Effects of Perfluorinated Compounds on GnRH Gene Expression in vitro

by

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Perfluorinated chemicals have been widely used in industry for their superior surfactant qualities. Because of their widespread usage and resistance to photolytic and biodegradation mechanisms, as well as their ability to undergo long-range atmospheric transport, these compounds have become ubiquitous in the world-wide water supplies. Of particular interest are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), which have been demonstrated to be biological endpoint products that are capable of acting as estrogen mimetics. As estrogen itself plays a pivotal role in reproductive function in mammals, this study aimed to determine if and to what extent perfluoro chemicals disrupt normal patterns of gene expression of the neurohormone gonadotropin-releasing hormone (GnRH). By use of stably transfected secreteable reporter systems in immortalized GT1-7 neurons, a widely accepted in vitro model of GnRH neurons, effects of perfluoro chemicals on GnRH transcriptional activity were determined at concentrations of 10 and 20 µM PFOS or PFOA, as well as dose-response curves following in vitro exposure to 50 µM, 500 nM, 5 nM, 500 pM and 5 pM concentrations of PFOS, and 100 mM, 1 mM, 100 µM, 1 µM and 100 nM concentrations of PFOA. Apparent up-regulation of GnRH gene expression was observed in 1mM, 10µM, and 1µM concentration treatments of PFOA, and 5nM, 500pM and 5pM concentration treatments of PFOS. Apparent down-regulation of GnRH gene expression was noted in 50µM and 10µM PFOS, as well as 100mM PFOA. TUNEL assays were also performed to determine whether changes observed in gene expression rates could be attributed to induction of apoptosis. Increased percentages of cells in the apoptotic phase were observed in 100mM, 1mM, 1µM and 100nM PFOA treatment groups, as well as 50µM PFOS, vs. DMSO control. Apparent reduction in percentage of apoptotic cells was noted in 100µM PFOA, and 500nM, 5nM, 500pM and 5pM PFOS treatments.

Perfluorinated compounds (PFCs) have been widely used in industry for their properties as superior surfactants[1-3]. Because of their unique ability to reduce surface tension, perfluorinated compounds possess a wide range of commercial and industrial applications[2,4,5]. These chemicals have been used in many consumer products including non-stick cookware, textile treatments, flame retardants, cosmetics, and grease-resistant food packaging[2,3,6,7].

The basic chemical structure of PFCs makes the incredibly stable in the environment. While the length of the alkyl chain is variable and several substituent groups are possible, all PFCs are fully or partially saturated with fluorine[4,8]. Because of the high electronegativity of fluorine atoms, electron density is drawn away from the carbon backbone of the alkyl, forcing adjacent carbon atoms to share electron density and further reinforcing these carbon-carbon bonds[4]. Fully-saturated fluorocarbons, such as those used in the manufacture of Teflon™ products, are immiscible in both water and non-polar organic solvents, and consequently form a third phase when mixed[2,4,5]. Perfluorooctanoic acid (PFOA, Fig. 1A) and perfluorooctane sulfonate (PFOS, Fig. 1B), however, contain shorter alkyl chains and hydrophilic substitutions, making them highly mobile in the aqueous phase[2,4,6].
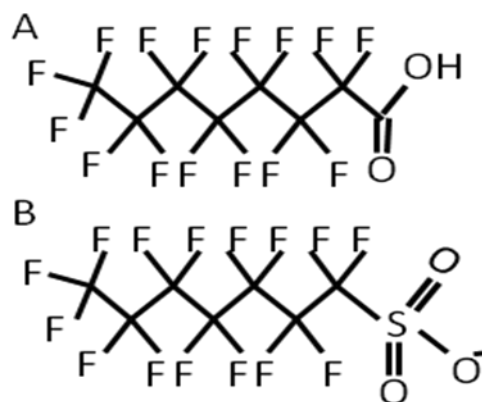

Figure 1. Chemical structures of perfluorooctanoic acid (A) and perfluorooctane sulfonate (B).

PFOA and PFOS have been classed as persistent organic pollutants, abundant in the atmosphere and capable of long-range atmospheric transport[9-12]. Consequently, these chemicals have now been detected in wildlife and surface waters as remote as the North Pacific and Arctic Oceans[2,12-15]. Extensive environmental contamination is likely due to the fact that PFCs are highly resistant to typical routes of degradation, including direct and indirect photolysis, biodegradation, and microbial degradation[2,5,9,15-18]. Though some chemical breakdown of perfluorotelomer alcohols through transformation reactions involving hydroxyl radical have been observed, the most frequent breakdown products are PFOA and PFOS[9,11,18]. Recent studies have also observed higher concentrations of PFCs, which bind to serum albumin and various cellular receptors, in the serum and liver of fish and predatory animals[13-15,19,20]. These experimental findings suggest biomagnification, bioaccumulation, and greater exposure in aquatic animals[13-15,21].

Despite the 3M Corporation voluntary phase-out of perfluorochemical use in 2002, PFOA and PFOS are still at detectable concentrations in public drinking water supplies[2,5,17,22-24]. Humans are exposed to PFCs on a daily basis through a number of routes, including drinking water, off-gassing of chemical at normal cooking temperatures from non-stick cookware, and stain-resistant coatings on carpets and textiles. Children are typically reported as having 5 to 10 times higher rates of exposure per body weight due to *in utero* exposure, breast milk as a primary source of nutrient sustenance, close association with carpets and textiles, hand-to-mouth transfer and dust ingestion[3,7]. Epidemiological studies have reported average blood serum concentrations of PFOA and PFOS ranging from less than 2 ng/mL serum to over 1600 ng/mL serum in high-exposure areas, and approximately 33.1 ng/mL PFOS and 4.5 ng/mL PFOA in the general population[6,23,25]. Given the average serum concentration of chemicals, the experimental determination that PFCs are able to cross the blood-brain barrier, and the

propensity of these chemicals to be secreted in breast milk, there are ample reasons for assessing the possibilities for developmental toxicity in humans[3,7,24,26-28]. The previously reported chemical body load, in conjunction with experimentally determined half-lives of PFCs ranging from 4 to 9 years in humans, lends credence to concerns for possible health implications following chronic exposure[1,7,29]. Several studies have been conducted *in vivo*, *ex vivo*, and *in vitro* to determine possible biological endpoints of exposure to PFCs including immunotoxic effects, developmental and teratological effects, neurotoxicity, and endocrine disruption.

Immunotoxic effects, including cell cycle arrest and apoptosis in the spleen and thymus, have been observed in mouse models, and significant reductions in total IgM and IgY were reported following *in ovo* exposure of white leghorn chickens to PFOS[22,30-32]. Further studies in bottle-nose dolphins, which carry loads of PFOS 20 to 40 times higher than that of humans, showed increased numbers of lymphocytes, increased B-cell proliferation, and suppression of lysozyme activity[33]. Alterations have also been observed in both innate and adaptive immune responses through PFCs' influence on neutrophil activities, such as reactive oxygen species secretion and chemotaxis[22,31,32,34].

Developmental toxicity has been the most frequently researched biological endpoint of PFC exposure. Several studies report decreased body weight in neonates and highly increased mortality[1,7,24,28,30,35-37]. High exposure to PFOS and PFOA in utero yielded pups with breathing difficulties and increased rates of mortality, though losses observed in PFOA treatment groups were fewer than those observed following treatment with PFOS[38]. Of those pups that survived, several developmental deficits were observed including reduced weight gain, altered nutritional status, brain asymmetry, delays in sexual maturity, dental abnormalities, and adrenal and hepatocellular hypertrophy[1,17,24,28,38,39]. Additionally, *in ovo*

studies conducted in white leghorn chickens reported significant changes in liver ALT and LDH levels[1,30]. Epidemiological studies in humans, though subject to confounding by maternal physiological variation and much lower plasma concentrations of PFCs, have reported no significant association between *in utero* exposure and human developmental delays[40-42].

Neurotoxicological studies conducted in both rats and mice, based on post-mortem homogenization of neural tissue and subsequent analysis by HPLC, have demonstrated that PFOS and PFOA do not alter chemical concentrations of norepinephrine, dopamine, serotonin, glycine, 4-aminobutylic acid, or glutamic acid in the brain[27,54-56]. Though these findings could have implications for neurotransmitter signal conduction, the data is not supportive of actual secretion or blood concentration within the brain tissue, and so does not reflect the actual secretory status of these cells. Additional studies in PC12 cells, however, report decreased DNA synthesis following exposure to PFCs, with subsequent differentiation changes suggestive of disruptions in neuronal differentiation, including decreased dopamine phenotype and increased acetylcholine phenotype, following treatment with PFOS [57]. While no data is yet available as to whether these compounds actually have effects on differentiation at the level of the brain, altered neuronal signaling may also be as a potential source of reproductive toxicity.

Hormonal changes in biological systems have also been demonstrated as a consequence of chronic exposure to PFOA and PFOS. Several studies have reported decreased free thyroid hormone, possibly due to competitive binding to thyroid hormone transport protein transthyretin, and thyroid hypertrophy in rats, mice, and monkeys following PFOS exposure[39,43,44]. Recent studies in tilapia hepatocytes, male rare minnows and zebrafish have reported that PFOA and PFOS can induce

the production of vitellogenin, a compound synthesized in response to estrogen exposure, as well as initiate oocyte formation within the testes, indicating that PFCs act as xenoestrogens under certain conditions[45-47]. Decreased testosterone synthesis and elevated estradiol levels in male rat *in vivo* studies, as well as in vitro studies, have also been reported following treatment with PFCs, though these findings contradict data collected in yeast culture exposed to perfluorochemicals that report no estrogenic effects on human estrogen receptors α and β [49,52-57].

Following the discovery of estrogenic properties associated with PFCs, several scientists have investigated the potential for reduction in fertility post-exposure. In rats given daily intraperitoneal injections of 0, 1, or 10 milligrams of PFOS per kilogram of body weight for two weeks, most subjected to treatment did not undergo normal estrous cycling. Thirty-three percent of the high dose group were in persistent diestrus, meaning that these animals did not ovulate and were infertile, and only 42% underwent normal estrous cycling. In the low dose group that received only 1 mg/kg body weight daily, only 66% had a normal estrous cycle[26].

Though estrus cycle changes have been observed, it is still unclear how these effects are exerted. One system of molecular mechanisms potentially vulnerable to disruption by xenoestrogens is gene expression and secretion of gonadotropin-releasing hormone (GnRH), which is released in pulses approximately every 1-2 hours during the metestrus phase of the female estrous cycle in rodents[58]. This pulsatile secretion is vital for the function of GnRH, which is to stimulate the pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). FSH and LH, in turn, stimulate the gonads to synthesize androgens, convert them to estrogens, and secrete these hormones into the bloodstream. Estrogen then acts as an inhibitor for its own production by

turning off the LH and FSH signals at the level of the hypothalamus[59-63].

On the afternoon of proestrus, the phase of the female cycle where the female rodent will ovulate, the regular pulsatile secretions of GnRH are interrupted by a large surge. This surge is believed to be generated by estrogen's contribution to the daily neuronal signals released by the SCN, and the subsequent LH surge serves as the push to cause release of the egg from the ovary. Interestingly, this surge is very time-dependent and will only occur in the afternoon of proestrus, approximately 24-hours after a threshold dose of estrogen has been released from the ovaries[43,64-66].

In light of recent data reporting estrogen's involvement in GnRH surges observed on the afternoon of proestrus, reports of neuronal differentiation interference, and given the lack of evidence for neurotransmitter interference following exposure to these chemicals, mechanisms by which PFCs disrupt neuronal signaling are still unclear[27,67]. It has previously been demonstrated that GnRH pulsatility is subject to GABAnergic, adrenergic, and glutamatergic signaling, which ties back to possible neurotoxic effects on neuronal differentiation. Disruption in stimulatory signals conducted to GnRH neurons could be to blame for the *in vivo* disruptions in estrus cycling previously cited, but it is also possible that the interference in reproductive cycling is originating from the level of hormone signaling within the hypothalamo-pituitary-gonadal axis.

To further investigate neuroendocrine signal interference generated by this class of chemicals, subclonal lines of GT1-7 immortalized neurons taken from the mediobasal hypothalamus of transgenic mice were created. These subclonal lines contains a secretable luciferase reporter derived from the marine copepod *Metridia longa*. The transcription is dependent upon promoter activation of estrogen-responsive gene *GnRH* (GnRH-MetLuc) [67]. By treating these cells with PFCs, transcriptional effects of exposure can be quantified and observed via culture media analysis without mandatory lysis of the original cell population. Through the generation of the GnRH-MetLuc cell lines, various aspects of the molecular mechanisms governing the reproductive axis could be investigated within the cells of the brain responsible for the neuroendocrine control of reproduction.

Furthermore, TUNEL assays were conducted using regular GT1-7 neurons to detect DNA fragmentation following treatment with several doses of PFCs. By evaluating the propensity for cells to enter the apoptotic phase prematurely, we can say for sure whether any observed down-regulation of GnRH expression is simply due to cell death, or if the chemical exposure is causing the decrease in transcription.

Materials and Methods

Cell Culture

GT1-7 neurons were obtained from Dr. Pamela Mellon's lab. Cells were cultured in Dulbecco's Modified Eagles' Medium (DMEM, Cellgro) containing 4.5% glucose, 10% fetal bovine serum (Gemini BioProducts), and 1% penicillin and streptomycin (Gibco). All cultures were maintained in conditions of 5% CO2 at 37°C.

Stable Transfection

A commercially produced vector of metridia luciferase (MetLuc, Contech) was purchased and inserted into cell DNA following restriction digest.

GnRH-MetLuc subclones were created using pBSK (Invitrogen) as an intermediate vector. The *Metridia* luciferase vector was first inserted into the intermediate vector using *HincII* (New England BioLabs). *Xho* and *Xba* (New England BioLabs) restriction sites were used to excise the firefly luciferase (FLuc) gene from GnRH-FLuc vector, and also for subsequent insertion of MetLuc to create the final vector product, GnRH-MetLuc.

Luciferase Assay

Stably transfected GnRH-MetLuc were seeded to 24-well plates in parallel. At approximately 90% confluency, cells were treated with 50% FBS/50% DMEM serum shock for two hours. Serum shock was then removed and media replaced with either DMSO vehicle, EtOH vehicle, 100 pM estradiol, 10 μM PFOA, 20 μM PFOA, 10 μM PFOS, or 20 μM PFOS in serum-free DMEM. Reference readings were taken at 6 or 12 hours following treatment application, then again at 24 and 48 hours. A time course was also run with estradiol and media analysis performed at 6-hour intervals to show changes in gene expression over time.

Per Clontech luciferase assay protocol, 10X luciferin substrate stock solution was mixed using substrate buffer. Substrate/reaction buffer was diluted to a 1X concentration, mixed by pipette, and allowed to sit for 15 minutes before use. Fifty-microliter samples were taken from each well and applied to a 96-well plate, then 5 μL of 1X substrate/reaction buffer was added to each before reading with the luminometer.

Dose-Response

Cells were cultured in parallel and treated at 90% confluency with 50 μM, 500 nM, 5 nM, 500 pM, and 5 pM doses of PFOS, 100 mM, 1 mM, 100 μM, 1 μM, and 100 nM concentrations of PFOA, or DMSO vehicle in triplicate. Dose-response was determined via luciferase assay, as previously described, at 0 hours, 24 hours, and 48 hours.

BCA Protein Assay

Following final 48-hour media collections, cell cultures were washed with 1X phosphate-buffered saline and lysed with RIPA buffer with 50x protease inhibitor cocktail (ThermoScientific). Albumin standards were prepared per kit instructions to generate the standard curve, then 25 uL aliquots of standards and samples plated to 96-well plate in duplicate. Working reagent was prepared per protocol using RIPA buffer as background, and 200μL added to each well. The plate was allowed to incubate at 37°C for 30 minutes. Sample plates were then cooled to room temperature before reading at an absorbance of 562nm in the Opsys spectrophotometer, per manufacturer protocol.

TUNEL Assay

HT TiterTACS assay kit for quantitative detection of apoptosis in cells was obtained from Trevigen. Cells were grown at a density of $9x10^4$ cells/well in a 96-well plate and treated with 50 μM, 500 nM, 5 nM, 500 pM, and 5 pM doses of PFOS, 100 mM, 1 mM, 100 μM, 1 μM, and 100 nM concentrations of PFOA, or DMSO vehicle. Positive controls were generated using TACS-nuclease per kit instructions, and manufacturer protocol followed to fix, wash, and tag cells. Colorimetric analysis was conducted at 630nm following 30 minute incubation in the dark, then again at 450nm after stop reaction treatment for an additional 30 minutes with 0.2 N HCl per manufacturer protocol.

Luciferase/BCA Assay Data Analysis

Technical triplicates of raw luciferase readings were normalized to total protein, as determined by BCA assay. Normalized data was then averaged and standard deviation calculated to give error. Fold-increase of GnRH gene expression in cells treated with PFCs was calculated by treating controls (EtOH, DMSO, or E2) as zeroes.

TUNEL Assay Data Analysis

Absorbance from technical replicates of each treatment was averaged, then percent-change of PFC treated cells vs. DMSO control calculated by treating control values as zeroes.

Results

Effects of PFC exposure on GnRH gene expression vs. estrogen and ethanol vehicle controls

Figure 2 shows the effects on GnRH gene expression of treatment with two concentrations of PFOA and PFOS compared to two controls. Although GnRH gene expression appeared to be similar to the control following 10 µM and 20 µM PFOS treatment, both 10 µM and 20 µM PFOA caused evident up-regulation of gene expression at the 48-hour time point.

Further analysis of normalized data was performed to determine up- or down-regulation of GnRH gene expression vs. ethanol and estradiol controls (Fig. 3). By calculating fold-increase of each PFC treatment over E2 and EtOH, the true extent of expression changes was illustrated. Fold-increase calculations revealed up-regulation in cultures treated with 10 µM PFOA. A 2.3-fold increase in gene expression was noted at 24 hours compared to

EtOH, and 1.5-fold increase noted at 48 hours. When compared to E2 controls, 10 µM PFOA maintained a consistent fold-increase of approximately 1.7 over the entire time course (Fig. 3A, 3B). Slight to moderate up- and down-regulation in the 20 µM P FOA and 20 µM PFOS was also observed, but appeared to be resolved at the end of the time course. Slight up-regulation did occur in 20 µM PFOA-treated cells after 48 hours, but only a 1.3-fold increase was noted as compared to EtOH. When compared to E2 controls, a 1.5-fold increase occurred at 24 hours which dropped to a 1.1-fold increase at 48 hours. Up-regulation of GnRH gene expression in 20 µM PFOS-treated cells (1.25-fold vs. EtOH, 1.8-fold vs. E2) was noted at 24 hours, but no evident difference was observed at 48 hours. Comparative change in gene expression vs. controls is illustrated in Figure 4. This clearly illustrates the fold-increase data by treating EtOH and E2 individually as zeroes.
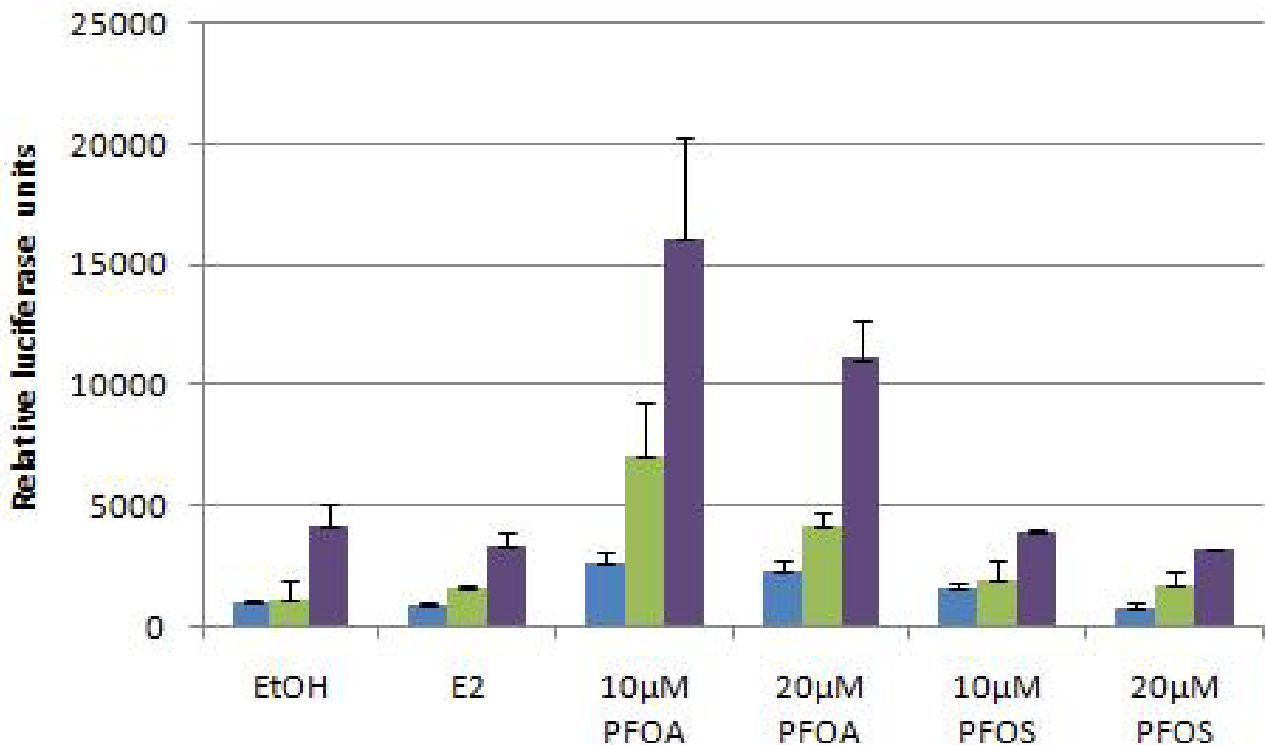

Figure 2. *GnRH* gene expression at 12-, 24-, and 48-hours following treatment of cell cultures with 100 pM estradiol, 10µM PFOA, 20 µM PFOA, 10 µM PFOS, 20 µM PFOS, or EtOH vehicle. *Blue bars,* 12 hour reading; *green bars,* 24-hour reading; *purple bars,* 48-hour reading. Values reported are averaged triplicates of luciferase signal normalized to total protein, indicating *GnRH* gene expression relative to cell mass present.
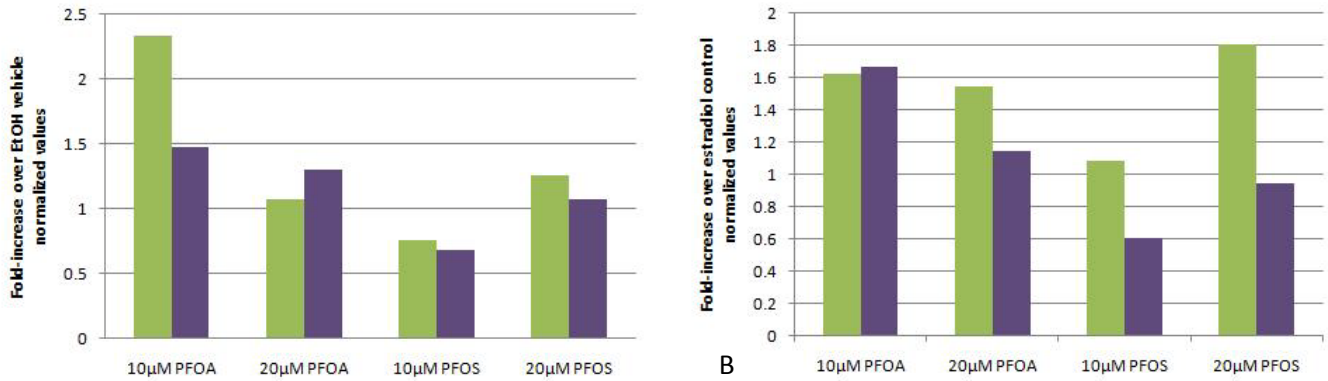
Figure 3. Fold-increase of normalized PFOA and PFOS treatments over controls. A) Fold-increase of 10 µM PFOA, 20 µM PFOA, 10 µM PFOS, and 20 µM PFOS treatments over EtOH vehicle. *Green*, fold-increase at 24 hours over 12-hour reference; *purple*, fold-increase at 48 hours over 12-hour reference. B) Fold-increase of 10 µM PFOA, 20 µM PFOA, 10 µM PFOS, and 20 µM PFOS treatments over estradiol control. *Green*, fold-increase at 24 hours over 12-hour reference; *purple*, fold-increasee at 48 hours over 12-hour reference.
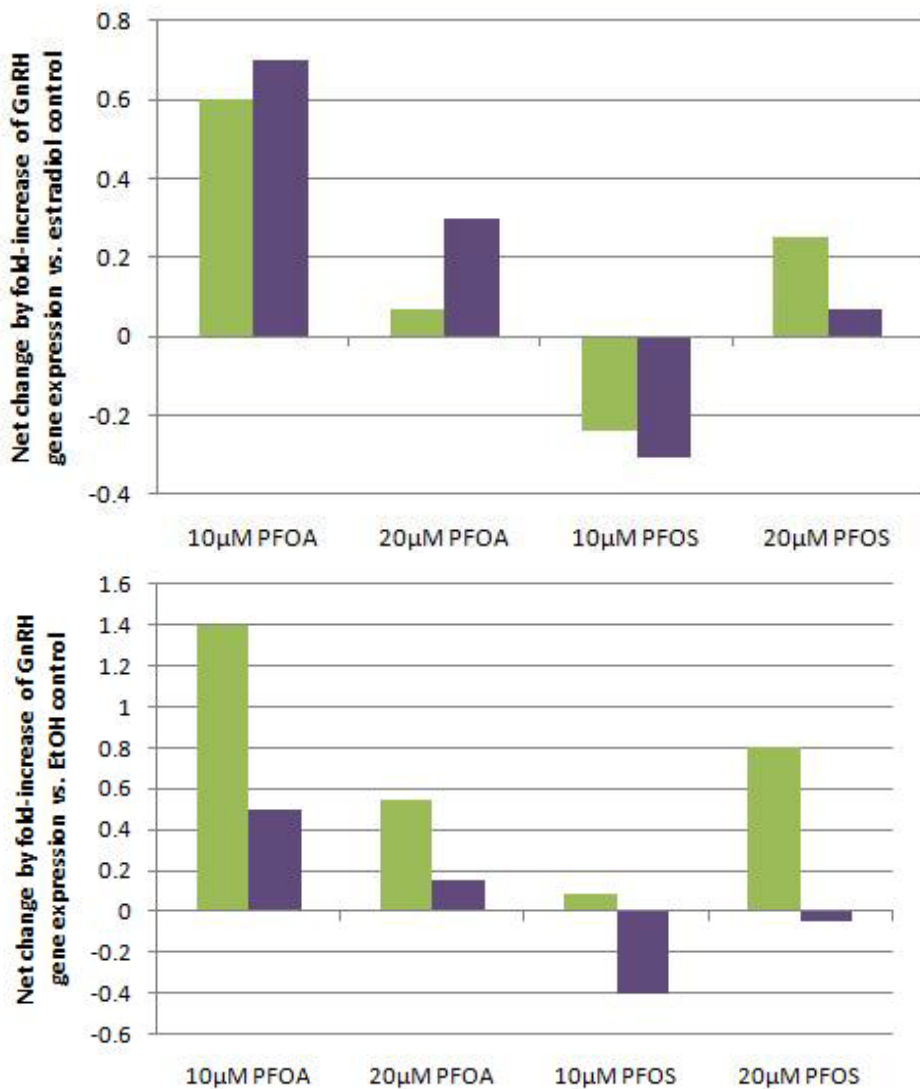

Figure 4. Net change of GnRH gene expression in cultures treated with 10 µM PFOA, 20 µM PFOA, 10 µM PFOS, and 20 µM PFOS as compared to EtOH (A) and estradiol (B) controls. Calculated from fold-change, treating controls as zeroes and reporting fold-increase or – decrease. A) Net change in GnRH gene expression vs. EtOH control. *Green*, difference in net change from 12 to 24 hours PFC treatment vs. EtOH; *purple*, difference in net change from 12 to 48 hours PFC treatment vs. EtOH. B) Net change in GnRH gene expression vs. estradiol control. *Green*, difference in net change from 12 to 24 hours PFC treatment vs. estradiol; *purple*, difference in net change from 12 to 48 hours PFC treatment vs. estradiol.

Dose-Response

In order to further examine the effects of PFOA and PFOS on GnRH gene expression, we performed a dose-response assay using several molar concentrations of these chemicals. As illustrated in Figures 5 and 6, both compounds had profound effects. Apparent up-regulation of *GnRH* was observed in the 1mM PFOA, 1µM PFOA, and 5µM PFOS treatments, and smaller up-regulations with 5nM PFOS and 500pM PFOS. No apparent difference between expression resulting from treatment with DMSO vehicle vs. 100 µM PFOA, 100nM PFOA, or 500nM PFOS was noted, although the highest dose of each perfluorochemical (100mM PFOA and 50µM PFOS) caused notable down-regulation (Fig. 6).

Individual dose-response curves were generated for each chemical to more clearly illustrate the experimentally determined effects on gene expression. A bimodal curve, with the most marked up-regulation occurring at 48 hours after culture incubation with 1mM and 1µM PFOA, was generated in lieu of the standard sigmoidal curve (Fig. 7). Although peaks and nadirs over 24 hours are much less dramatic than those over 48 hours, the general dose-response is similar for both intervals.

Figure 5. Dose-response of *GnRH* expression following treatment of cell cultures with 100mM, 1mM, 100 µM, 1µM and 100nM concentrations of PFOA, or DMSO control. Values reported are averages of triplicate luciferase signals normalized to total protein, indicating cumulative *GnRH* gene expression over a 48-hour time course. *Blue*, 0 hour; *green*, 24 hour; *purple*, 48 hour.
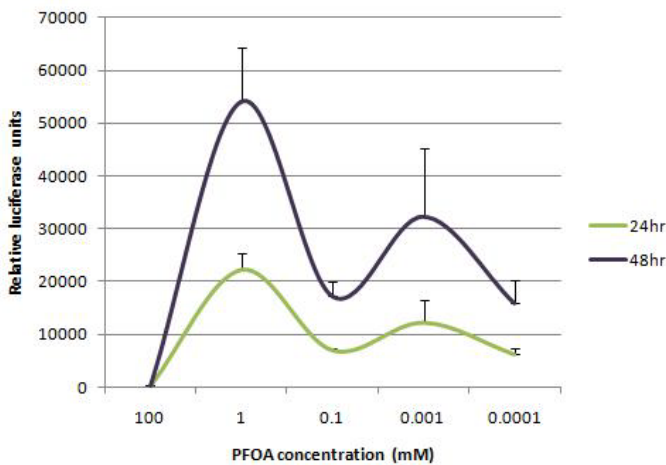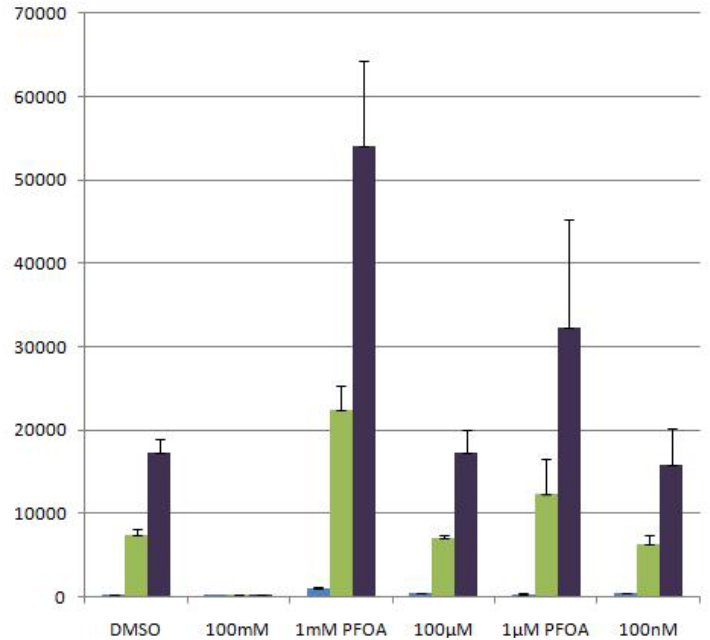
Figure 7. Dose-response curve generated for PFOA-treated GT1-7 cells; graphic representation of dose-response for PFOA, determined for both 24-hour and 48-hour analyses, over concentrations ranging from 100mM to 0.0001 mM. *Green*, 24 hour; *purple*, 48 hour.
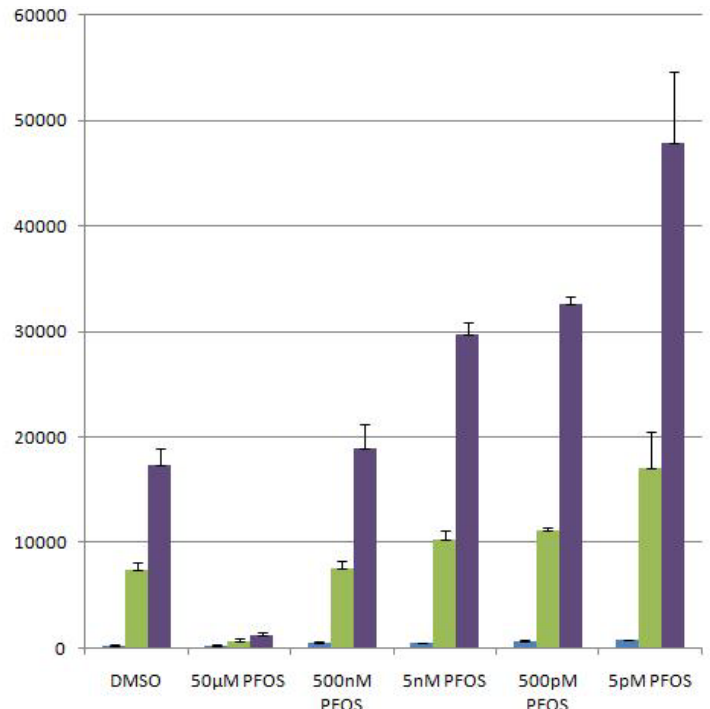
Figure 6. Dose-response of *GnRH* expression following treatment of cell cultures with 50 µM, 500nM, 5nM, 500pM and 5pM concentrations of PFOS, or DMSO control. Values reported are averages of triplicate luciferase signals normalized to total protein, indicating cumulative *GnRH* gene expression over a 48-hour time course. *Blue*, 0 hour; *green*, 24 hour; *purple* 48 hour.
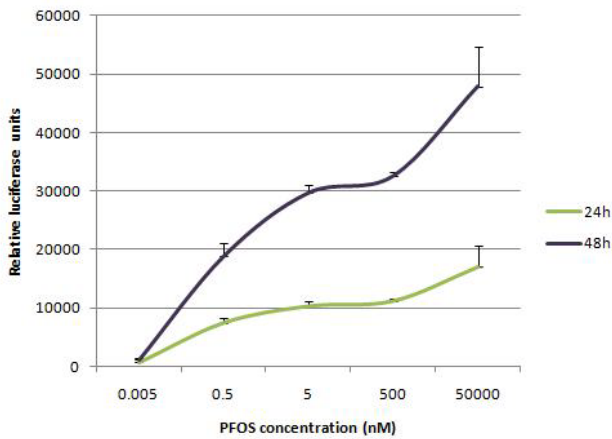
Figure 8. Dose-response curve generated for PFOS treated GT1-7 cells; graphic representation of dose-response for PFOS, determined for both 24-hour and 48-hour analyses, over concentrations ranging from 50,000nM to 0.005nM. *Green*, 24 hour; *purple*, 48 hour.

Treatment with PFOS, on the other hand, generated a much more classic dose-response curve. A steady increase in gene expression was observed with increasing PFOS concentrations at both 24 and 48 hours (Fig. 8).

TUNEL assay

Because of the potential initiation of apoptosis due to DNA fragmentation secondary to treatment of cell cultures with xenobiotics and the possibility that this could be a confounding factor in determination of up- or down-regulation, a TUNEL assay was performed to evaluate these effects.

The percentage of cells undergoing programmed cell death was increased following incubation in 100mM PFOA (31.4%), 1mM PFOA (41.7%), 1µM PFOA (16.2%), 100nM PFOA (18.6%), and 50µM PFOS (7.3%). No notable difference between the percentages of cells in the apoptotic phase after treatment with DMSO, 100µM PFOA (-8.4%), 50µM PFOS (7.3%), 500nM PFOS (-1%), or 5nM PFOS (-4.6%) was observed. Interestingly, 500pM and 5pM doses of PFOS caused an apparent decrease in the percentage of cells entering apoptosis by 37.7 and 20.4%, respectively (Fig 9).

Because the data representing GnRH gene expression is also dependent upon the number of cells present and capable of secretion, a
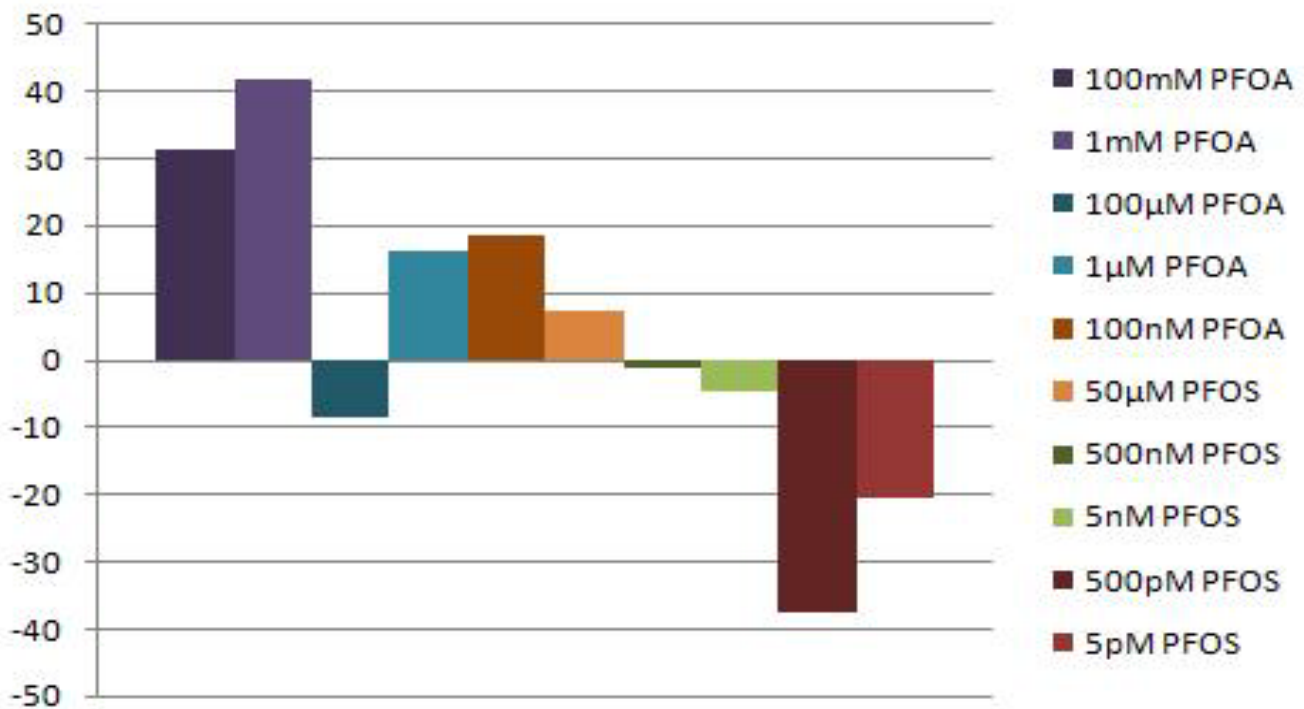

Figure 9. Percent change in number of cells with DNA nicks, indicative of entrance into apoptotic phase, treating value for DMSO control as 0, following treatment with 100mM, 1mM, 100µM, 1µM, and 100nM PFOA, and 50µM, 500nM, 5nM, 500pM, and 5pM PFOS. *Purple, aqua, brown,* varied doses of PFOA; *orange, green, red,* varied doses of PFOS.

summary of these data was made to compare apparent up- or down-regulation determined by assay to cell apoptosis rates in culture (Table 1), and to clarify whether observed changes in transcription resulted from cell apoptosis.

Discussion

The initial luciferase testing conducted with moderately dosed PFOA and PFOS served to confirm our hypothesis that PFCs exert effects on *GnRH* gene expression. Subsequent comparisons were made to determine the extent of transcriptional effects observed in cells treated with PFCs vs. those observed with physiologically relevant concentrations of estrogen, which yielded mixed results.

Our observation that 10μM PFOA caused a 2.3-fold upregulation of GnRH gene expression served as a reference point from which further testing parameters were determined. To gain a better understanding of relevant in vitro doses of PFCs, a dose-response was conducted using a wide range of molar concentrations. Surprisingly, PFOA and PFOS did not have the same patterns of effects on GnRH, which may suggest that there is more than one mechanism by which these chemicals induce their effects.

PFOS elicited strong effects on transcription rates at much lower dose concentrations with no trend toward up- or down-regulation with increasing dose. Presumable inhibition of *GnRH* gene transcription was observed with 50μM PFOS treatment without notable change in DNA nicks as determined by TUNEL assay. Apparent up-regulation of GnRH gene expression in 5nM, 500pM and 5pM PFOS treatment groups, which also demonstrated lower rates of apoptosis induction *in vitro*, could be indicative of major changes in transcription in addition to a protective effect of the PFC. It is also possible, however, that the reduced number of DNA nicks detected are due to lower numbers of cells present in culture. To help eliminate this possibility, protein assays should be conducted in cell populations used to determine apoptosis

rates such that the quantification of DNA nicks can be compared to total cell protein present.

PFOA treatments were not as unpredictable in their effects on transcription, eliciting apparent up-regulation in most cases where effects were seen. Up-regulation of GnRH was observed in 1mM and 1 μM PFOA treatment groups, which also demonstrated higher incidence of DNA nicking by TUNEL assay. Although 100mM treatments resulted in down-regulation of *GnRH*, this dose also caused approximately 30% more cells to enter apoptosis over DMSO control, and it is likely that the effects on gene expression could be wholly attributed to programmed cell death. Interestingly, the most profound up-regulation of *GnRH* gene expression occurred at the next lowest dose (1μM PFOS), which also caused the highest percentage (40%) of cells to enter apoptosis compared to DMSO controls.

Epidemiological studies report mean serum levels of PFOS as approximately 33.1ng/mL, which is roughly equivalent to a 66nM concentration[25]. Though the reported serum levels in the general population are definitely relevant to those concentrations used in this study, it is difficult to determine exactly what chemical concentration is being seen by the GnRH neurons themselves. Doses flanking that observed in the general population did not elicit similar effects *in vitro*. No effects on GnRH gene expression or cell propensity to enter apoptosis were noted following 500nM PFOS treatment. The lower 5nM concentration, however, elicited apparent up-regulation of GnRH without inducing increased apoptotic rates.

Similarly, general population serum concentrations of PFOA are approximately 11nM[25]. The same difficulty of estimating concentrations seen by the GnRH neurons applies, and the closest dose tested (100nM PFOA) did not reveal changes in *GnRH* gene expression, though marked increases (20% over control) in DNA nicking were observed.

Because of the lack of trends within the data, it is difficult to say what kinds of effects PFCs could have on the HPG axis. Since these chemicals do seem to interfere with *GnRH* gene expression, it is possible that exposure and bioaccumulation could have very serious implications for mammalian reproductive function and other pathologies that result from neuroendocrine disruption. *In vivo* testing for polycystic ovaries or precocious puberty, known biological endpoints of GnRH signal disruption, could be helpful in determining possible ramifications of PFC exposure for the human population beyond those suggested by experimental data that reports estrus cycle disruption[26].

There are many practical applications for introducing secreteable reporter vectors into model *in vitro* cell lines. Clearly, the generation of GnRH-MetLuc subclones has made it possible to quantify changes in gene expression over time without confounding factors that come from using different cell populations throughout a time course. This novel system has been used to demonstrate estrogen's role in the regulation of *GnRH* expression in the hypothalamus[67], and so is also an ideal model for testing the potential neurotoxic effects of perfluorochemicals on the hypothalamo-pituitary-gonadal (HPG) axis at the level of the hypothalamus. Though this model is ideal for the determination of gene expression rates, it does not allow for the determination of cell secretory status. More expression and more protein doses not necessarily mean more secretion. Further testing by radioimmuno assay would help determine whether these cells are just generating a lot of protein product or if they are actually secreting these products out of the cell.

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