feedback to hypothalamus	Lerner, 1964
U11555A (2-[*p*-(6-methoxy-2-phenylinden-3-yl) phenoxy]-triethylamine, hydrocloride)	0.25–6.0 mg/kg/d–oral (d28–32)	0.5 mg/kg/d ↑ ova released during PMSG induced ovulation; 1.5–6.0 mg/kg/d ↓ ova released	Possible inhibition of E negative agonist effect on ER/CNS, feedback on LH; most likely pituitary, and ovary	Coppola and Perrine, 1965
U11555A	0.5 mg/kg/d–sc (d18–21)	Advanced VO	Possible inhibition of E negative feedback on LH; most likely agonist effect on hypothalamic feedback	Coppola and Perrine, 1965

TABLE 4 (continued)
Chemicals That Alter The Onset of Puberty by Disrupting Steroid Function

Treatment	Dose-route (duration)	Effect	Putative/suggested mechanism(s)	Ref.
Phytoestrogens				
Coumestrol	0.01% diet (d21–24 or d22–60)	Advanced VO and 1st estrus	Agonist feedback effects	Whitten and Naftolin, 1992
Coumestrol	5–80 mg/kg–oral d21–23	Incr. in uterine wt. 20–80 mg/kg	Agonist effect at estrogen receptor	Baker et al., 1999
Environmental chemicals				
Methoxychlor	25–200 mg/kg/d–oral (weaning – onward)	Advanced VO, 25–200 mg/kg/d, advanced 1st estrus and onset of cycles (25 mg/kg/d); persistent vaginal estrus (200 mg/kg/d)	Feedback to hypothalamus	Gray et al., 1988, 1989
Methoxychlor	500 mg/kd/d–oral or sc, (d21–23)	Advanced VO with 500 mg/kg/d–oral but not with sc route	Feedback to hypothalamus	Odum et al., 1997
Lindane (γ-HCH)	5–40 mg/kg/d–oral (d21–110 or 125)	Delayed VO (10 and 40 mg/kg/d): ↓ number of animals with regular 5-d estrous cycle w/ all doses from 51–90 d	↓ LH or ↓ prolactin	Cooper et al., 1989
4-*tert*-octylphenol	100–200 mg/kd/d–oral (Weaning – VO)	Advanced VO (100–200 mg/kg/d)	Feedback to hypothalamus	Gray and Ostby, 1998
4-*tert*-octylphenol	50–200 mg/kg/d-oral (d21-34)	Advanced VO 200 mg/kg/d	Feedback to hypothalamus	Laws et al., 2000
p-nonylphenol	25–100 mg/kg/d–oral (d21–34)	Advance VO 50 and 100 mg/kg/d	Feedback to hypothalamus	Laws et al., 2000
Bisphenol A	50–400 mg/kg/d–oral (d21–34)	No effect on VO	—	Laws et al., 2000
Bisphenol A	400–800 mg/kg/d–oral, 600–800 mg/kg/d–sc	No effect on VO with oral dosing; advanced	Feedback to hypothalamus	Ashby and Tinwell, 1998

	(d21–23)	VO with 600–800 mg/kg/d -sc		
Bisphenol A	5–150 mg/kg/d–oral, (d21–23)	↑ Progesterone receptors, no uterotrophic effect	Possible involvement of alternate pathway rather than classic ER pathway	Gould et al., 1998
Aromatase inhibitors				
Fadrozole	0.6–6.0 mg/kg–oral (d21–40)	Delayed VO, ↓ uterine wts. all doses	Inibition of estradiol production	Marty et al., 1999
Cytochrome P450 inhibitors				
Ketoconazole	50 and 100 mg/kg–oral (d21–40)	Delayed VO, ↑ uterine wts 100 mg/kg	Inhibition of steroid synthesis	Marty et al., 1999
Androgens, agonists				
Testosterone	Silastic implant with 50% T (d12–VO)	Advanced VO followed by 12–17 d of persistent diestrus before regular estrus cycling	CNS effect as well as direct effect on vaginal epithelium	Nass et al., 1984
Testosterone	Silastic implant with 100% T (d15–30)	Advanced VO; Constant estrus with small polyfollicular ovaries at 86 d	CNS effect as well as direct effect on vaginal epithelium	Bloch et al., 1995
Testosterone	2–6 mg/ml, silastic implant, (d28–1st estrus)	Advanced VO; did not advance 1st ovulation	Direct effect on vaginal epithelium probably mediated by local aromatization of testosterone (T)	Mathews et al., 1987
Testosterone	60–120 μg/rat–sc (d16–25, d17–19, d18–19, d18–24)	Increased ovarian weight (d16–17, 120 μg)	Effects are time and dose dependent	Marton & Endroczi, 1973
Testosterone	0.1 mg/rat, sc; (d21, d21–23, d21–30) 0.01 mg/rat, perivaginal, (d21–23)	Advanced VO (d21–23; d21–30), advanced 1st estrus (d21-23); delayed 1st estrus (d21-30)	Effects are time and dose dependent; direct effect of T on CNS	Zarrow et al., 1969
Testosterone ± progesterone	100 μg/rat,sc ± 2 mg progesterone/rat, sc (d22)	T↑ ovarian wt. after HCG augmentation; P inhibited response	T increased output of pituitary FSH	Naqvi and Johnson, 1970

TABLE 4 (continued)
Chemicals That Alter The Onset of Puberty by Disrupting Steroid Function

Treatment	Dose-route (duration)	Effect	Putative/suggested mechanism(s)	Ref.
Dehydroepiandrosterone (DHA)	60 mg/kg–sc (d27–47)	Precocious ovulation followed by ovulatory failure, ↑ prolactin	Restoration of cyclicity after DHA withdrawal suggests ovulatory failure due to androgen	Knudsen et al., 1975
Dehydroepiandrosterone (DHA)	60 mg/kg–sc (d27–29)	↑ uterine wt. and surge of FSH, LH and prolactin with early ovul. on d30	Aromatization of DHA and effect on CNS	Knudsen and Mahesh, 1975, Parker and Mahesh, 1977
Dehydroepiandrosterone + cyanoketone	60 mg/kg–sc 71 mg Cyanoketone/kg, sc (d 27–29)	Cyanoketone blocked DHA ↑ in uterine wt. and early ovulation	Cyanoketone inhibited the conversion of DHA to estrogen	Knudsen and Mahesh, 1975
Dihydrotestosterone (DHT)	0.5–60 mg/kg/d–sc (d27–29)	No early ovulation or potentiation of PMSG-induced gonadotropin release	DHT cannot be aromatized to estrogen	Knudsen & Mahesh, 1975
3α-diol (5-α-adrostane-3α, 17β-diol)	50 μg-silastic implant (d22–26), 100 μg-silastic implant, (d22–45)	Pin-hole VO, 1st estrus delayed (100 μg)	Possible inhibitory effect on the onset of puberty	Kramer and Meijs-Roelofs, 1982
3α-diol	0.5–3.0 mg/kg–sc, (d13–30; d33–35)	↓FSH (d13–30, all doses), (d33–35, 3.0 mg/kg)	Possible inhibitory effect on gonadotropins	Meijs-Roelofs et al., 1982
3α-diol	Silastic implant, (d22–52)	Advanced VO, but no effect on estrus cyclicity; ↓pituitary wt. and Pit. LH	Possible antagonist effect on E2 function and inhibitory effect on gonadotropins	Ruf, 1983
3α-diol	0.5, 2, 10 mg/ml-silastic implants, (d30–1st diestrus)	Delayed VO and 1st diestrus (10 mg/ml)	Dose is several times higher than normal prepubertal 3α-diol	Ojeda et al., 1984
3α-diol	0.2–10 mg/ml–silastic implants, + 7.5 IU PMSG-sc (d29–32)	Did not prevent PMSG-induced LH surge or ovulation	Suggests that 3α-diol may not play a role in the steroidal negative feedback prior to pubertal onset	Ojeda et al., 1984

3β-diol (5-α-adrostane-3β, 17β-diol)	50 μg/rat/d-sc (d22–26) 100 μg/rat/d–sc, (d22–1st estrus)	Pin-hole VO several days prior to delayed 1st estrus (100 μg/rat/d–sc)	Possible inhibitory effect on 1st ovulation with exposure to higher doses	Kramer and Meijs-Roelofs, 1982
3β-diol	50 μg/rat/d–sc (d22–26)	Advanced VO but no effect on 1st estrus	Possible inhibitory effect on 1st ovulation	Eckstein, 1975
3β-diol	Silastic implants (d22-52)	No effect on VO	—	Ruf, 1983
Weak Androgen				
Danazol	750 mg/kg/d–sc (d24–28)	↓PMSG induced ovulation	↓ LH surge and possible direct effect on ovary	Tamura et al., 1991
5α-reductase inhibitor				
4-MA (17β-*N,N*-diethyl-carbamoyl-4-methyl-4-aza-5α-androstane-3-one)	25–50 mg/kg,–sc (d26–1st estrus)	↓ 3α-diol but no effect on VO or 1st estrus	Suggests that 3α-diol alone does not regulate the onset of puberty	Ojeda et al., 1984
Progestins-antagonist				
RU-486	1 mg/kg/d–sc (d21–32)	No change in age of VO or 1st estrus	Progesterone antagonist	Trimino & Aguilar, 1992

Note: All data from rats. Abbreviations: CNS, central nervous system; sc, subcutaneous; T, testosterone.

TABLE 5
Summary of Effects on EDSTAC Endpoints on Pubertal Development and Thyroid Function

Chemical	Growth	Age at Vaginal Opening	Vaginal cytology[a]	Tissue Weight[b]	Hormones[c]	Histology[d]
GnRH						
Agonist (sustained treatment)		↑				
Antagonist		↑			↓ LH, FSH	
Dopamine						
Agonist		↑			↓ PRL	
Antagonist		↓			↑ PRL	
Estrogen						
Agonist	↓	↓	CVE, PD, PE*	↑ U	↑ LH, FSH	Hypertrophy of uterine luminal and glandular epithelium
Weak agonist[e]		↓	IR (PD, PE)*	↑ U		
Antagonist		↑				NE on uterine luminal epithelium[f]
Androgen						
Agonist (aromatizable)		↓	IR (PD,PE)*		↑ FSH	
Antagonist		NE				
Progestin						
Agonist						
Antagonist		NE[g]				
Thyroid hormones						
Hypothyroidism	↓	↑	IR (PD)		↑ TSH; ↓ T3, T4	↓ TFCA; ↑ TFCH
Hyperthyroidism					↓ TSH; ↑ T3, T4	

Note: NE: no effect observed.

[a] Vaginal cytology: CVE, cornified vaginal epithelium; IR, irregular 4–5 day estrous cycles; PD, persistent vaginal diestrus; PE, persistent vaginal estrus.
[b] Tissue weights: P, pituitary; U, uterus; O, ovaries; L, liver; K, kidneys; A, adrenals.
[c] Hormones: LH, luteinizing hormone; FSH, follicle stimulating hormone; PRL, prolactin; T3,T4, thyroid hormones; TSH, thyroid stimulating hormone.
[d] Histology: TFCA, thyroid follicular colloidal area; TFCH, thyroid follicular cell height.
[e] Weak agonist: environmental chemicals such as Methoxychlor, 4-tert-octylphenol, *p*-nonylphenol.
[f] Based on studies of ICI 182,780; Mixed agonist/antagonist such as tamoxifen can induce change in uterine histology.
[g] Based on one study of RU486.

* The specific change in vaginal cytology will depend on the concentration and length of treatment.

et al., 1994; Wen et al., 1994; Kuiper et al., 1996, 1997; Paech et al., 1997; Petersen et al., 1998) and provide for the possibility that environmental chemicals may cause subtle alterations in steroid receptor function. In this regard, the α and β forms of the estrogen receptor show similar binding affinities for various compounds, but can be dissimilar in their ability to bind to some environmental chemicals (Kuiper et al., 1998). In addition, results from *in vitro* studies suggest that modifications in transcriptional activity may occur through distinct signaling pathways, depending on the formation of homo- or heterodimers of the receptor isoforms (Wen et al., 1994; Cowley et al., 1997; Giguère et al., 1998). While the biological significance of these receptor isoforms is not totally understood, their distribution has been shown to vary with tissue (Kuiper et al., 1997; Shughrue et al., 1997) and endocrine status (Camacho-Arroyo et al., 1998; Sharma et al., 1999; Szabo et al., 2000).

In addition to the classic intracellular steroid receptors, estrogen and progesterone receptors associated with the cell membrane have been identified (see Golden et al., 1998 or Watson and Gametchu, 1999 for reviews). Membrane steroid receptors mediate rapid cellular changes, occurring within seconds to minutes, and initiate nongenomic cellular changes. Typical changes include ion fluxes, glucose transport, activation of second messenger systems, and an altered firing of neurons.

3. Environmental Estrogens

There has been a growing body of literature showing that plant estrogens are capable of binding to the estrogen receptor (e.g., Martin et al., 1978; Nelson et al., 1984; Hopert et al., 1998) and exerting weak estrogenic responses in mammals. For example, coumestrol (Medlock et al., 1995; Baker et al., 1999), equol (Tang and Adams, 1980), and genistein (Santell et al., 1997) have been found to increase uterine weight. Moreover, genistein has been reported to affect the development of the sexually dimorphic nucleus of the brain medial preoptic area (Faber and Hughes, 1993) and alter the GnRH-induced release of LH in ovariectomized rats (Hughes, 1987). However, inconsistencies in the data do exist that have been influenced by such things as age (Levy et al., 1995), dosing regimen, the relationship between times of exposure and assessment, and the route of exposure (Whitten et al., 1994; Baker et al., 1999). Also, a directional difference has been reported between estradiol and at least one phytoestrogen (coumestrol) in the regional CNS expression of ER subtype mRNA (Patisaul et al., 1999). Whether such an effect has some functional significance remains to be demonstrated.

At present, the great bulk of those studies exploring an effect of the phytoestrogens on reproductive maturation have employed gestational or early neonatal exposures. Whitten and Naftolin (1992) did investigate the influence of prepubertal dietary exposures to coumestrol (0.01% concentration, d21-24 or d22-60) and found a 4-day acceleration of VO for the longer exposures (Table 4). Estrous cycles began once VO had occurred, and in the longer-treated animals did show some irregularities by about 17 weeks of age. Baker et al. (1999) have also reported that oral administration of this compound (5 to 80 mg/kg) on days 22 to 24 caused significant elevations in uterine weight at concentrations of 20 mg/kg and above.

The organochlorine pesticide methoxychlor (2,2-*bis*(4-methoxyphenyl)-1,1,1- trichloroethane) stimulates estrogenic activity presumably through the ability of at least one of its metabolites to bind to the intracellular estrogen receptor (Bulger et al., 1978a, 1978c). The principal active metabolite is 2,2-*bis*(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is rapidly formed from methoxychlor by *o*-demethylation. Methoxychlor (25 mg/kg/d) administered by gavage beginning at weaning was found to advance VO by an average of 6 days, along with corresponding shifts in the appearance of first estrus and the onset of estrous cycles (Gray et al., 1988, 1989). In females receiving higher doses of methoxychlor (50 to 100 mg/kg/d), VO was advanced by 7 days and the length of the estrous cycles was increased. In addition to exhibiting a comparable shortening of the time to VO, continued treatment with the highest dose of methoxychlor (200 mg/kg/d) induced a persistent cornification in the vaginal smear that continued through a 9-week period of observation (Gray et al., 1989). In these studies, significantly lower body weights were observed with all doses of methoxychlor, an effect consistent with estrogenic activity. A comparison of the effects of methoxychlor on uterine weight and VO

following a 3-day oral or subcutaneous dosing regimen was reported by Odum et al. (1997). Methoxychlor was uterotrophic following both oral or subcutaneous exposures (500 mg/kg/d for 3 days), although an advancement in VO (60% of the animals) occurred only in the oral treatment group.

Bisphenol A (BPA) is a plasticizer with estrogenic properties that is used in the production of epoxy resins and polycarbonate. It has been found to compete with estradiol for binding to estrogen receptors and can induce the synthesis of progesterone receptors in human mammary cancer cell (MCF-7) culture (Krishan et al., 1993). BPA also has been reported to increase prolactin secretion and gene expression (Steinmetz et al., 1997) and trigger a uterotrophic response in rats. Ashby et al. (1998) and Laws et al. (2000) observed increased uterine weights in immature rats following three doses of BPA (200 to 800 mg/kg/d) when administered by oral gavage or subcutaneous injection. However, no change in VO was detected following three doses of 400 to 800 mg/kg/d by oral gavage (Ashby and Tinwell, 1998) or continuous treatment with 100 to 400 mg/kg/d from weaning until 34 days of age (Laws et al., 2000). An advancement in VO was, however, observed in four of seven animals in response to subcutaneous injections of concentrations between 600 and 800 mg/kg/d (Ashby and Tinwell, 1998). Interestingly, Gould et al. (1998) reported an increase in progesterone receptors (an estrogen-induced alteration), but no uterotrophic response in immature female rats given 5 to 150 mg/kg/d BPA for 3 days by oral gavage. Together these data suggest that in addition to a differential effectiveness seen with particular routes of exposure, the observed effects of BPA may be mediated not only through the classic estrogen receptor pathway, but could involve alternate pathways as well.

A number of other compounds in the environment have been reported to affect estrogenic activity. The alkylphenol 4-tert-octylphenol was found to increase uterine weight in prepubertal rats (10 mg/d, s.c., Bicknell et al., 1995; 100 to 400 mg/kg, oral, Gray and Ostby, 1998; Laws et al., 2000). An advancement in VO (200 mg/kg, oral, Gray and Ostby, 1998; Laws et al., 2000) has also been found, along with disruptions in estrous cyclicity (20 to 40 mg/dose, s.c., Blake and Ashiru, 1997; 200 mg/kg, Laws et al., 2000), alterations in pituitary hormone levels (80 mg/dose, s.c., Blake and Boockfor, 1997), and a stimulation of prolactin gene expression (1 to 100 μM, *in vitro*, Abraham and Frawley, 1997). All of these observations imply an estrogen-like mechanism of action. Similarly, another alkylphenol, *p*-nonylphenol, has been reported to be uterotrophic in immature rats (Lee and Lee, 1996; Odum et al., 1997; Laws et al., 2000) and induce both an increase in mammary gland development (100 mg/kg, oral, Odum et al., 1999) and advance the age at VO (50 mg/kg, oral, Laws et al., 2000). The mycotoxin zearalenone has also been observed to be uterotrophic under different treatment paradigms (Mirocha et al., 1978; Sheehan et al., 1984), but there is no evidence that sexual maturation in the female rat is affected by prepubertal exposures.

4. Alterations in Steroid Synthesis

In addition to changes in receptor function, it is well established that pharmaceuticals and environmental chemicals can disrupt steroidogenesis in male (e.g., Hirsch et al., 1987a; Gray et al., 1991) and female rats (e.g., Leung and Armstrong, 1979; Cummings et al., 1997). Cholesterol, the precursor for steroid biosynthesis, is converted to estradiol and progesterone through a number of enzymatic reactions catalyzed by mixed function oxidases that utilize cytochrome P-450, molecular oxygen, and NADPH along with several dehydrogenases (Ojeda, 1988). Small amounts of androgens are also synthesized in the female that are generally aromatized to estrone and estradiol. Chemicals can disrupt steroid biosynthesis either by inhibiting any of these enzymatic reactions, or by altering the intramitochondrial transport of cholesterol. For example, the fungicide fenarimol (α-(2-chlorophenyl)-α-(4-chlorphenyl)-5-pyrimidinemethanol) is a non-steroidal inhibitor of aromatase activity that will block estrogen synthesis (Hirsch et al., 1987b). Its analog, LY56110 (α,α-*bis*(4-chlorophenyl)-5-methylpyrimidine), has been found to suppress a testosterone-induced increase in uterine weight (Hirsch et al., 1987b), although there has been no evidence that vaginal opening is affected following prepubertal administration. Cummings et al. (1997) reported a reduction in serum progesterone and the number of implantation sites in rats following early gesta-

tional exposure to ketoconazole, an antifungal agent known to inhibit several enzymatic reactions in the steroid biosynthetic pathway. Recently, Marty et al. (1999) evaluated the effects of ketoconazole (100 mg/kg, oral) and the aromatase inhibitor fadrozole (0.6 and 6 mg/kg, oral) on pubertal development using the EDSTAC-recommended d21-40 exposure paradigm. They observed that both compounds at the administered concentrations delayed the onset of VO.

Alterations in estrogen metabolism following exposure to environmental chemicals may also occur. Bradlow et al. (1995) reported an increase in the ratio of 16 alpha-hydroxyestrone to 2-hydroxyestrone in MCF-7 cells following exposure to *o,p*-DDT and other environmental chemicals. It has also been suggested that activation of liver cytochrome P450 systems involved in estrogen clearance may be affected by environmental exposures and in some cases may reduce endogenous serum estrogen concentrations (Welch et al., 1971). Specifically, these authors have shown that chlordane decreased the concentration of tritiated estrone in the uterus of sexually immature female rats and reduced the hormone's uterotrophic action.

5. Pharmaceutical and Environmental Antiestrogens

Pharmaceuticals developed for use as antifertility agents or antiestrogenic cancer treatments have also been tested for their ability to alter sexual maturation (Table 4). The alkanoic acid derivatives, E696 (3-(3,4-di-hydro-6-methoxy-2napthy)2-dimethyl pentanoic acid) and E785 (3-(3,4-dihydro-6-methyoxy-2-naphthyl)2-dimethyl hexanoic acid), were originally developed as antifertility agents and possess estrogenic activity. Both were observed to induce VO, as well as vaginal epithelial cell cornification, in immature rats within 5 days of an oral dose of 5 μg/kg (Singh and Kamboj, 1980). Antiestrogens possessing partial agonist activity such as MER-25 (ethamoxytriphetol, 5 mg/kg in feed, Lerner, 1964), MRL-41 (clomiphene citrate, 0.5 mg/kg, oral), and U1155A (2-[*p*-(6-methoxy-2-phenylinden-3-yl) phenoxy]-triethylamine hydrochloride) (Coppola and Perrine, 1965) have been reported to cause premature VO. At lower doses, the latter two antiestrogens have also been found to cause a superovulation in juvenile rats (Coppola and Perrine, 1965).

The triphenylethylene antiestrogen tamoxifen (1.0 mg/kg, s.c.) is known to increase uterine weight in immature rats, although the maximal stimulation is less than that observed with estradiol (Wakeling, 1989). The same dose of tamoxifen exerts a partial inhibitory effect on the uterotrophic response to estradiol benzoate when the two chemicals are administered concurrently. Similarly, tamoxifen treatment (0.1 to 10 mg/kg, 14 days, oral) in intact adult rats has been found to reduce the effectiveness of endogenous estradiol, resulting in a decrease in uterine weight and a disruption of estrous cyclicity (Wakeling and Bowler, 1988). A partial agonist effect is evident following neonatal exposure (1 to 25 μg/rat/days 4 to 6, s.c.), when an acceleration of VO can be observed (Wakeling and Bowler, 1988). Also, a prepubertal exposure (10 μg/rat, days 20 to 24) has been reported to cause hypertrophy of the uterine luminal and glandular epithelium (Branham et al., 1996).

The tissue-selective estrogen agonist raloxifene (LY156758; [2-(4-hydroxyphenyl)-6-hydroxybenzo [b]thien-3-yl] [4-[2-(1-piperidinyl)ethoxy]phenyl]methanone hydrochloride), although observed to be uterotrophic in rats following a 3-day exposure to 0.1 mg/kg (oral), did not induce VO (Ashby et al., 1997) within the same dosing period. Administration of ICI 164,384, an antiestrogen with negligible agonist activity *in vivo*, blocked the uterotrophic response induced by estradiol benzoate (0.5 μg/rat, s.c.), tamoxifen (1.0 mg/kg, s.c.) or raloxifene (0.1 mg/kg, oral) in immature rats (Wakeling, 1989; Ashby et al., 1997).

Lindane (γHCH; 1,2,3,4,5,6-hexachlorocyclohexane), the γ-isomer of hexachlorocyclohexane, is a broad spectrum insecticide that has been argued to disrupt estrogenic activity. In Y1 mouse adrenal tumor cells it has been reported to inhibit steroidogenesis by disrupting the intramitochondrial transport of cholesterol (Zisterer et al., 1996). Cooper and co-workers (1989) have shown that lindane (10 and 40 mg/kg/d, oral) delayed VO and the appearance of regular estrous cycles in immature rats when treatment was initiated at 21 days of age. With continued treatment, none of the lindane treatment groups (5 to 40 mg/kg/d) displayed regular estrous cycling patterns. Extended periods of leukocytic (e.g., persistent diestrus) or cornified

(e.g., persistent estrus) smears were observed in all doses. Additional studies evaluated the short-term effects of lindane in prepubertal females (dosed from 21 to 27 days of age with 30 mg/kg/d, oral) with or without E2B (10 μg/kg, s.c.) 6 or 30 h prior to necropsy. In these animals, lindane significantly reduced the uterotrophic response to estrogen at 30 hours. Additionally, the E2B-stimulated increases in serum LH and prolactin were diminished with lindane treatment, suggesting that the pituitary response to E2B was blocked. These effects are probably not mediated through an alteration of estrogen receptor function, as Laws et al. (1994) have shown that lindane does not alter circulating estradiol levels, intracellular estrogen receptor binding affinity, receptor number, or the functional response to estrogen measured by the induction of progesterone receptors.

6. Androgen Aromatization

A physiological role for aromatizable androgens in the onset of puberty has not been clearly determined, even though serum testosterone concentrations increase during the onset of puberty in the female rat. Mathews et al. (1987) reported that serum testosterone concentrations begin to rise during early proestrus and reach a nine-fold increase at the time of the first LH surge. Serum androstenedione concentrations also increased at the time of the first LH surge, but dehydroepiandrosterone concentrations remain undetectable throughout the onset of puberty. Using steroid implants in immature rats (28 days of age) to approximate the physiological concentrations of testosterone found during early proestrus, these authors reported that testosterone advanced VO but not first ovulation. Rather, there was a reduction of LH secretion, resulting in a delay of first ovulation. These studies are in agreement with earlier reports demonstrating the ability of testosterone to advance VO (Tramezzani et al., 1963; Zarrow et al., 1969) but differ with regard to its ability to induce early ovulation. Differences between the studies may be a result of variations in dose and time of exposure. Zarrow et al. (1969) observed that while a single dose of 0.1 mg of testosterone on day 21 failed to alter the age of puberty onset, the same dose during days 21 to 23 advanced both VO and first estrus. Moreover, a 10-day exposure (d21 to 30) advanced VO, but extended the time to the appearance of first estrus (Table 4). Although serum estradiol concentrations were not increased in intact immature female rats following an administration of physiological levels of testosterone (Mathews et al., 1987), the observation that aromatase-like activity increases in the vaginal epithelium of immature rats prior to the onset of puberty suggests that local estrogen produced by aromatization may mediate changes in the timing of VO by acting directly on the vaginal epithelium (Lephart et al., 1989). Table 5 includes a summary of these androgen-associated effects.

Dehydroepiandrosterone is another aromatizable androgen that has been reported to induce precocious puberty in female rats. Exposure to dehydroepiandrosterone for 3 days beginning on day 27 produced increased uterine weight within 6 h of injection and stimulated gonadotropin release and ovulation by day 30 (Knudsen and Mahesh, 1975; Parker and Mahesh, 1977). Conversion of this androgen to estrogen may be the mechanism mediating these effects, as treatment with cyanoketone, an inhibitor of 3β-hydroxysteroid dehydrogenase (which converts dehydroepiandrosterone to estrogen), blocked the onset of precocious puberty. Additionally, treatment with the nonaromatizable androgen, dihydrotestosterone failed to alter the onset of puberty (Knudsen and Mahesh, 1975). As concentrations of dehydroepiandrosterone are undetectable during pubertal development (Mathews et al., 1987) and are only minimally altered in immature rats during PMSG-induced ovulation (Parker et al., 1976), the probability that this androgen plays a physiological role in the onset of puberty is low. Nevertheless, these studies suggest that high concentrations of aromatizable androgens can alter puberty onset, which could be of importance during toxicological exposures.

7. Postpubertal Effects Following Peripubertal Exposures

Long-term reproductive effects following peripubertal exposure to estrogen or aromatizable androgens have been evaluated. Nass et al. (1984) reported that regular estrous cyclicity is disrupted during adulthood following exposure to estrogen,

diethylstilbestrol (DES), or testosterone from day 12 through VO. These authors reported that although VO was advanced with each treatment when compared with controls, first ovulation did not occur until after a period of 12 to 17 days of diestrus vaginal smears. Once first ovulation occurred, all animals began to display regular estrous cycles. However, within 30 days the percentage of animals exhibiting regular cycles had dropped to 59, 0, and 59% for E2, DES, and testosterone-treated animals, respectively. The number of regular estrous cycles in the treated animals continued to decline with age as many of these animals displayed a persistent vaginal estrus. Evaluation of gonadotropin release at 150 days of age reflected a diminished response to GnRH, which may have been associated with the alteration in cycling status. Bloch et al. (1995) have reported that exposure to testosterone (silastic implants to maintain testosterone plasma concentrations of 0.66 ± 0.04 ng/ml) during days 15 to 30 in the female rat advanced VO, with 8 of 10 females showing vaginal estrus on at least 80% of the days monitored from VO until 79 days of age. When the animals were ovariectomized at 86 days of age, the ovaries from the treated females were reduced in weight and possessed a pale appearance characteristic of polyfollicular ovaries. In addition, serum FSH and LH concentrations were reduced in E2 and progesterone-primed ovariectomized testosterone-treated animals. Similar results were reported for immature female rats treated with dehydroepiandrosterone (Knudsen et al.,1975). Following a precocious ovulation on day 30, serum prolactin remained elevated and vaginal smears were disrupted.

8. Steroid Metabolites and Derivatives

The effects of 5α-androstane-3β,17β-diol (3α-androstanediol; 3α-diol) on puberty onset have been evaluated extensively because the serum concentration of this steroid decreases around the time of first ovulation (Ojeda et al., 1984). This, along with its ability to reduce gonadotropin release (Kraulis et al., 1981), fueled the idea that the onset of puberty may be partly regulated by a reduction in serum 3α-diol concentrations. An earlier report had shown that daily subcutaneous injections of 3α-diol sulfate (100 μg/100g bw) from d21 onward could advance VO (Ravid and Eckstein, 1976). Similarly, Kramer and Meijs-Roelofs (1982) observed that daily exposures to 3α-diol (100 μg) from 22 to 45 days of age resulted in a 'pinhole' type of VO, although estrous cyclicity did not begin until 6 days after dosing was discontinued. Suppressed FSH release (Meijs-Roelofs et al., 1982) and an inhibition of the LH surge (Kraulis et al., 1981) were also noted following exposure to 3α-diol. Subsequent studies by Ojeda et al. (1984) have demonstrated that administration of physiological concentrations of 3α-diol to juvenile rats does not alter the age of puberty. Neither the age at VO nor first ovulation was changed in immature rats after exposure to the 5α-reductase inhibitor 4-MA (17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one), which caused a premature reduction in ovarian and serum 3α-diol concentrations.

Danazol, a synthetic derivative of 17α-ethynyltestosterone, has been commonly used as a therapeutic agent for endometriosis and benign fibrocystic breast disease. It has been shown to disrupt pituitary gonadotropin secretion (Dmowski, 1979), inhibit ovarian steroidogenesis (Steingold et al., 1986), and bind to androgen and progesterone receptors (Ikegami et al., 1986). Following treatment of PMSG-primed immature females with 750 mg/kg/d from days 24 to 28, there was a reduction in the percentage of animals ovulating on day 29 (Tamura et al., 1991). The magnitude of the LH surge was also reduced when evaluated on day 28. While ovulation was restored with injections of human chorionic gonadotropin, the number of oocytes released remained significantly lower than controls, suggesting that the inhibitory effects of danazol on ovulation may be mediated by multiple mechanisms. Both a suppression of gonadotropin release, as well as a direct effect on prostaglandin-mediated ovulatory events appeared to be contributing factors.

9. Progesterone

Although progesterone is known to enhance the LH surge in the immature and adult ovariectomized rat (Ramirez et al., 1980; Ying and Greep, 1971), the role of progesterone in the appearance

of the first preovulatory surge appears to be minimal. Serum concentrations of progesterone prior to the onset of puberty are moderately increased when compared with serum concentrations associated with subsequent LH surges (Dohler and Wuttke, 1975). Indeed, studies using RU-486 have shown that this progesterone antagonist has no effect on the age of VO or first estrus in immature rats treated from day 21 through puberty (Table 4) (Trimino and Aguilar, 1992). However, in terms of environmental exposures, chemicals that mimic the action of progesterone could possibly have an effect on the onset of puberty. This has been demonstrated by Naqvi et al. (1969), who used progesterone to block the estrogen-induced advancement of VO and the onset of regular estrous cycles in immature rats. Similarly, the early increase in pituitary and plasma LH in prepubertal rats following exposure to estradiol can be blocked by progesterone (Ramirez and Sawyer, 1965). The inhibitory effects of progesterone on the increase in pituitary FSH (as measured by hCG-augmented ovarian weight changes) following a single day 22 dose of estradiol benzoate or testosterone propionate were evaluated by Naqvi and Johnson (1970). In these studies, the administration of progesterone 1 h prior to estrogen completely abolished the rise in FSH due to E2 alone. Progesterone pretreatment in the testosterone group delayed the effect of the androgen on FSH for approximately 84 h.

B. Non-Steroid-Related Alterations or Indirect Effects on Steroid-Related Processes

A primary site of action for those chemicals that are able to affect sexual maturation following prepubertal administration is the brain. More specifically, the target is those hypothalamic regions responsible for generation of the neural signals that control the release of pituitary hormones. Alternatively, some chemicals are thought to affect one or more ovarian processes necessary for first ovulation to occur. For those compounds with known mechanisms of action, the majority can be classified according to their effects on peptide hormone releasing factors and/or neurotransmitter regulatory mechanisms. A representative summary of these compounds and their effects on measures of pubertal development has been included in Table 6.

1. GnRH Antagonists/Analogs

Because the pubertal process, including first ovulation, is dependent on the activation of the GnRH pulse generator, an inhibition of GnRH activity will cause a marked developmental delay. For example, subcutaneous injections of the GnRH antagonist, Org. 30276 (100, 250, or 500 μg/100 g bw) on days 28, 31, 34, and 37 showed a delay in VO and first ovulation after two exposures at the highest dose (Meijs-Roelofs et al., 1990). The circulating levels of gonadotropins were also significantly decreased. A continued decrease and longer delays were seen with additional injections. Similar effects are seen when a GnRH analog is administered in an extended fashion (as opposed to a pulsatile exposure). For example, VO was delayed and the appearance of estrous cyclicity suppressed when immature female rats were exposed to elevated concentrations of the GnRH analog [D-Leu^6, des-Gly-NH_2^{10},Pro-ethylamide^9]-GnRH by twice daily subcutaneous injections (3 μg × 2) from days 22 through 77 (Johnson et al., 1976). In humans, the GnRH analog leuprolide acetate (Lupron®) has been used as a treatment for precocious puberty and will initially result in an increased production of both LH and FSH for a period of about 7 to 10 days. This is followed by a desensitization of the pituitary to its stimulatory action, causing a dramatic and sustained drop in the production of biologically active LH and FSH for as long as treatment is continued. This alteration in gonadotropin secretion is the basis for those effects on VO seen in the rat (see Table 5 for general summary).

2. Effects on Neurotransmitter Activity

As previously mentioned, a number of neurotransmitters are now known to participate in the onset of puberty in the rat, and a suppression or augmentation in their activity can alter normal pubertal development. Significant delays in VO were observed in response to a single injection of the catecholaminergic neurotoxicant 6-hydroxydopamine into the brain ventricular system on day 23 (Ruf and Holmes, 1974), underscoring a role for noradrenergic input during this time. In contrast, a

TABLE 6
Chemicals Affecting Sexual Maturation via an Alteration in Hypothalamic-Pituitary Functions

Treatment	Dose-route (Duration)	Effect	Putative/suggested mechanism(s)	Ref.
GnRH activity				
Org30276	100–500 μg/kg BW–sc (d28,31,34, and 37)	Delayed VO	GnRH antagonist-gonadotropin at 250 & 500 mg/kg	Meijs-Roelofs et al., 1990
GnRH analog	3 μg twice daily–sc (d22–77)	Delayed VO	Down-regulation of pituitary sensitivity to LH	Johnson et al., 1976
Dopamine/Prolactin Secretion				
Prolactin	5 μg sc–2/d (d20-onward)	Advanced VO	Believed PRL affected ovarian aromatase activity	Kawagoe and Hiroi, 1989
Prolactin	Unspecified concentration by cannula into median eminence (d23)	Advanced VO	Suggest effect on symapthetic ovarian input	Advis et al., 1982
Pimozide	10 and 30 mg/l-DW (d22–30)	Advanced VO	Dopamine receptor blocker-↓ dopamine caused hyperprolactinemia	Advis et al., 1981c
Pimozide	Unspecified concentration by cannula into medial preoptic area (d28-onward)	Advanced VO	↓ DA decreased sensitivity of neg. E2 feedback	Docke et al., 1987
Sulpiride	0.5 g/l–DW (d22–1st diestrus after VO)	Advanced VO	↑ PRL sensitization of ovaries to low levels of gonadotropins	Advis and Ojeda, 1978
Bromocryptine	40 μg/ml–DW (d20-onward)	Delayed VO	Dopamine receptor agonist—believed ↓ prolactin affected ovarian aromatase activity	Kawagoe and Hiroi, 1989
Bromocryptine	20 and 100 mg/ml–DW (d22-onward)	Delayed VO/1st diestrus, decr. ovarian response to hCG	↓ ovarian sensitivity to gonadotropins	Advis et al., 1981a

TABLE 6 (continued)
Chemicals Affecting Sexual Maturation via an Alteration in Hypothalamic-Pituitary Functions

Treatment	Dose-route (Duration)	Effect	Putative/suggested mechanism(s)	Ref.
Excitatory amino acid involvement				
NMDA	20 mg/kg–sc (d24–VO)	Advanced VO	Glutamate recept. stimulation-↑ LH pulses (non-NMDA EAA recept. antag.kainate or DNQX — no effect)	Smyth/Wilkinson, 1994; Brann, et al., 1993
NMDA	18 mg/kg–ip (d21–)	No effect on VO	—	Mena-Valdivia et al., 1995
N-methyl-DL-aspartic acid (NMA)	20 mg/kg–Minipump pulsed 1 min. every 30 min-4 h/d (d26–29)	Advanced VO	Glut. Recept. stimulation-↑ LH pulses	Urbanski and Ojeda, 1987
MK-801	0.2 mg/kg–sc (d28–1st Ov) 0.1 mg/kg–2/d (d35–1st Ov) 0.1 mg/kg–2/d (d30–38)	Delayed 1st ovulation	Glutamate Receptor blocker-suppressed LH	Meijs-Roelofs et al., 1991
MK-801	0.2 mg/kg–ip (d21–30)	Delayed 1st ovulation	Glutamate Receptor blocker-suppressed LH	Veneroni et al., 1990
Ethanol (EtOH) + NMA	3 g/kg (EtOH) oral–1/d (d26–VO) 40 mg/kg (NMA) sc–2/d (d26–VO)	Adv. VO+ (EtOH+NMA) Adv. VO++ (Sal + NMA)	EtOH-suppressed GnRH release and attenuated NMA-induced puberty	Nyberg et al., 1993
Ethanol				
EtOH	5% (intragastric cannula) (d29–37)	Interval from VO-diestrus was delayed	Believed effect due to ↓ GH and LH	Dees and Skelley, 1990
Food intake/metabolism (leptin and NPY)				
NPY	10–20 mg/kg icv- (once d30)	Advanced VO and 1st ovul.	NPY potentiates initiation of LH surge	Minami and Sarkar, 1992
NPY (food-restricted rats)	18 μg/d icv-minipump (d60–66), off food restrict–d61	No VO by d66; all non-NPY controls by d66	Extended dosing—NPY was an inhibitory signal	Gruaz et al., 1993

Leptin (mice)	4 μg/g–ip (d21–29)	Advanced VO	Leptin provides metabolic signal for the initiation of puberty	Chehab et al., 1997
Leptin	6.3 μg/g/d–ip (d23–1st vaginal E)	Attenuated delay in VO seen in pair-fed controls	Leptin effect on initiation of puberty	Cheung et al., 1997b
Opiates				
Morphine	2 and 8 mg/kg–sc (d22–VO) pituitary grafted and sham-operated rats	Delayed VO, prevented advanced VO due to grafts (with 8 mg)	Enhancement of inhibitory opiate signaling—overcame effect of hyperprolactinemia on puberty	Reis & Reis, 1992
Morphine	100–800 mg/l–DW (d22–VO +5 d) [intake 5–40 mg/kg/d]	Delayed VO (800 mg/l)	Likely effect on hypothalamic regulatory mechanisms	MacDonald and Wilkinson, 1991
Naltrexone	2.5 mg/kg–4× per day–sc (d28–32)	Advanced 1st ovulation	Removal of LH from hypothalamic restraint	Meijs-Roelofs and Kramer, 1989
Naloxone	0.5 mg/kg ip (d21–)	Advanced VO	Removal of LH from hypothalamic restraint	Mena-Valdivia et al., 1995
Cannabinoids				
Δ-9-tetrahydrocannabinol	10 mg/kg–ip–2/d (d27–1st vag. E) (d24–1st vag. E)	Delay VO, no effect	Altered hypothal.-pituitary regulation	Field and Tyrey, 1984 Field and Tyrey, 1990
Δ-9-tetrahydrocannabinol	1 μg/kg–ip (d22–VO)	Delay edVO, decreased number of oocytes, altered cyclicity	Alteration in hypothalamic regulation of pituitary function	Wenger et al., 1988
Additional types of chemical insult				
Lead acetate	6 mg/ml DW (d24–74)	Delayed VO, disrupted estrus cycles (monitored d60–74)	Proposed dual effects of lead-hypothal.-pituitary (affecting LH) and gonads (steroidogenesis)	Ronis et al., 1996
Fish oil (rich in eicosapentaenoic acid, ECPA)	212 g EPCA/kg-feed (d22–1st diestrus after VO)	Delay in appearance of 1st vaginal estrus	Suggested ↑ intake of ECPA PGE2 and lowered GnRH release	Zhang et al., 1992

TABLE 6 (continued)
Chemicals Affecting Sexual Maturation via an Alteration in Hypothalamic-Pituitary Functions

Treatment	Dose-route (Duration)	Effect	Putative/suggested mechanism(s)	Ref.
2,3,7,8-tetrachloridibenzo-*p*-dioxin (TCDD)	0.3–60 μg/kg-oral (d22)	Reduced percentage of animals ovulating. Prevented prevulatory fall in estradiol (>3 μg/kg)	Direct ovarian insult affected hypothalamic-pituitary control of ovulation	Li et al., 1995
Guanethidine (sympathetic noradrenergic neurotoxicant)	20 mg/kg–ip (d21–65)	No effect on VO	Sympathetic adrenergic receptors not involved in initiation of pubertal events	Trkulja and Lackovic, 1996
Trihexyphenidyl	2 mg/kg–ip (d21–65)	Delayed VO	Blockade of muscarinic receptor-cholinergic involvement in puberty	Trkulja and Lackovic, 1996
Propantheline	15 mg/kg–ip (d21–65)	Delayed VO	of muscarinic receptor-cholinergic involvement in puberty	Trkulja and Lackovic, 1996
Pyrazine derivatives				
Tetraethyl pyrazine	100 mg/kg–sc (d21–VO)	Delayed VO	Speculated effect on gonadotropin release; structure-activitybe relationships could not be determined	Yamada et al., 1989
2,5-dimethyl pyrazine		No effect on VO		
2,5-diethyl pyrazine		No effect on VO		
Triethyl pyrazine				
Mirex	0.4–50 mg/rat (d28) PMSG primed	Reduced number eggs ovulated Effect reversed by hCG	Effect on neuroendocrine control of LH	Fuller and Draper, 1975

Note: Abbreviations: DW, drinking water; ip, intraperitoneal; icv, intracerebroventricular; sc, subcutaneous.

more specific alteration by mediobasal hypothalamic implants of the dopamine receptor blocker, pimozide, on day 28 advanced both VO (from day 38 to day 35) and first ovulation (Docke et al., 1987). This shift was hypothesized to be due to a reduction in the regional sensitivity to the negative feedback influence of estradiol at this time. Alternatively, a similar implantation of the specific dopamine agonist apomorphine enhanced the LH-suppressing effect of estradiol. Bromoergocryptine, also a dopamine receptor agonist, was systemically administered in the drinking water (20 and 100 μg/ml) from day 22 until the appearance of the first cornified vaginal smear. It delayed the appearance of both vaginal opening and first ovulation (Advis et al., 1981a), effects consistent with the findings for apomorphine and comparable to results reported by Kawagoe and Hiroi (1989). Such increases in dopaminergic activity within the hypothalamus will enhance the tonic inhibitory action of this transmitter on prolactin secretion, inducing hypoprolactinemia in the animals. Because concomitant subcutaneous injections of prolactin prevented the pubertal alterations, it is likely that these effects on dopaminergic neurotransmission were mediated by the impact on pituitary release of this hormone. Additional studies have demonstrated that prolactin administered either directly to the median eminence (day 23) or elevated by sulpiride exposure in the drinking water (0.5 g/l, days 22 until the first diestrus after VO) caused a marked advancement in both VO and first ovulation (Advis and Ojeda, 1978; Advis et al., 1982), emphasizing the involvement of this pituitary hormone in the onset of puberty. In this regard, it is also interesting to note that an increase in prolactin levels by subcutaneous injections of ovine prolactin, or by pituitaries grafted underneath the kidney capsule, caused a marked rise in circulating leptin concentrations (Gualillo et al., 1999). While the effect on leptin appears to be indirect, it suggests a support by other factors in the pubertal effects seen in response to prolactin. Furthermore, it indicates that leptin is regulated by factors other than adiposity.

As mentioned above, there is evidence that the excitatory amino acids glutamate and aspartate participate in the process of puberty via an activation of the NMDA receptors. MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate) is a NMDA receptor antagonist that was able to delay VO (MacDonald and Wilkinson, 1990) and first ovulation (Veneroni et al., 1990; Meijs-Roelofs et al., 1991) when given by peripubertal subcutaneous injections (0.1 to 0.2 mg/kg bw). The effects were consistent with the finding that injections of NMDA (15 or 20 mg/kg) induced an early synchronization of VO over a 24-h period (MacDonald and Wilkinson, 1990; Smyth and Wilkinson, 1994). However, using an intraperitoneal route of exposure, Mena-Valdivia et al. (1995) reported no changes in VO with 18 mg/kg. The above induced alterations in VO appear to be limited to the EAA NMDA receptors, because kainate and the AMPA/kainate antagonist DNQX were without effect (Brann et al., 1993).

3. Leptin, Neuropeptide Y, and Reductions in Body Weight

At present, there is still a fair amount of uncertainty about the physiological roles that both leptin and NPY play in the initiation of pubertal development. A number of research groups have hypothesized that leptin, as a fat-derived hormone, acts as a permissive signal to the brain that sufficient energy stores exist to support the demands of the reproductive process (Ahima et al., 1997; Cheung et al., 1997b). In order to address this issue, Cheung and colleagues (1997b) investigated the effects of exogenously administered leptin (6.3 μg/g bw twice daily) in immature rats, using both ad libitum and food-restricted/pair-fed controls. The data showed that while leptin administration could attenuate delays in VO caused by food restriction in control rats, it could not advance VO beyond that seen for the ad libitum group. This indicates that leptin does not act as a rate-limiting factor, but only allows puberty to proceed if some threshold concentrations are available.

Current data indicate that NPY can act to amplify GnRH release and serve as an inhibitory metabolic signaling factor during times of restricted food intake. When given to immature females as a single intracerebroventricular injection during the later prepubertal period (Minami and Sarkar, 1992), NPY was observed to advance VO and first ovulation. However, in food-restricted rats, NPY was able to extend the delay in VO even when the dietary restrictions were discontinued, unlike controls that were seen to exhibit VO within days of full dietary restoration (Gruaz et al., 1993).

In toxicological studies, a relationship between alterations in body weight gain and pubertal development is not one that can be characterized by a simplified rule of thumb. Previous mention has been made of the fact that food restrictions will cause a well-documented delay in VO (e.g., Glass et al., 1976; Holehan and Merry, 1985; Bronson, 1987), and compounds that induce a general systemic toxicity may do the same. However, a decrease in weight gain caused by toxicant administration may have different effects, depending on the mechanism(s) of action involved. For example, estrogenic compounds administered at weaning will cause reductions in body weights, while advancing the day of VO when compared with controls (e.g., Marty et al., 1999). Also, a blockade of estradiol production with an aromatase inhibitor can have the opposite effect. Using Fadrozole for this purpose, Marty et al. (1999) showed that a delay in VO was accompanied by an increase in body weight. Such data clearly indicate that toxicant-induced restrictions in body weight gain are not in and of themselves predictive of delays in sexual maturation and that due consideration must be given to mechanisms of toxicant action that can have an overriding influence on this relationship.

4. Opioids/Cannabinoids

Endogenous opioid peptides are acknowledged as important components of the brain mechanisms regulating gonadotropin release (for review see Grossman, 1983; Pfeiffer and Herz, 1984). Data have also shown that during puberty there occurs a change in the sensitivity of the hypothalamic opioid-responsive neurons to challenge (Cicero et al., 1986). Both morphine and fentanyl are agonists at the opioid μ-receptors, and the administration of either in the drinking water from day 22 onward causes a delay in VO (MacDonald and Wilkinson, 1991). Moreover, there is some evidence in rats that the opioid receptor antagonist naloxone administered intraperitoneally (0.5 mg/kg) at postnatal day 25 was able to advance VO (Mena-Valdivia et al., 1995). Also, an advancement in first ovulation was seen following multiple daily injections of the antagonist naltrexone (20 mg/kg) during the prepubertal period (Meijs-Roelofs and Kramer, 1989), although later prepubertal injections were more effective that those given a few days earlier. The effects of these receptor antagonists are likely associated with an elevation in LH release (e.g., Blank et al., 1979) via a hypothalamic noradrenergic mechanism (Koh et al., 1983; Adler and Crowley, 1984).

The psychoactive component of marijuana, Δ-9-tetrahydrocannabinol (THC), is known to decrease circulating LH (e.g., Murphy et al., 1990). In the hypothalamus, it binds to a CB1 receptor, where the putative endogenous ligand is a long-chain fatty acid derivative, *N*-arachidonylethanolamine (more commonly known as anandamide). Prepubertal injections of THC (10 mg/kg, intraperitoneal, twice daily) have been observed to delay both first estrus and ovulation (Field and Tyrey, 1984, 1990). Wenger et al. (1988) administered even lower concentrations (1 g/kg/d) from day 22 up until the time of VO and reported a 2-day delay in VO, reductions in ova released at first ovulation, and subsequent irregularities in estrous cyclicity over the ensuing 6 weeks. The suppression of LH by both THC and a stable analog of anandamide (de Miguel et al., 1998) would seem to explain at least partly the induced pubertal alterations. The presence of hypothalamic CB1 receptors suggests that the LH effect is central in origin; however, there is also evidence for an action at the pituitary (Murphy et al., 1991).

5. Other Types of Chemical Insult

Groups of immature female rats given single oral doses of the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, 0.3–60 μg/kg on day 22 showed a decrease in the percentage of animals ovulating in response to priming with equine chorionic gonadotropin (Li et al., 1995). The ED50 for this effect was between 3 and 10 μg/kg. The authors also found that the typical fall in estradiol that accompanies the LH surge was absent, which would suggest that direct effects on the ovary caused alterations in the hypothalamic-pituitary regulation of ovulation.

Ethanol exposure has been shown to delay the onset of puberty in female rats (Bo et al., 1982; Dees and Skelley, 1990). Dietary administration delayed VO, prevented the establishment of estrous cycles, and kept the appearance of the vagina and uterus comparable to 20-day-old controls. In prepubertal females, ethanol has been found to diminish the release of GnRH *in vitro* (Hiney and Dees, 1991), and it may be that this effect in-

volves an alteration in the PGE2 (Hiney and Dees, 1991) or NMDA receptor-associated (Nyberg et al., 1993) mechanisms of GnRH secretion.

Early developmental exposures to lead frequently have been reported to cause marked alterations in reproductive functions (e.g., Kimmel et al., 1980; McGivern et al., 1991; Ronis et al., 1998). However, there is also some evidence that female rats exposed to lead acetate in the drinking water as late as day 24 exhibited some delay in VO (Ronis et al., 1996), although subsequent estrous cyclicity during extended administration appeared to be unaffected.

The involvement of PGE2 in the feedback effects of estradiol on GnRH release in the peripubertal female suggests that a suppression in concentrations of this prostanoid may affect the timing of puberty. Smith et al. (1989) used a semipurified diet deficient in essential fatty acids to decrease circulating levels of the prostaglandin precursor arachidonic acid in prepubertal females. They observed delays in VO and first ovulation, in addition to impairments in norepinephrine-stimulated hypothalamic PGE2 release and in the ability to generate an LH surge in response to E2-containing implants. Some evidence of an effect on puberty was also reported by Zhang et al. (1992), who made use of the finding that the fatty acid eicosapentanoic acid competes with the prostaglandin precursor arachidonic acid as a substrate for cyclo-oxygenase. By exposing 22-day-old females to diets containing fish (menhaden) oil high in eicosapentanoic acid, they reported hypothalamic PGE2 was decreased by about 15% and the appearance of first estrus delayed, although VO was not affected.

C. Induced Alterations in Thyroid Histology and Endocrine Indices of Thyroid Function

While the first descriptions of adverse health effects caused by an underlying alteration in thyroid function probably date back to Paracelsus' reports of goitrous cretinism in the sixteenth century, it was not until the mid-1800s that the role of the thyroid in this condition was discovered (see Welbourn, 1992 for review). By 1915, when Edward Kendall first purified the thyroid hormone thyroxine (T4), a Nobel Prize in Medicine had already been awarded to Swiss physician Emil Kocher for his work on thyroid physiology, pathology, and surgery. Thus, the medical importance of physiologically appropriate levels of thyroid hormone activity has long been understood.

The ability of pharmaceuticals and environmental chemicals to disrupt the function of the thyroid gland by altering the biosynthesis, secretion, or metabolism of thyroid hormones has been well documented in humans and rodent animal models (Capen, 1992, 1997). Such effects can be mediated by alterations in the uptake and iodine trapping mechanism, the organic binding of iodine, and coupling of iodothyronines to form T4 and T3, or the release of the thyroid hormones into the blood as well as their binding to the serum transport proteins (Capen, 1997). Additionally, changes in the peripheral metabolism of the thyroid hormones can be mediated through chemical-induced alterations in hepatic microsomal enzyme systems (Curran and DeGroot, 1991). Many of the compounds that disrupt the synthesis of T3 and T4 can be classified into three groups according to their structure — thioamides (e.g., propylthiouracil [PTU] and mercaptoimidazole), aminoheterocyclic compounds (e.g., tolbutamide), and substituted phenols (e.g., resorcinol and salicylamide) (Crisp et al., 1998). Also, calcium channel blockers, steroids, retinoids, chlorinated hydrocarbons, and polyhalogenated biphenyls have been shown to alter the peripheral metabolism of thyroid hormones (Capen, 1997). A variety of screening methods have been used to evaluate alterations in thyroid function, and these have been reviewed recently by DeVito et al. (1999).

Because many of the studies of toxicant-induced alterations in thyroid hormone function have focused on gestational and neonatal exposure, few examples of studies evaluating only pubertal development are available (see Table 7). The effects of hypothyroidism on the age of puberty onset were evaluated by Wilen et al. (1981) in immature rats fed PTU (0.001, 0.01, or 0.1%) from weaning through the day of vaginal opening. As indicated above, PTU disrupts the synthesis of T4 and T3. In this study, it reduced the growth rate in all treatment groups, but delayed the age of vaginal opening only in the highest group. Similar findings were reported by Marty et al. (1999) using oral administration (240 mg/kg, d21 to 40). A second experiment reported by Wilen et al. (1981)

TABLE 7
Chemicals That Alter T3, T4, or TSH Concentrations

Treatment	Dose-Route (duration)	Effect	Putative/Suggested Mechanism(s)	Ref.
Propylthiouracil (PTU)	0.001–0.1%–diet (weaning — VO)	↓ BW, T3, and T4 with all doses; delayed VO (0.1%)	Altered thyroid hormone synthesis	Wilen et al., 1981
Propylthiouracil	240 mg/kg–oral (d21–40)	↓ BW, ↓ thyroid wt.	Altered thyroid hormone synthesis	Marty et al., 1999
Extracts of subsurface soil with 2.5% PCBs	3–96 mg/kg/d–ip (d20–21)	↓ T4 and ↑ follicular epithelial cell height (36–96 mg/kg/d); ↓ follicular colloid area (12 mg/kg/d)	Altered thyroid hormone synthesis	Hansen et al., 1995
CB 47 (2,2′,4,4′-tetrachlorobiphenyl)	30 mg/kg/d–ip (weaning—22 or 25 d)	↓ T4 and ↑ follicular epithelial cell height, (2, 3, and 5 doses); ↓ follicular colloid area (3 and 5 doses)	Altered thyroid hormone synthesis	Saeed and Hansen, 1997
CB 110 (2,3,3′,4′,6-pentachlorobiphenyl)	3–96 mg/kg–ip (d21–22)	↓ T4 (48-96 mg/kg)	Altered thyroid hormone metabolism	Li et al., 1998
Aroclor 1242	total dose 120 mg/kg–ip divided into 2, 3, or 5 doses (weaning—22 or 25 d)	↓ T4 and ↑ follicular epithelial cell height	Altered thyroid hormone synthesis	Saeed and Hansen, 1997

Note: All data are from rat studies unless indicated. Abbreviations: BW, body weight; ip, intraperitoneal.

compared PTU treatment with pair-fed rats receiving the same amount of food consumed by the PTU-treatment group. The dose of PTU (0.01%) employed was sufficient to reduce growth rate and serum T4 and T3, but not to alter the age of vaginal opening and first estrus. Although the growth rates and body weights were similar between the two groups, vaginal opening was delayed in the pair-fed (undernourished) animals only. These results indicate that PTU may have additional effects on pubertal development that might protect against the delay induced by undernourishment or reduced growth rate.

1. Polychlorinated Biphenyls and Dioxins

It is well known that polychlorinated biphenyls (PCBs) and chlorinated dibenzo-*p*-dioxins (TCDD) disrupt thyroid hormone function in the rat by altering the metabolism (Curran and DeGroot, 1991) or the serum transport of T3 or T4 (Cheek et al., 1999). A reduction in circulating levels of T4 and T3 is very often associated with exposure to these compounds. Saeed and Hansen (1997) examined morphological changes in the thyroid glands of immature female rats after short-term exposure to 2,2′4,4′-tetrachlorobiphenyl (CB 47) and Aroclor 1242. Daily injections were begun at weaning and continued for 2 or 5 consecutive days. While serum T4 increased in control rats from day 22 to 25, treated animals showed a suppression in response to both treatments. Moreover, there were changes in thyroid morphology following the 5-day treatment. Smaller thyroid follicles attributable to a shrinkage of the colloid area and increased cell height were present, which is consistent with the movement of stored thyroid hormones from the colloid region stimulated by TSH in response to low circulating levels of T4 (Capen, 1992). Similar results were reported by Hansen et al. (1995) when weanling female rats were exposed on day 20 and 21 to a refined extract of subsurface soil containing 2.5% PCBs (trichlorobiphenyls and tetrachlorobiphenyls, traces of polychlorinated naphthalenes, 2,2-*bis-p*-chlorophenyl-1,1-dichlorethylene (DDE), and low levels of chlorinated dibenzofurans). When the animals were killed on day 22, serum total T4 and thyroid follicle colloid area were decreased (with a concurrent increase in cell height) in response to 36 to 96 mg/kg of the refined extract. A prepubertal intraperitoneal administration of a purified preparation of 2,3,3′,4′,6′-pentachlorobiphenyl (CB110, 48 to 96 mg/kg) and 2,3,3′,4′,5′-pentachlorobiphenyl (CB126) on days 21 and 22 also decreased serum T4 by the next day and caused an elevation in liver weight (Li et al., 1998).

IV. SUMMARY

From the foregoing discussion, it is clear that exposures to a variety of xenobiotics during the prepubertal period are capable of influencing the occurrence of those interrelated processes that underlie the transition to sexual maturity. Consequently, the proposed protocol, which employs an period of exposure from postnatal day 22 to 42, should be able to identify the vast majority of environmental compounds that have an impact on the endocrine events underlying sexual maturation, if due consideration is given to a number of experimental design issues. Existing data indicate that pubertal development can be readily altered by substances that affect estrogen activity (i.e., synthesis, receptor binding, and clearance). It is also clear that agents that influence central nervous system function will also modify the onset of puberty. Finally, those compounds able to cause alterations in thyroid activity are also identifiable, if the limitations for endocrine assessments are understood (see part V).

While statistically significant effects on sexual maturation can be determined, the biological significance of an alteration in any of the recommended pubertal endpoints to subsequent adult reproductive activity is equivocal. A larger magnitude of an effect at an environmentally relevant level of exposure would, of course, be of increased concern. However, the weight of evidence from a comprehensive assessment of data from the various T1S protocols should provide sufficient information to address an alteration in hormonal signaling. Given that an endocrine-disrupting chemical is defined according to a broadly based alteration in hormonal function, the data argue for inclusion of an assessment incorporating pubertal endpoints that can be combined with other protocols to form a

comprehensive battery that will identify potentially adverse endocrine-mediated effects induced by a diverse assortment of compounds.

V. TECHNICAL CONSIDERATIONS IN THE CONDUCT OF THE MODIFIED EDSTAC TIER I FEMALE PUBERTAL SCREENING PROTOCOL

In order to function as a usable tool for the assessment of endocrine-disrupting chemicals, the protocol should be both robust and sensitive. It should generate results that are comparable within a single laboratory and across a number of different sites. Moreover, the data should be consistent over a broad range of compounds. Toward this end, the following points of discussion are considered important to the success of the protocol (Table 2). They are predominantly design issues that should be addressed in order to reduce those sources of variance that will affect the collection and interpretation of the data. An abbreviated commentary on the individual subsections of the protocol is also presented in Table 8 that touches on some of the strengths, uncertainties, and potential weaknesses of the current version.

A. Dietary Phytoestrogens

Recently, a number of independent laboratories have published data showing that various commercially available rodent dietary formulations contain levels of isoflavone phytoestrogens in concentrations that are sufficient to induce characteristic estrogenic alterations in uterine weight and histology (Boettger-Tong et al., 1998; Thigpen et al., 1999a,b). Moreover, these concentrations can vary among different milled batches from a single supplier, introducing the potential for a further confound in whatever background levels of exposure are present. Data presented by Tansey et al. (1998) indicated that soybean estrogens provided in the diet could actually diminish the uterine effects of a pharmaceutical estrogen preparation. In weanling rats, dietary supplementation with a soy extract was reported to advance VO, but at a level (2.4% of a preparation containing 12% isoflavones and 35% saponins) that was well beyond concentrations normally present in the diet (Gallo et al., 1999). When comparisons were made between the offsrping of dams maintained either on a standard NIH rodent diet or one that was soy- and alfalfa-free without detectable isoflavones, no neonatal or pubertal differences were noted, with the exception of a possible effect on female postnatal day 1 anogenital distance (Casanova et al., 1999). Taken as a whole, these various findings would suggest that some consideration be given to the selection of a diet containing minimal or undetectable levels of phytoestrogenic activity, particularly because endocrine issues are a principal focus.

B. Route of Exposure

Although the recommended route of exposure for this protocol is by oral gavage, it should be noted that the effective dose for each test chemical may vary depending on the dosing route. Differences in absorption, metabolic activation, and/or elimination of the test chemicals occur following exposure by intraperitoneal or subcutaneous injections or by oral gavage (Klaassen and Eaton, 1991). Odum et al. (1997) have compared the effects of 17β-estradiol, 17β-estradiol benzoate, and ethynyl estradiol on uterine growth and the onset of vaginal opening in sexually immature rats following exposure by subcutaneous injection and oral gavage. In each case, the doses required to induce a uterotrophic response and vaginal opening were higher with exposure by oral administration when compared with subcutaneous injection. For example, a dose of 100 μg/kg of 17β-estradiol was required to induce a uterotrophic response vs. 5 μg/kg by subcutaneous injection. Conversely, chemicals such as methoxychlor that undergo metabolic activation in the liver (Bulger et al., 1978b) are generally more toxic following exposure by oral gavage. Gray et al. (1989) have reported that methoxychlor (25 mg/kg, oral) advanced vaginal opening by 6 days when dosing began at 21 days of age and continued through vaginal opening. Odum et al. (1997) have compared oral and subcutaneous exposure to a much higher dose of methoxychlor (500 mg/kg) for 3 days and reported a uterotrophic response in both groups, but vaginal opening was only observed in the those animals treated by oral gavage. Thus, because the pharmacokinetics for each test chemical will influence the outcome of the endpoints evaluated in this protocol, the selection of

TABLE 8
Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats: Overview and Comments

Purpose and applicability	The current version of the proposed pubertal protocol is intended to detect toxic induced alterations in sexual maturation and thyroid function. Compounds that influence thyroid activity or alter pubertal development by affecting steroid- or gonadotropin-mediated functions should be identifiable.
Required endpoints	• Age and weight at vaginal opening—day of VO (or vaginal patency) is a standard index of pubertal maturation. It is a straightforward, noninvasive assessment. Differences in weight may reflect a general toxic effect, or be a consequence of an estrogen-like action (see section on Technical Considerations). • Growth—a retardation in weight gain may itself affect VO. • Serum thyroxine (T4) and thyroid-stimulating hormone (TSH)—Indicative of functional status of the hypothalamic-pituitary-thyroid axis. Time of sample collection may result in some variability in TSH data due to diurnal rhythmicity (see Technical Considerations). • Uterine and ovarian weights and histology—can provide some valuable data for intrepretation of other endpoints. Uterine and ovarian measures require information about cycling status (see Appendix B). Further descriptions about uterine histological requirements are needed in the protocol. Ovarian histological evaluations coupled with cycling data can help characterize the nature of a toxic impact on reproductive function (see Technical Considerations). • Liver, kidney, pituitary, and adrenal weights—ancillary measures that can provide general information about the systemic impact of toxicant exposure. An increase in pituitary weight can also suggest an estrogenic effect. • Vaginal cytology—A non-invasive type of assessment that contributes valuable data about reproductive status in the post-pubertal female.
General Conditions **1. Diet**	There is currently an ongoing debate about the influence of the choice of diet on those endpoints that can be altered by estrogenic activity. The presence of phytoestrogenic compounds in a broad range of commercially prepared dietary formulations may represent a confounding factor in the interpretation of test data (see Technical Considerations).
2. Animal Housing Recommendations	The currently recommended parameter of room lighting (14 h:10 h light:dark photoperiod), temperature (20–24°C), and humidity (40–50%) have commonly been employed for rodent housing in both Toxicology and Reproductive Physiology. The dosing and necropsy times (0700–0900 h and 1300–1500 h, respectively, can potentially detect alterations in baseline hormonal values, but will not allow evaluation of any effects on midafternoon preovulatory endocrine events, such as the proestrous gonadtotropin or prolactin surges (1600–1800 h), if these nonrequired endpoints should become of concern.
Juvenile Female Rats: Group Assignment	• Both in-house breeding and procurement of timed pregnant dams from a commercial supplier are listed as options. For each, it is currently recommended that litters be culled to 8 to 10 pups on postnatal day 3 or 4, but it is not specified how this should be done. Should the number of females per litter be maximized, reducing the sum total of litters needed? Currently, the only information provided is that sufficient litters be produced to ensure that enought females are available for the study. Since interlitter differences can be a concern, some further guidelines could be provided about the relationship between the number of treatment groups and the minimum number of litters. • Animals are assigned to treatments so that there are similar mean body weights and variances. A weight-ranked assignment is indicated, with a range limited to 8 g above or below the group mean. It may well be that this number is based

TABLE 8 (continued)
Protocol for Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats: Overview and Comments

	on one or more standard deviations from a database mean; however, this is not clear.
Experimental Design	• Two replicate blocks are recommended to assess reproducibility of results. Included are control and at least one treatment group with dosage levels at or just below the maximum tolerated dose (MTD). The MTD is broadly defined as the dose that a test animal can tolerate without any adverse physical effects. The original concept written in 1976 by the National Cancer Institute involved consideration of a variety of factors, including weight gain or loss, clinical and biochemical signs of toxicity, organ weights and function, absorption, distribution, and excretion characteristics. Most studies have used reductions in body weight (generally a body weight gain that is 10% less than controls) as a single criterion. However, it is not clear in the present guidelines whether this is the case. The utility of the MTD in toxicological assessments for carcinogenic and non-cancer-related endpoints has been the subject of considerable debate (e.g., Haseman, 1985;[a] Weideman, 1993;[b] Ziegler, 1993[c]) and will not be revisited here. However, the section on technical considerations does reflect on the influence of estrogenic compounds on food consumption and body weight and the relevance of such changes to the present experimental design.
	• Given that options for the choice of rat strain are included, it is important to know the strain in which the MTDs were determined. Otherwise, there is the possibility in selecting dosages that a strain may be exposed at a concentration above its MTD.
Treatment	Oral treatments in corn oil are administered (0700–0900 h) from d22 through 42 in a volume of 2.5 ml/kg body weight. Body weights are recorded daily and dosing volumes are adjusted accordingly
Vaginal opening	The day of complete vaginal opening is recorded, along with the appearance of 'pin hole' openings or vaginal threads. Body weights are also recorded at this time. For statistical purposes, the day of complete opening is the data point employed, although the other two measures can suggest alterations in sexual maturation. For a more extensive discussion of vaginal opening in the rat, the reader is referred to Clark (1999). After complete VO, vaginal lavages are begun for evaluations of estrous cyclicity (see Technical Considerations).
Necropsy	
1. Euthanasia	Alternatives are presented for the method of euthanasia. Decapitation is recommended if pituitary hormone determinations are to be performed. It would preclude any stress-associated effects on hormonal concentrations as a result of exposure to inhaled or injected agents. This is critical for measures of prolactin, because it is promptly responsive to stress. However, it is unlikely that TSH (or T4) would show such rapid alterations during pharmacological euthanasia. Consequently, if those are the only assays to be performed, the scientific justification for decapitation is no longer apparent, and CO2 can be used. Nevertheless, environmental stressors are still of concern and should be minimized (see Technical Considerations).
2. Blood/Tissue Collection	• Serum that is separated from collected blood is stored in siliconized tubes to prevent steroid adherance to the tube walls. This would be more of a problem if estradiol (or progesterone) were to be measured.
	Weights are taken for ovaries, uterus, liver, kidney, pituitary, and adrenals. It is assumed that all are to be trimmed of any adhering fat. Weights for both fluid filled and emptied uteri are recorded to assess potential estrogenic alterations. Also, it is likely that whole pituitary weights are to be recorded instead of separate anterior and posterior measures.

TABLE 8 (continued)

	• Ovaries and uteri (along with the thyroid) are then fixed for subsequent paraffin embedding, sectioning and hemotoxylin-eosin staining. Further information about section thicknesses and criteria for evaluation would be helpful.
Statistical Analysis	It is recommended that multiple analyses of covariance (MANCOVA) be performed on the acquired data, using body weight at weaning as the covariate. Because animals are preselected at weaning for uniformity in body weights, the usefulness of a covariate analysis is debatable.
Data Summary	• Guidelines specify tabular data displaying the means and standard errors of the mean for all the obtained endpoints. Additional presentations of covariance adjustments for body weight can be provided. Separate tables for collected raw data are to be appended.
	• A summary of histological findings is also specified, although as mentioned above no guidance is given for the parameters of concern. Some information should be provided as to whether histological or histopathological assessments (or both) are to be performed; in this regard, there should also be some indication of whether the data presented are to be qualitative or quantitative in nature.

Note: [a]Haseman, JK. (1985). Issues in carcinogenicity testing: dose selection. *Fund. Appl. Toxicol.* **5:** 66–78. [b][Weideman, M]. (1993). Toxicity tests in animals. Historical perspectives and new opportunities. *Environ. Hlth. Perspect.* **101:** 222–225. [c][Ziegler, J]. (1993). Toxicity tests in animals: extrapolating to human risks. *Environ. Hlth. Perspect.* **101:** 396–406.

doses for the protocol should be based on the maximum tolerated dose (MTD, see Table 8) following oral doses to minimize the chance of false-negative results.

C. Changes in Body Weight

One of the endpoints included in the protocol is a determination of the effects of toxicant administration on growth. The exposure of a cohort of rats to an estrogen presents an interesting dilemma. One of the classic effects of estradiol administration is a reduction in food intake and a lower body weight compared with controls (e.g., Reynolds and Bryson, 1974; Wade, 1975; Donohoe et al., 1984), a phenomenon that may in part be a consequence of an enhancement in leptin concentrations (Brann et al., 1999). Alternatively, the virtual elimination of circulating estradiol by ovariectomy will stimulate appetitive behavior and cause an increase in weight. Consequently, a drop in body weight following dosing with a putative estrogen may not necessarily represent a general toxic effect. Because a fall in body weight of 10% or more has frequently been a criterion for a disinclusion of subjects from an ongoing study, this factor is something that should be considered in the implementation of any such protocol.

D. Endocrine Assessment

1. Sample Collection

Along with the nervous system, endocrine factors serve as an internal communications system within the body. In this capacity, hormones may act as inducers or repressors of protein synthesis and thus regulate the rate of enzyme production and influence the activity of various metabolic pathways. They may also affect metabolic activity by regulating mechanisms of inter- and intracellular transport, thereby controlling the availability of a variety of substrates. Consequently, assessments of circulating hormonal concentrations can frequently add important information to an evaluation of toxicant-induced functional alterations. However, in order to maximize reliability and obtain hormonal data that are both valid and useful, a number of factors must be taken under consideration.

a. The Shifting Endocrine Milieu in the Post-Pubertal Female

At the present time, measurements of serum thyroxine (T4) and TSH are the only required hormonal endpoints. The final EDSTAC report (U.S. EPA, 1998c) had further specified that the assess-

ments of estradiol and prolactin were to be optional. This recommendation received subsequent concurrence by a joint internal EPA committee from the Office of Research and Development and the Office of Pesticide Programs and Toxic Substances (see Introduction). While the inclusion of various hormonal determinations can often provide useful information about reproductive functioning, restricting the required endponts to T4 and TSH was a reflection of the perceived relative value of other endocrine measures within the exposure and sampling parameters established for the present protocol.

In toxicological studies, serum or plasma samples are typically obtained at necropsy, because multiple in-life blood sampling can be quite labor-intensive, especially in a study involving large numbers of animals. However, the release of pituitary peptide hormones throughout the day is pulsatile in nature and grouped data consisting of single-point samples always reflect the variability inherent in the peak-to-valley excursions in circulating concentrations. The secretion of growth hormone represents a more extreme example, with recurring peak heights in the adult male often 200-fold or more over values at the nadir (e.g., Millard et al., 1981; Miki and Shizume, 1986). Although maximum elevations in the female are lower, the peak-to-valley differences are still large (Terry et al., 1977). Even in the pubertal female, these peaks can reach levels well above baseline (Gabriel et al., 1992). Along with such daily patterns of change are marked hormonal shifts that occur at specific times during the estrous cycle. The proestrous afternoon surges of FSH and LH are well-characterized examples of such alterations that are functional endocrine events. FSH provides the hormonal impetus for development of a ovarian cohort of arrested primordial follicles. The surviving members of this cohort will then be subsequently subjected to stimulation by an LH surge to trigger the final stages of follicular and oocytic maturation that culminate in ovulation.

When using single-point endocrine assessments, it is important to understand the nature of these cyclic fluctuations in circulating concentrations and how the time/day of sampling may affect the levels seen. The current version of the protocol does not specifiy a time of day at which blood collections are to be obtained, although earlier iterations indicated a window between 1300 to 1500 h. As a required endpoint, TSH concentrations can provide useful information about the activity of the hypothalamic–pituitary–thyroid axis. However, the hormone does show a diurnal rhythm in the rat, with circulating levels peaking around noon and then decreasing to a nadir around midnight (Leppaluoto et al., 1974; Fukuda et al., 1975; Jordan et al., 1980). This peak in TSH is then followed 3 to 4 h later by a slight rise in T3 and T4 concentrations (Ottenweller and Hedge, 1982). Therefore, single-point control thyroid hormone samples taken under standardized conditions should be fairly consistent within a group. However, TSH from blood collected between 1300 and 1500 h may be more variable.

As mentioned above, a designation of other hormonal endpoints as "optional" in the female pubertal protocol was a reflection of the interpretive value of such measures under the recommended parameters and the understanding that a variety of measures are influenced by factors apart from a direct effect of toxicant exposure. Estradiol concentrations, for example, fluctuate over the estrous cycle. Consequently, for such data to be meaningful, it is critical to document whether the animals under treatment are in fact cycling and, if so, to identify the particular stages of the cycle at which the samples are obtained. Prolactin measurement, another optional endpoint, presents an additional problem. Marked elevations in circulating prolactin concentrations do occur during the estrous cycle late on the afternoons of proestrus and estrus (Esber et al., 1976; Goldman et al., 1985; Haisenleder et al., 1989). It is possible then that blood samples taken at the time of necropsy could reflect a surge-associated elevation, which has been observed in a proestrous/estrous female to range from 1300 to 1900 h. Moreover, prolactin is a hormone that is very responsive to stress. For this reason, assessments of circulating prolactin concentrations necessitate that the conditions present around the time that the animals are killed must be carefully controlled (see below).

b. Uterine Weights

During the estrous cycle, rising levels of circulating estradiol will cause a marked elevation in uterine weight on the day of proestrus. The effect reflects increases in both the fluid content and cel-

lular proliferative activity. A variety of estrogenic compounds have been found to have similar effects (e.g., Bulger et al., 1985; Grunert et al., 1986; Smith and Quinn, 1992; Ashby et al., 1997). Consequently, in obtaining weights for both fluid-filled and empty uteri after the recommended 20 days of dosing, one must (as with hormonal endpoints) be cognizant of whether the animals are cycling and, if so, the particular day on which the rats are killed. Unlike uterotrophic evaluations in toxicant-exposed ovariectomized animals, cyclic changes in uterine weight could cause marked increases in the variability within groups that preclude an accurate assessment of any treatment-related uterine alterations.

c. Characterization of the Estrous Cycle

The daily characterization of vaginal cytology in the rat is a noninvasive and reliable method for evaluating estrous cyclicity. In the post-pubertal female, temporal shifts in the appearance of cells present in a vaginal lavage is a direct reflection of the changes in circulating concentrations of estradiol and progesterone and may indicate toxicant-induced alterations in reproductive functioning. Under commonly employed lighting conditions, the first 4 to 5-day cycle will occur about 7 to 10 days after vaginal opening. In untreated animals, it repeats in a regular fashion and reflects the maturation and rupture of successive waves of ovarian follicles. Cycle length is influenced by the duration of progesterone secretion from ovarian follicles and corpora lutea (e.g., Sanchez-Criado et al., 1996). It is generally separated into three distinct phases, commonly termed metestrus/diestrus (typically 2 to 3 days), proestrus (1 day) and estrus (1 to 2 days). Ovulation from mature follicles occurs during the early morning hours on estrus in response to stimulation by the late afternoon proestrous LH surge.

The identification of changes in vaginal cytology over the cycle is not difficult, although, as with many things, accuracy increases with experience. Because the epithelial cells respond to cyclic alterations in estradiol levels that exhibit a rise over diestrus, it is beneficial to perform daily lavages at a comparable time during the day. Lavage samples can be read in fresh smears or fixed and stained, although for archival purposes the latter method would be indicated. For a review of vaginal cytological assessments in toxicology studies, the reader is referred to Cooper and Goldman (1999).

d. Stress

The responsiveness of a variety of hormone to novel and/or stressful situations represents a major concern for studies in which endocrine endpoints are of interest. It is well established that the adrenal corticosteroids and prolactin undergo dramatic and rapid increases under stressful conditions. Currently, to address any stress associated with pharmacological anesthesia prior to euthanizing the animals, it is recommended that if pituitary hormonal assessments are to be performed animals be killed quickly by decapitation in a room separate from the housing area. Otherwise, the females may be euthanized under CO2. While there is evidence that stress can alter TSH and the thyroid hormones (e.g., Riegle and Meites, 1976; Langer et al., 1983; Armario et al., 1986; Gala, 1990; Wyatt et al., 1995), they are not likely to respond within such a brief time frame. Nevertheless, consideration must be given to those existing environmental conditions prior to the time at which blood samples are obtained. For example, cages should not be scheduled for changing on the morning preceding necropsy, or should test animals be transferred to a holding area during this time. Exposure to such stressors before sample collection can induce an unwanted amount of variability in the data, and the chances of making a type II error (falsely accepting a no-effect) in the statistical evaluation of the data are markedly enhanced.

2. Conduct of the Immunoassays

Immunoassays should be performed under the standards of Good Laboratory Practice. Unknown samples should always be run in duplicate, and it is advisable to prepare standards in triplicate. Multiple pairs of quality control (QC) samples from a common pool are run interspersed within each assay to establish inter- and intra-assay coefficients of variation, which for the assays concerned typically run 10% or less. Complete ready-to-use kits are available for the steroid hormones from a number of commercial vendors. These generally have

yielded compatible results and offer a number of purchasing options. Materials for measurement of rat pituitary peptide hormones can be commercially obtained, although the large database for these hormones is almost exclusively generated from materials supplied by the National Hormone and Pituitary Program under the auspices of the National Institute for Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDKD). The latter materials are available to government, university, and other not-for-profit organizations. Other laboratories may have to acquire assay kits from commercial sources for in-house work. However, it should be understood that the use of different reference preparations for assay standards can generate different values for the same QC samples. Moreover, such assays may have very discrepant sensitivities for detecting low levels of circulating hormones. At the very least, such commercial kits should be revalidated against the NIADDKD materials.

E. Histological Assessments

Typically, toxicological studies include a histopathological examination of the different tissues and organs of concern. In addition, this protocol extends the evaluations to include histological assessments that may reflect treatment-related functional changes in uterine, ovarian, and thyroid tissues imposed by varying degrees of hormonal perturbation.

1. Uterus

Uterine histology has been used extensively to detect estrogenic changes in sexually immature or adult ovariectomized rats following treatment with exogenous estrogens, phytoestrogens, and estrogen agonists (e.g., O'Conner et al., 1996; Reel et al., 1996). An induction of uterine growth is associated with hypertrophy of the uterine luminal and glandular epithelium within 12 to 24 h after exposure to estrogen. During this time, the epithelium can increase to six to eight cell layers, when compared with two to three layers in the unstimulated uterus (Padykula et al., 1981; O'Conner et al., 1996). Methods for quantifying epithelial cell height (Branham et al., 1993, 1996; Sourla et al., 1998) and uterine endometrial stromal cell proliferation (Martin and Claringbold, 1958; O'Conner et al., 1996) have been well documented.

Although uterine histology is useful for characterizing estrogenic activity in the absence of endogenous estrogen, the utility of this endpoint in cycling females is questionable without considering the hormonal profile of the animal at the time of necropsy. Recently, Spornitz et al. (1999) have provided a detailed summary of histological changes in the uterine epithelium during the estrous cycle and correlated those changes with serum steroid and gonadotropin concentrations. Using the proposed female pubertal protocol, animals are killed at a given age rather than on a particular day of the estrous cycle. Therefore, uterine histological data should be considered in context of the cycling status of the animal (i.e., whether estrous cycles are present, and if so the particular day of the cycle on which the female is evaluated) and the levels of circulating sex steroids.

2. Ovaries

Ovarian histological assessments should also take into account the functional (i.e., cycling) status at the time of necropsy, because this may markedly affect the appearance of the ovaries. Histological evaluations are useful in determining the extent to which ovarian development has been affected by treatment and at the same time contribute information on any observed alterations in cycling status. For example, animals dosed with a potent estrogen, such as estradiol or diethylstilbestrol (DES), would show early vaginal opening and persistently cornified smears. However, the ovaries of such animals would be atrophic (e.g., Pinilla et al, 1993; Biegel et al., 1998). Alternatively, animals displaying periods of extended diestrus may be either pseudopregnant or anestrus, conditions that can be differentiated by histological examination. Pseudopregnant ovaries will contain prominent corpora lutea (e.g., Miyagawa et al., 1975; Smith et al., 1975), while in an persistently anestrous animal the ovaries appear atrophic with few primary follicles (Huang and Meites, 1975; Cooper et al., 1993).

3. Thyroid

Morphological changes in the thyroid gland have been described that are correlated with thy-

roid hormone synthesis and secretion. Reduced circulating levels of T4 and T3 following a chemical or physiological insult generally induce a compensatory release of TSH from the pituitary (Capen, 1997; 1998; Kasza et al., 1978). The secretion of TSH leads to proliferative changes in the thyroid follicular cells such as hypertrophy and hyperplasia. Prolonged TSH excretion is correlated with an increased incidence of thyroid tumors in rodents (Capen, 1992). However, morphological changes in the thyroid follicular cells can be observed after short-term exposure, where modest to substantial reductions in serum T4 have occurred. Saeed and Hansen (1997) report increased thyroid follicular cell height (9 to 10–12 μm) and decreased colloid area (1100 to 800–900 μm^2) in weanling female rats with modest serum T4 concentrations following two doses of 2,2′,4,4′-tetrachlorobiphenyl. Thus, an evaluation of these parameters may be helpful in detecting an early or transient response to a thyroid toxicant, especially in cases where a compensatory release of TSH and stored T4 may have temporarily masked a reduction in thyroid hormone synthesis at the time of necropsy (Saeed and Hansen, 1997; Hansen et al., 1995; Capen, 1998). For a more comprehensive discussion of toxicant-induced alterations in thyroid endpoints, the reader is referred to DeVito et al. (1999).

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