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—Full Paper—

Short-Term Hormone Release from Adult Female Rat Hypothalamic and Pituitary Explants is not Altered by 2,3,7,8-Tetrachlorodibenzo-p-dioxin

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Abstract. 2, 3, 7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has adverse effects on reproduction, in part due to direct actions at the ovary. It is unclear whether effects are further mediated by glands that regulate ovarian function. We investigated whether effects of TCDD are mediated via the hypothalamic-pituitary axis. Hypothalamic and pituitary tissues were cultured in medium with and without TCDD. TCDD did not alter GnRH release from hypothalamic samples. It continued to be pulsatile with no differences in the average peak frequency, average peak amplitude, or baseline GnRH release. TCDD did not alter GnRH-induced release of gonadotropins from pituitary samples. There were no differences in average peak amplitude or baseline release. AhR, ARNT or ERα mRNA copy numbers in cultured pituitaries were not affected by TCDD. Our data suggest that TCDD effects on ovarian function are not mediated through the hypothalamic or pituitary release parameters tested in this study.

Key words: Hypothalamus, Pituitary, 2, 3, 7,8-Tetrachlorodibenzo-p-dioxin (TCDD), GnRH, Gonadotropins

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Recently, the public has become increasingly concerned about pollutants within our environment. Individuals are exposed to a wide range of chemical pollutants on a regular basis. Further, approximately 10–15% of couples are considered infertile [1]. Based on correlational data, it appears that exposure to various environmental contaminants may at least in part contribute to this alarming infertility rate by altering the reproductive endocrinology of the individual [1, 2]. Environmental pollutants might constitute one possible cause of spontaneous abortions [2] and birth defects.

The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent modulator of reproduction and is an endocrine disruptor. Our laboratory and others have shown that some of these effects are due to direct actions of TCDD at the ovary [3, 4]. However, since the

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hypothalamus regulates anterior pituitary function and the anterior pituitary in turn regulates ovarian function, TCDD could alter ovarian function indirectly by modulating hypothalamic and/or anterior pituitary function. The aim of this study was to investigate whether acute exposure to TCDD could compromise hypothalamic or anterior pituitary function *in vitro*, thus altering reproductive function in female Sprague-Dawley rats.

Presently, there are only limited data to suggest that TCDD exerts direct actions at the hypothalamus. It is even controversial whether or not the proteins (aromatic hydrocarbon receptor [AhR] and AhR nuclear translocator [ARNT]) that mediate the effects of TCDD are present in the hypothalamus. Kainu *et al*. [5] were unable to identify either in rat hypothalamus or brain stem and yet others [6–8] have reported their presence in the hypothalamus. There is some evidence that suggests that the hypothalamus, at least in part, mediates the weight-reducing effects of TCDD [9, 10]. There is also evidence that suggests that the hypothalamus, at least in part, mediates the changes in reproductive function caused by TCDD. Chaffin *et al*. [11] showed that hypothalamic estrogen receptor (ER) mRNA was increased while hypothalamic ER DNA-binding activity was decreased in females rats exposed to TCDD in utero and through lactation. Although the concentration of hypothalamic AhR mRNA was not altered by in-utero and lactational exposure to TCDD, the AhR DNA binding ability was reduced in the hypothalamus [6]. In contrast, Petroff *et al*. in an immortalized hypothalamic cell line, have observed no effect of TCDD on GnRH accumulation *in vitro* or GnRH gene promoter activity [12]. These data imply that TCDD exerts direct actions at the hypothalamus. We therefore hypothesize that TCDD could alter the frequency and/or amplitude of GnRH secretion from the hypothalamus in the female Sprague-Dawley rat, thereby potentially altering LH/FSH secretion at the anterior pituitary and subsequent reproductive function.

There are data to support a direct action by TCDD at the anterior pituitary. Li *et al*. [13] reported that TCDD treatment of immature rats resulted in an immediate and dramatic rise in serum LH and FSH concentrations. The gonadotropin concentrations were elevated in a dose-dependent fashion 24 h following treatment [14]. Using pituitary cultures, Li *et al*. [14] also demonstrated that TCDD induced a dose-dependent release of LH.

In male rats treated with TCDD, basal LH was not increased despite reductions in plasma androgen concentrations [15]. LH concentrations increased dramatically in TCDD-treated rats that were castrated, implying that this phenomenon was sex-steroid dependent [15]. This response was similar to that in control rats that were castrated. It was concluded that TCDD was able to increase the potency of sex-steroids as feedback inhibitors of LH [15,16] by altering pituitary GnRH receptor number and responsiveness to GnRH [16]. Similarly, Gao *et al*. [17] reported that exposure to TCDD blocks ovulation in female rat.

Thus, there is ample evidence to support the hypothesis that TCDD exerts direct effects at the anterior pituitary. We hypothesized that TCDD could disrupt reproductive function by altering the response of the anterior pituitary to a repeated challenge of GnRH. In particular, we were interested in whether TCDD could alter constitutive or induced release of the gonadotropins, FSH and LH. In addition, we hypothesized that TCDD would modify AhR, ARNT and estrogen receptor (ER)α mRNA populations thus altering AhR- and ER-mediated signal transduction. TCDD typically reduces AhR mRNA or protein concentrations [18–20] such that the tissue may become less responsive to subsequent assaults by TCDD. Others have not seen reductions in AhR mRNA following TCDD exposure [21–23] indicating that the modulation of AhR is very complicated and may be species-, tissue-, time- and dose-dependent.

Therefore the aims of the present study were to evaluate the effects on pulsatile GnRH release by rat hypothalamic slices and gonadotropin release by rat pituitary slices in vitro by TCDD delivered in a flow-through, perifusion system that emulates the animal's physiology *in vivo*.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at UW-Milwaukee approved all experimental protocols. Healthy adult female rats

ranging in weight from 233–319 g (Sprague-Dawley albino; Sasco, Madison, WI, USA) were used in this study. Upon arrival, animals were housed three to a cage in a room with constant temperature (22 ± 2 C), humidity (55 ± 5%) and light cycle (12L:12D; lights on 0700 h). The animals were provided with commercial food and water *ad libitum*. Estrous cycles were monitored daily by analyzing vaginal smears [24]. We used only animals showing at least two consecutive, normal four-day cycles immediately prior to the experimental cycle. Rats were anesthetized with CO_2 and killed by decapitation between 0730 and 0830 h on metestrus and proestrus, and the hypothalamus or the pituitary removed. Nine rats were euthanized on each day for each experiment. Trunk blood was also collected and allowed to clot. Serum was separated by centrifugation and stored at –20 C until analysis with RIA.

Tissue preparation

For the first experiment, the hypothalamus was removed and divided in half longitudinally at the midline [25]. The hypothalamus was identified as the tissue bordered laterally by the hypothalamic fissures, anteriorly by a cut approximately 2 mm anterior to the anterior aspect of the optic chiasm and posteriorly by the rostral portion of the mamillary bodies [25]. Cuts were made to an approximate depth of 2.5 mm. Each hemihypothalamus was placed in medium (Krebs-Ringer bicarbonate buffer containing 2.2 mM $CaCl_2$, 154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl, 6.0 mM $NaHCO_3$, 10 mM glucose, 2 mM HEPES, 0.1% BSA and 0.006% bacitracin, pH=7.4).

For the second experiment, the pituitary was removed and divided in half longitudinally at the midline. Tissue was placed in medium [M199 buffer containing 26.2 mM $NaHCO_3$, 684 μM L-glutamine, and 1 × antibiotic/antimycotic pH=7.2 [26].

Perifusion procedures

For both experiments, an Endotronics Accusyst S cell perifusion system (Cellex Biosciences, Minneapolis, MN, USA) was used during the procedure. Tissues were placed into chambers. Medium was warmed to 37 C and was pumped through the chambers at a flow rate of 100 μl per minute. Medium was supplemented with vehicle (0.1% dioxane; Sigma, St. Louis, MO, USA) or vehicle with TCDD (3.1 nM; Cambridge Isotope Laboratories, Andover, MA, USA). A dose of 3.1 nM TCDD was chosen because this is an environmentally relevant dose and this dose elicited effects on estrogen production by breast cancer cells [27] and human and rat ovarian cell cultures [3, 28]. One half of each hypothalamus or pituitary was perifused with TCDD-media while the other half was perifused with vehicle-medium. Carbon dioxide was allowed to flow into the chamber area to maintain the pH of the medium in the first experiment. Oxygen (95%)/CO_2 (5%) flowed into the chamber area to maintain the pH of the medium in the second experiment. Medium collected during the first 20 min was discarded to allow the tissue to acclimate to the conditions of the culture. Thereafter, one-ml fractions were collected at 10-min intervals using a refrigerated Gilson FC205 fraction collector (Middleton, WI, USA). The experiment was maintained for six hours. In the second experiment, pituitary tissues were challenged with 10^{-6} M GnRH for ten minutes once per hour to stimulate gonadotropin release. All cultures were challenged with 56 mM KCl thirty minutes before the end of the procedure to evaluate bolus release of GnRH or FSH/LH in the hypothalamic and pituitary cultures, respectively. Medium samples were frozen at –20 C until further analysis with RIA. At the end of the culture period, tissues were frozen at –80 C until further analysis using competitive reverse transcriptase-PCR.

Radioimmunoassay

To verify that rats were euthanized on the appropriate day of the estrous cycle, serum estradiol-17β (E_2) and progesterone (P_4) concentrations were determined using RIA kits (Diagnostic Products, Los Angeles, CA, USA). Assays were validated for rat serum by demonstration of parallelism between rat serum samples in serial dilution versus authentic standards and by demonstration of recovery in samples that were "spiked" with known concentrations of steroids. The limits of detection were 20 pg/ml and 0.1 ng/ml for E_2 and P_4, respectively. The intra-assay coefficients of variation were derived from six replicate aliquots and were less than 13.6 and 8% for E_2 and P_4, respectively. Each unknown sample was assayed in duplicate.

GnRH concentrations in hypothalmic perfusates

Table 1. Primers used for amplification of the target mRNA in RT-PCR reaction

Name of the gene product	Forward primer (5′-3′)	Reverse primer (5′-3′)	Competitive primer (5′-3′)
AhR	CCGACGCGTCGAGAT CTCCAGCCCTTTCTCTCC	CCGCTCGAGGTC GATGTCTTTGAA GTCAAGGCTACC	CCGACGCGTCGAGATCTCCAGCC CTTTCTCTCCGCTCCCCAGTCAAC CCCGAGTAAGGATTCTTTC
ARNT	GAACAAGATGACAG CTTACATCAC	GTGGACTACCACAAA GTGAGGGTC	GAACAAGATGACAGCTTACATCA CCGATCTTACGCATGGCTTTTCTC
ERα	AATTCTGACAATCGA CGCCAG	GTGCTTCAACATTCTC CCTCCTC	AATTCTGACAATCGACGCCAGAG GGAAACATGATCATGGAGTC
Cyp1A1	GTTCCCAAAGGTCTG AAGAG	CATATGGCACAGATG ACATTGG	

were determined with RIA using GnRH (Richelieu Biotechnologies, Montreal, PQ, Canada) labeled with ^{125}I. Standards were also purchased from Richelieu Biotechnologies, Anti-GnRH (R1245; Dr. Terry M. Nett, Colorado State University, Fort Collins, CO, USA) was the primary antibody and goat anti-rabbit gamma globulin (Biogenesis, Brentwood, NH, USA) was the secondary antibody. Assays were validated for rat by demonstration of parallelism between rat perifusion media in serial dilution versus authentic standards. The limit of detection was 0.5 pg/ml. The average intra-assay coefficient of variation, derived from six replicate aliquots in each assay, was 7.2%. The inter-assay coefficient of variation was 6.0%. Each unknown sample was assayed in duplicate.

FSH and LH concentrations in pituitary perfusates were determined with RIA, using NIDDK-rFSH-I-8 and NIDDK-rLH-I-9, labeled with I-125. NIDDK-rFSH-RP-2 and NIDDK-rLH-RP-3 were used to generate the standard curve. NIDDK-anti-rFSH-S-11 and NIDDK anti-rLH-11 were the primary antibodies and goat anti-rabbit gamma globulin (Biogenesis, Brentwood, NH, USA) was the secondary antibody. Assays were validated for rat by demonstration of parallelism between rat serum samples in serial dilution versus authentic standards. The limits of detection were 0.5 and 0.33 ng/ml for FSH and LH, respectively. The intra-assay coefficients of variation were derived from three replicate aliquots in one assay, and were always under 10% for both FSH and LH. Each unknown sample was assayed in duplicate.

GnRH pulse detection

The pulsatile release of GnRH from hypothalamic cultures was determined using the PULSAR computer algorithm [29]. The cut-off criteria for G1, G2, G3, G4 and G5 were 3.8, 2.6, 1.9, 1.5 and 1.2, respectively [25]. The intra-assay coefficient of variation for GnRH was described by the formula $y=3.38 \times + 3.14/100$ [25]. GnRH release was determined by evaluating average peak height, average peak interval, average peak frequency and baseline GnRH. Peak heights were determined by subtracting the nadir preceding the GnRH peak from the peak value. Peak interval was determined as the time that elapsed between one peak and the beginning of the next peak. Peak frequency was determined to be the number of spontaneous GnRH pulses per hour. Baseline GnRH was determined by averaging the nadirs as determined when calculating peak heights.

Gonadotropin response

Gonadotropin release from the pituitary cultures was stimulated by GnRH (10^{-6} M) challenges once per hour. The gonadotropin concentrations in the three samples obtained just prior to each challenge were averaged and designated as "pre-challenge baseline". The highest concentration found within the three samples following each challenge was designated as the "peak". The difference between the concentration at the peak and the pre-challenge baseline was the "peak amplitude". The peak amplitude was designated to be zero if that difference was negative. Pre-challenge baseline values were averaged to find the average baseline gonadotropin concentrations.

Cloning of rat RNA fragments

Total RNA was isolated from rat liver using Trizol reagent (Gibco BRL, Bethesda, MD, USA). Primers used to amplify the RNA fragment were

generated by Integrated DNA Technology (Coralville, IA, USA). (Table 1: Rat AhR primer [28, 30]; rat ARNT primer [28]; rat ERα primer [31]; rat cytochrome P4501A1 (cyp1A1) gene expression primer [28]). The fragment was amplified using an Access RT-PCR system (Promega Biotech, Madison, WI, USA). The PCR product was separated from contaminating fragments by gel electrophoresis and was electroeluted from the gel. DNA was precipitated with 3 M sodium acetate and 100% ethanol and resuspended in DNase-free water. DNA fragments were ligated into pGEM T-EZ vector (Promega) or the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). Ligation products were used to transform JM109 High Efficiency Competent cells (Promega). Cells were plated on LB/ampicillin (Sigma) plates coated with 50 μl of 2% X-Gal in dimethyl formalin (DMF; Sigma) and 100 μl of 40 mM IPTG (Gibco BRL), and incubated overnight at 37 C. White colonies were transferred to new plates and grown overnight at 37 C. Mini-cultures were inoculated in LB/ampicillin media and grown overnight while shaking at 37 C. Plasmid DNA was isolated using a DNA-PureTM Plasmid Mini-Prep kit (CPG Lincoln Park, NJ, USA). The plasmid DNA was sequenced (Automated DNA Sequencing Lab., University of Wisconsin-Milwaukee) and the sequence was compared with published sequences in Genbank (http://www2.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast). Glycerol stocks were made by mixing a volume of the LB/ampicillin miniculture with an equal volume of 30% glycerol.

Clones containing fragments used to make competitor RNA were made in a similar manner except that the templates for the fragments were plasmid DNA and the forward primers were extended such that 10% of the fragment of interest was omitted (Table 1).

Competitive reverse transcriptase-PCR

Total RNA was isolated from rat hypothalamus and pituitary using Trizol reagent. Total RNA was stored at –80 C until further analysis.

Competitor RNA was made using plasmids containing the cloned competitor fragment. The competitor fragment (sense RNA) was transcribed in vitro using SP6 or T7 RNA polymerase (Promega). After transcription, the DNA template was digested with RQ1 Rnase-free DNase (Promega). Transcripts were extracted with phenol:chloroform (1:1).

The quantity and purity of all RNA was evaluated by absorption at 260 nm and 280 nm on a spectrophotometer. The integrity of the RNA was evaluated by gel electrophoresis. Competitor RNA was aliquoted and frozen at –80 C until needed.

Competitor RNA and tissue RNA were combined in tubes and amplified using the Access RT-PCR system and the original primers. To verify that the samples were not contaminated with DNA, we attempted to amplify fragments without adding reverse transcriptase. PCR products were resolved on 2% agarose gels by electrophoresis. The net intensity of the two ethidium bromide-stained DNA bands was determined (EDAS; Kodak Electrophoresis Documentation and Analysis System). Copy numbers were estimated using the regression equation $y=\beta_0 + \beta_1(\ln x) + \beta_2(\ln x)^2$ where y is the ratio of the intensity of the product and competitor bands and x is the competitor copy number [28]. From the regression curve, we determined the theoretical equivalence point (*i.e.* the value of x when y is equal to one [28]. The correlation coefficients were ≥0.887.

Statistical analyses

GnRH data were spline transformed while FSH and LH data were log-transformed prior to statistical analysis. RNA and RIA data were analyzed using one-way ANOVA and Tukey's multiple-comparison test. Independent T-Tests were also performed on specific pairs. For statistical purposes, values below the limit of detection for the assay were set at the limit of detection. $P<0.05$ was considered to be statistically significant.

Results

Serum E_2 was significantly increased by 33% on proestrus over that on metestrus. Further, in the hypothalamus experiment, serum P_4 was significantly reduced by 50% on proestrus versus on metestrus validating day of cycle. We purified RNA from our cultured hypothalamic tissues. Using semi-quantitative RT-PCR, we were able to amplify CYP1A1 mRNA in only one of three assayed vehicle-treated hemihypothalami, while we amplified CYP1A1 mRNA in three of four assayed TCDD-treated hemihypothalami (data not

Table 2. Pulsatility of GnRH release by hypothalamic explants

	Proestrus-Control	3.1 nM TCDD	Metestrus-Control	3.1 nM TCDD
Average peak interval (min.)	67.7 ± 14.4[1]	68.2 ± 6.7	76.3 ± 8.7	95.5 ± 30.0
Average peak frequency (no. peaks/6 h)	3.7 ± 0.6	4.8 ± 0.3	3.7 ± 0.4	3.6 ± 0.6
Average peak amplitude (ng/ml)	1.1 ± 0.2	1.5 ± 0.4	2.0 ± 0.7	1.0 ± 0.1
Baseline (ng/ml)	2.4 ± 0.9	2.2 ± 0.7	1.8 ± 0.5	2.2 ± 0.6

There were no differences in the average peak interval between pulses, average peak frequency, average peak amplitude, or baseline GnRH release. [1]Data are expressed as mean ± SEM for nine replicates.

Table 3. Average baseline and average peak amplitude concentrations of FSH (ng/ml) in culture medium collected from GnRH-challenged pituitaries *in vitro*

	Vehicle	3.1 nM TCDD	Average
Average baseline			
Metestrus	3.7 ± 1.0[1]	3.4 ± 0.6	3.6 ± 0.6
Proestrus	4.1 ± 1.0	4.5 ± 1.1	4.3 ± 0.9
Average peak amplitude			
Metestrus	1.9 ± 0.5	2.9 ± 1.3	2.4 ± 0.7
Proestrus	2.1 ± 0.3	2.1 ± 0.8	2.1 ± 0.5*

[1]All values are expressed as mean ± SEM of nine replicates. *Denotes significant difference between samples taken on metestrus and proestrus.

Table 4. Average baseline and average peak amplitude concentrations of LH (ng/ml) in culture medium collected from GnRH-challenged pituitaries *in vitro*

	Vehicle	3.1 nM TCDD	Average
Average baseline			
Metestrus	8.3 ± 2.1[1]	5.8 ± 2.1	7.1 ± 2.1
Proestrus	28.1 ± 4.0	29.3 ± 5.3	28.7 ± 4.5*
Average peak amplitude			
Metestrus	17.2 ± 6.1	10.8 ± 4.9	14.0 ± 5.5
Proestrus	31.0 ± 4.7	29.6 ± 6.1	30.3 ± 4.6*

[1]All values are expressed as mean ± SEM of nine replicate samples. *Denotes significant difference between samples taken on metestrus and proestrus.

shown). This shows that TCDD exerts an effect in our system within 6 h.

GnRH release was pulsatile. It was typically released every 60–90 min. Neither TCDD nor the day of the estrous cycle exerted an effect on the pulsatility of GnRH release as there were no differences in the average peak frequency, average peak amplitude or baseline GnRH release (Table 2). A majority of the hypothalamic tissues responded to the KCl challenge with bolus release of GnRH that was similar in height to the spontaneous peaks. In a few samples, the KCl did not elicit a response. If the tissue had been exhibiting a pattern of periodic GnRH release, then the data were still included in the analysis.

TCDD did not alter gonadotropin average peak amplitude or baseline gonadotropin release from the pituitary. Data were therefore pooled to allow us to verify that the animals used were in metestrus or proestrus. The average FSH peak amplitude was significantly higher on metestrus than proestrus (Table 3). The average LH peak amplitude and baseline LH release were significantly higher on proestrus than metestrus (200 and 400%, respectively) (Table 4). KCl elicited bolus release of one or both gonadotropins in approximately half of the pituitary cultures. The response was typically similar to the GnRH-stimulated gonadotropin

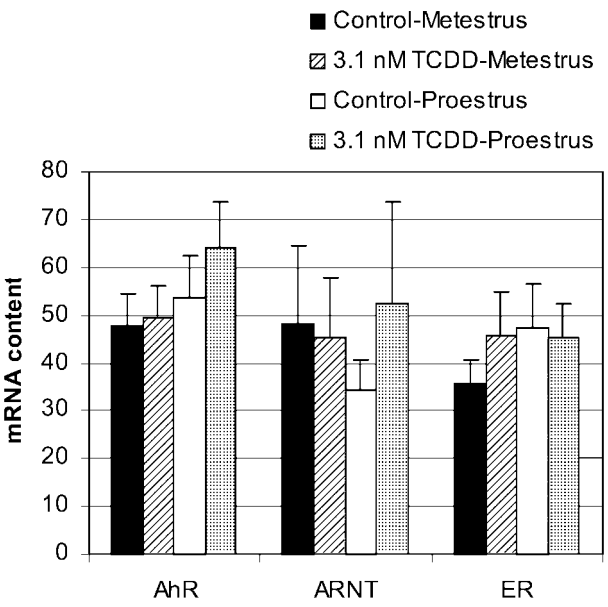

Fig. 1. Graphic representation of pituitary AhR, ARNT and ERα mRNA copy number. AhR and ARNT are $\times 10^3$ copies/30 ng RNA. ER is $\times 10^5$ copies/30 ng RNA. All values represent mean ± SEM of nine replicates.

release. The samples that did not respond to KCl typically did not show a return to baseline gonadotropin concentrations following the previous peak; therefore, all of the data were included in the analysis.

AhR, ARNT, and ERα fragments were successfully cloned and competitive RNA fragments were synthesized. For AhR, we amplified a fragment that was 499 and 444 bases for the target and competitor RNA, respectively. For ARNT, we amplified a fragment that was 615 and 556 bases for target and competitor, respectively. For ERα, we amplified a fragment that was 344 and 309 bases for target and competitor, respectively. Neither day of cycle nor TCDD exposure affected AhR, ARNT or ERα mRNA content (Fig. 1).

Discussion

TCDD did not alter any of the parameters of hypothalamic or pituitary function that we measured, lending support to our hypothesis that much of TCDD's effects is at the ovary directly. However, this does not completely refute the hypothesis that TCDD exerts detrimental effects on hypothalamic or pituitary function because not all possible endpoints were evaluated. We found that TCDD did not affect the following aspects of episodic release of GnRH; there were no differences in the average peak interval between pulses, average peak frequency, average peak amplitude or baseline GnRH release. Our GnRH secretion patterns were similar to those described by Woller *et al*. [25], since GnRH release was episodic with pulses occurring every 60–90 min. We found that TCDD exerted no effect on GnRH-induced release of FSH or LH from the pituitary, as there were no differences in peak amplitude of gonadotropin release or baseline release. This lack of effect is further supported by a recent report by Petroff *et al*. [12], in which the authors did not see an effect of TCDD on the release of GnRH from a hypothalamic neuronal cell line or on GnRH gene promoter activity in these cells. Finally, TCDD showed no effect on pituitary AhR, ARNT or ERα mRNA copy number.

The presence of both AhR and ARNT protein within a tissue is necessary for TCDD to exert a direct effect since those are the proteins that mediate its effects [32, 33]. The AhR and ARNT are quite ubiquitous in that their mRNA and protein have been identified in most tissues analyzed [19, 30, 32, 34, 35], therefore it seems evident that TCDD has the potential to exert its effects at many loci. Although Kainu *et al*. [5] were not able to localize either AhR or ARNT mRNA to the hypothalamus or the brain stem of the rat using *in-situ* hybridization, AhR mRNA has been identified in the hypothalamus by slot-blot hybridization [6]. AhR and ARNT have even been localized to specific nuclei within the hypothalamus and brainstem using a sensitive *in-situ* hybridization histochemical technique [7] Further, Huang *et al*. [8] quantified AhR and ARNT mRNA in the rat hypothalamus. Both are expressed in the anterior and posterior pituitary [8]. Although we did not measure any changes in AhR or ARNT mRNA, our data do confirm the presence of these molecules. Since both AhR and ARNT are expressed in the hypothalamus and pituitary, they both remain potential targets for TCDD's actions

One well-characterized effect of TCDD exposure is the induction of CYP1A1 [36]. TCDD induces CYP1A1 mRNA expression in the hypothalamus, as well as in the posterior and anterior pituitary [8] of rat. We were able to amplify CYP1A1 mRNA in

one of three assayed vehicle-treated hemihypothalami, while we amplified CYP1A1 mRNA in three of four assayed TCDD-treated hemihypothalami. This indicates that TCDD is capable of exerting a physiologic effect within our hypothalamic samples. We have previously shown that this dose of TCDD increases levels of CYP1A1 mRNA by 3–6 h [31]. For this reason, we believe that the lack of effect that we observed in the GnRH parameters was real. In another study, TCDD did not alter AhR mRNA and increased ARNT mRNA after 28 days following dosing [8]. Together, these results suggest that one reason we did not observe effects of TCDD in our system was because too little time elapsed to allow for TCDD to exert its effects. This experiment needs to be repeated by either using a range of doses on animals that were administered TCDD *in vivo* for different periods of time prior to the experiment or in tissue that had been exposed *in vitro* to TCDD for various time periods prior to medium collection. It is also possible that the dose (3.1 nM) of TCDD was too low to elicit a response in hypothalamic or pituitary tissues even though it elicited changes in cultures of other cell lines [3, 28] and it is an environmentally relevant dose [27].

Pitt *et al*. [37] did not uncover any changes in the maximal release or the time of maximal release of ACTH from perifused anterior pituitary explants dosed with TCDD following a corticotropin-releasing hormone challenge. Pitt *et al*. [37] suggested that there is a great amount of genetic variability within the rat as evidenced by the large range in reported LD50 (22–300 μg/kg), which may explain why these authors did not find significant changes.

Our data also revealed a wide range of variability among individuals with regard to AhR and ARNT mRNA and protein concentrations in various tissues. These data are supported by results obtained from other laboratories as well [35]. This might indicate that some individuals are more sensitive than others to the effects of dioxin. To determine whether this is the case, one would have to determine AhR protein concentrations prior to the experiment. Individuals could then be grouped to allow for comparisons among individuals with high-versus-low AhR and ARNT protein levels (high-versus-low responders).

We found no changes in ERα mRNA content. We chose to measure ERα mRNA because this is the predominant receptor subtype found in the pituitary [38]. Although ERβ is present in the pituitary, it is present only in low concentrations [38]. It is certainly possible that TCDD alters the mRNA content of the β subtype of ER, but we did not measure ERβ mRNA concentrations. TCDD could alter feedback of estrogen at the pituitary by altering either ERα or ERβ. Gao *et al*. [17] observed that the ovulatory rate, which is typically reduced by TCDD exposure, returned to normal in rats that were also treated with high doses of estrogen. The addition of estrogen triggered the release of the FSH and LH surge [17]. Apparently TCDD changes the estrogen threshold such that the concentration of serum estrogen required to elicit the gonadotropin surge is ten times greater than normal [17]. Our data would suggest that the reduced ability of the pituitary to respond to estrogen following TCDD exposure observed by Gao *et al*. [17] is mediated via a mechanism other than the ERα. On the other hand, we were also unable to detect any changes in ERα as a consequence of day of the estrous cycle. This appears to contradict results reported by Childs *et al*. [39]. They observed that while only 5% of gonadotropes expressed ERα or ERβ on metestrus, by the morning of proestrus 10% of the gonadotropes expressed ERα or ERβ [39]. In our study, we did not differentiate among pituitary cell types. It is possible that changes in ER content within specific populations of pituitary cells could alter function. Further, the Child's study [39] was not a quantitative study in that although the authors determined which cells expressed ERα or ERβ, they did not measure receptor number. These differences in assay sensitivity and experimental design make it difficult to compare these studies directly. Although we found no changes due to exposure to TCDD, we did observe expected differences in the response of the pituitary to GnRH depending upon the day of the estrous cycle. This was in agreement with Smith *et al*. [40] who also found that GnRH-stimulated release of LH *in vitro* was greater on proestrus than metestrus. On the other hand, Fox & Smith [41] found that *in-vivo* LH peak amplitudes were similar between metestrus and proestrus. Although our tissues were collected in the morning on proestrus, prior to the LH surge, the cultures were typically maintained until after 1600 h. Since we anticipate the LH surge to occur between 1300 and 1700 h in our colony [24], we

expect that the LH surge accounts for the increase in LH peak amplitude that we observed. The slight but significant reduction in GnRH-stimulated FSH peak amplitude on proestrus versus metestrus may be due to the fact that the FSH surge is not always exactly concomitant temporally with the LH surge [42]. The changes in LH are also more robust than those in FSH.

In conclusion, although we did not show alterations due to TCDD in the several parameters that we analyzed, the hypothalamus and pituitary still remain potential targets for TCDD. Our data neither supported nor negated the hypothesis that TCDD exerts detrimental effects on hypothalamic or pituitary function since they do not preclude other actions of TCDD at the hypothalamus or pituitary. In cultures of adult rat hypothalami and pituitaries, we were unable using TCDD to elicit changes in gonadotropin-releasing hormone and gonadotropin secretion, respectively. This suggests that the effects of TCDD on estrogen secretion are due to direct actions at the ovary. However, since young animals are far more sensitive to dioxin exposure than are adults [43] and all humans carry a body burden of TCDD, long-term rather than acute exposure to TCDD may need to be assessed in order to evaluate potential chronic reproductive hazards posed by this toxicant.

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