Effects of perfluoroalkyl compounds (PFCs) on the mRNA expression levels of thyroid hormone-responsive genes in primary cultures of avian neuronal cells

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfilment of the requirements
For the M.Sc. degree in Chemical and Environmental Toxicology

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Abstract

There is a growing interest in assessing the neurotoxic potential and endocrine disrupting properties of perfluoroalkyl compounds (PFCs). Several studies have reported *in vitro* and *in vivo* effects related to neuronal development, neural cell differentiation, pre- and post- natal development and behaviour. PFC exposure altered hormone levels (e.g. thyroid hormone, estrogen, and testosterone) and the expression of hormone-responsive genes in mammalian and aquatic species. Hormone-mediated events are critical in central nervous system development and function, especially those controlled by thyroid hormones (THs).

The studies presented in this thesis are the first to assess the effects of PFCs on primary cultures of neuronal cells in two avian species; the domestic chicken (*Gallus domesticus*) and herring gull (*Larus argentatus*). The following TH-responsive genes were examined using real-time RT-PCR: type II iodothyronine 5'-deiodinase (D2), D3, transthyretin (TTR), neurogranin (RC3), octamer motif binding factor (Oct-1), and myelin basic protein (MBP). Several PFCs were shown to alter mRNA expression levels of genes associated with the TH pathway in avian neuronal cells. It was determined that short-chained PFCs (<8 carbons) altered the expression of TH-responsive genes to a greater extent than long-chained PFCs (≥8 carbons). Although several significant changes in mRNA expression were observed in TH-responsive genes following PFC exposure in chicken embryonic neuronal (CEN) cells (Chapter 2), there were fewer changes in herring gull embryonic neuronal (HGEN) cells (Chapter 3). The mRNA levels of D2, D3, TTR, and RC3 were altered following treatment with several short-chained PFCs in CEN cells. Oct-1 and RC3 expression were induced following treatment with several short-chained PFCs in HGEN cells. These studies are the first to report that PFC exposure alters mRNA expression in primary cultures of avian neuronal cells and provide insight into the possible mechanisms of action of PFCs in the avian brain.

Résumé

L'étude du potentiel neurotoxique des composés perfluoroalkylés (PF) et de leurs propriétés de perturbateurs endocriniens suscite un intérêt croissant. Plusieurs travaux ont permis de constater *in vitro* et *in vivo* des effets liés au développement neuronal, à la différenciation des cellules neuronales ainsi qu'au développement et au comportement prénatal et postnatal. L'exposition aux PF a modifié des concentrations d'hormones (ex. hormone thyroïdienne, œstrogène et testostérone) et l'expression de gènes hormonodépendants chez des espèces mammaliennes et aquatiques. Le développement et le fonctionnement du système nerveux central font intervenir des phénomènes hormonodépendants importants, plus particulièrement ceux qui dépendent des hormones thyroïdiennes (TH).

Les travaux présentés dans cette thèse sont les premiers à porter sur les effets des PFs dans des cultures primaires de cellules neuronales de deux espèces aviaires, soit le poulet domestique (*Gallus domesticus*) et le goéland argenté (*Larus argentatus*). Divers gènes TH-dépendants ont été examinés au moyen de la RT-PCR en temps réel : l'iodothyronine 5'-désiodase de type II (D2), la D3, la transthyrétine (TTR), la neurogranine (RC3), le facteur 1 fixant l'octamère (Oct-1) et la protéine basique de la myéline (MBP). Plusieurs PF ont influé sur le degré d'expression de l'ARNm de gènes associés à la voie métabolique de la TH dans les cellules neuronales aviaires. Les PF à chaîne courte (< 8 carbones) ont modifié l'expression des gènes TH-dépendants dans une plus grande mesure que les PF à chaîne longue (≥ 8 carbones). Dans les cellules neuronales d'embryon de poulet, les PF ont donné lieu à plusieurs changements non négligeables de l'expression de l'ARNm des gènes TH-dépendants (chapitre 2); dans les cellules neuronales embryonnaires de goéland argenté, par contre, les changements étaient moins nombreux (chapitre 3). Les concentrations d'ARNm de D2, D3 TTR et RC3 ont changé après exposition à divers PF à chaîne courte dans les cellules neuronales d'embryon de poulet. Par ailleurs, l'expression des gènes Oct-1 et RC3 a été déclenchée par divers PF à chaîne courte dans les cellules neuronales de goéland argenté. Ces travaux sont les premiers à révéler les effets de l'exposition aux PF sur l'expression

de l'ARNm dans les cultures primaires de cellules neuronales aviaires en plus de nous éclairer sur les possibles mécanismes d'action des PF dans le cerveau des oiseaux.

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List of Abbreviations

APFO	ammonium perfluorooctanoate
BMF	biomagnification factor
CaM	calmodulin
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cDNA	complementary DNA
CEH	chicken embryonic hepatocyte
CEN	chicken embryonic neuronal
CYP1A	cytochrome P4501A enzyme
D1	type I iodothyronine deiodinase
D2	type II iodothyronine deiodinase
D3	type III iodothyronine deiodinase
DMSO	dimethyl sulfoxide
GAP-43	growth associated protein-43
GD	gestation day
FTOH	fluorotelomer alcohol
HGEN	herring gull embryonic neuronal
HPT	hypothalamic-pituitary-thyroid
IRD	inner ring deiodination
LTP	long term potentiation
MBP	myelin basic protein
MCT8	monocarboxylate transporter 8
MW	molecular weight
N-EtFOSE	N-ethyl-N-methyl-perfluorooctane-sulfonamido ethanol
NOEC	no observable effect concentration
NTC	no template control
Oatp1c1	organic anion transporting polypeptide 1C1
Oct-1	octamer transcription factor-1
ORD	outer ring deiodination
PBDE	polybrominated diphenyl ether
PCB	polychlorinated biphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl
pCREB	cAMP-response element binding protein
PFBA	perfluorobutanoic acid
PFBS	perfluorobutanesulfonate
PFC	perfluoroalkyl compound
PFCA	perfluoroalkyl carboxylate
PFDA	Perfluorodecanoic acid
PFDoA	perfluorododecanoic acid
PFHpA	perfluoroheptanoic acid
PFHpS	perfluoroheptanesulfonate
PFHxA	perfluorohexanoic acid
PFHxS	perfluorohexanesulfonate
PFNA	perfluorononanoic acid
PFOA	perfluorooctane acid

PFOS	perfluorooctane sulfonate
PFSA	perfluoroalkyl sulfonate
PFuDA	perfluoroundecanoic acid
PKC	protein kinase C
POUHD	POU homeodomain
PPAR	peroxisome proliferation-activated receptor
PND	postnatal day
RT	reverse transcriptase
rT3	reverse T3
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SMRT	silencing mediator for retinoid and thyroid hormone receptor
snRNA	small nuclear RNA
T2	3,3'-diiodothyronine
T3	Triiodothyronine
T4	thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TH	thyroid hormone
TR	thyroid hormone receptor
TRE	thyroid hormone response element
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TTR	transthyretin
UT	untreated
WW	weight wet

Publication plan

Paper 1: Chapter 2 and 3: Effects of perfluoroalkyl compounds (PFCs) on the mRNA expression levels of thyroid hormone-responsive genes in primary cultures of avian neuronal cells

Authors: Viengtha Vongphachan, Cristina Cassone, Dongmei Wu, Suzanne Chiu, Doug Crump and Sean W. Kennedy (Toxicological Sciences, 2011. in press)

Acknowledgements

First of all, I would like to thank Dr. Sean Kennedy for giving me the opportunity to work under his guidance and supervision at the National Wildlife Research Centre (NWRC). Thank you for being so supportive, encouraging, and understanding, I really appreciate everything that you have done for me. I would also like to thank my committee members, Dr. Vance Trudeau and Dr. John Stead.

My thesis would not have been possible without the help and support from the entire Kennedy lab. I am very grateful for the friendships that I have made during my time at NRWC. I would like to thank Doug Crump for always having his office doors open – thank you for your patience in answering my endless questions, helping me with troubleshooting, teaching me the cell culture techniques, and proofreading my thesis. Thanks to Cristina Cassone and Suzanne Chiu for helping me perform qPCR assays. A particular thanks to Dongmei Wu for designing the herring gull primers and probes and validating them with qPCR. A big thank you goes out to Jason O'Brien and Nathan Hickey for being there to answer my questions and providing technical advice. I would also like to thank Stephanie Jones, Kim Williams, Reza Farmahin, Lukas Mundy, and Gillian Manning. Thanks to Jason O'Brien, Wouter Gebbink, and Franscisco Ucan-Marin for reviewing parts of this thesis. A special thank you goes to Jessica Hervé, Cristina Cassone, and Caroline Egloff for being so supportive and reassuring.

I would like to thank my friends and family for all of their love and support. This thesis would not have been possible without the encouragement from my parents, Soukanh and Ket, and my sister, Joanne. I am also indebted to my friends who always support and believe in me, special thanks goes to Christina, Trish, Nikki, Jenn, Shannon, Kelsey, Jasmine, and Shalika. I would also like to thank all the Sisters of Delta Psi Delta Sorority for all of their support and encouragement (there are too many of you to name, but you know who you are)!

Last but not least, I would like to thank Corey for all of his support throughout the years. You are my biggest fan, but also my biggest distraction. You have definitely seen the good and the bad of this entire Masters experience. Thank you for putting up with me and helping me through the tough times. I really could not have done it without you!

Chapter 1
Introduction

1.1. Perfluoroalkyl compounds (PFCs)

1.1.1. General information

Perfluoroalkyl compounds (PFCs) are a class of ubiquitous synthetic contaminants found in the environment. These compounds consist of a carbon chain (4-14 carbons) attached to a charged functional group that is completely saturated in fluorine atoms. The most environmentally relevant PFCs are the perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs), which contain carboxylate and sulfonate functional groups, respectively. The complete saturation of fluorine atoms coupled with the charged functional group produces a highly stable compound with an extremely low surface tension. The hydrophobic fluorine-carbon backbone and the hydrophilic charged functional group are responsible for the partly lipophilic and hydrophilic properties of these compounds. This unique molecular arrangement gives PFCs their desirable water-, oil- and soil- resistant properties.

PFCs are found in many industrial and commercial products including, but not limited to, the lining of non-stick cookware, food wraps and containers, popcorn bags, aqueous fire-fighting foams and industrial lubricating agents (Jensen and Leffers 2008). However, the characteristics that make PFCs attractive to industrial and commercial sectors – their low surface tension and strong bonds – are the same properties that lead to accumulation in the environment. The stability of the fluorine-carbon bond is one of the strongest bonds in nature/chemistry and renders PFCs virtually non-biodegradable and resistant to metabolic degradation.

The two most environmentally relevant PFCs are the eight-carbon chained perfluorooctane sulfonate (PFOS) and perfluorooctane acid (PFOA) (Lau et al. 2007). Following the discovery of PFOS in non-occupationally exposed individuals and wildlife, the major manufacturing company, 3M, announced a voluntary phase out of PFOS in

2000 that was completed by 2002 (Andersen et al. 2008). The voluntary phase out of PFOA is still underway. The eight major PFC producers have committed to completely eliminate facility emissions and product contents of PFOA and related chemicals by 2015 (Lau et al. 2007). However, new and emerging PFCs, which include short- (<8 carbons) and long-chained PFCs (> 8 carbons) are currently being manufactured as replacement alternatives for PFOS and PFOA.

1.1.2. Sources of contamination

The total global PFCA emissions released into the environment from 1951-2004 was estimated to be approximately 3,200-7,300 tonnes (Prevedouros et al. 2006). Furthermore, the estimated global release of PFOS and its derivatives from 1970-2002 was approximately 42,250 tonnes (Paul et al. 2009). PFCs are released directly into the environment from manufacturing processes and from consumer and industrial product uses. For example, PFCAs, which are found in aqueous fire-fighting foams, are dumped directly into the ocean and soil by the military and refining industries during emergencies and training exercises (Prevedouros et al. 2006). The disposal of consumer products and manufacturing wastes also contribute to the environmental release of PFCs. Following their disposal, solid PFC waste products end up in landfills, are recycled, or incinerated (Prevedouros et al. 2006).

PFCs can also be released indirectly via the degradation of PFC derivatives and intermediates in the environment or during manufacturing processes. PFCs are the final degradation products of a wide range of volatile precursors. For example, perfluorooctylsulfonamides such as N-ethyl-N-methyl-perfluorooctane-sulfonamido ethanol (N-EtFOSE) and fluorotelomer alcohols (FTOHs) may be transformed to PFOS and various PFCAs, respectively (Paul et al. 2009;Prevedouros et al. 2006). There are over 600 intermediate manufacturing steps associated in manufacturing these products and unintended PFC by-products may be formed during chemical processing (Paul et al. 2009;Prevedouros et al. 2006).

1.1.3. Global distribution

Given the widespread use, persistent nature, and high production of PFCs, it is not surprising to discover their global detection in various environmental and human matrices. In aquatic environments, PFCs are detected in the low ppb (μg/L) and ppt (ng/L) range, depending on how far the sampling areas are from point sources. PFC concentrations are typically higher in more urbanized and industrialized regions. For example, PFC concentrations were two- to four- orders of magnitude higher in coastal waters than offshore (Yamashita et al. 2005). These compounds have also been detected in remote locations; PFCs and several intermediates were measured in snow and ice caps in the High Arctic (2-3ng/L of PFOA) (Martin et al. 2004;Young et al. 2007). Furthermore, PFCs and their precursors have been measured in indoor and outdoor air samples and in house dust samples in Canada (Kubwabo et al. 2005;Shoeib et al. 2006).

PFCs have been identified in many human matrices such as blood, umbilical cord blood, milk, seminal plasma, and liver (Apelberg et al. 2007;Fei et al. 2008;Guruge et al. 2005;Olsen et al. 2003). PFC concentrations are the highest in industrialized areas. Individuals living in countries that manufacture PFC-based products such as the United States, Japan, China, and Korea, had greater PFC concentrations in their blood serum, most likely due to local emissions (Suja et al. 2009). PFC concentrations were also higher in the serum of occupationally exposed workers (1.32 μg/g PFOS and 1.78 μg/g PFOA) than those reported in the general population (Olsen et al. 2003).

The pattern of PFC distribution in animal species is complex and varies among species and locations. High PFOS levels were reported in the livers of polar bears from the Canadian Arctic (mean: 3.1μg/g ww) (Bossi et al. 2005;Martin et al. 2004). Other top predators such as fish-eating birds also tend to accumulate high concentrations. For example, PFOS plasma concentrations in bald eagles reached levels up to 2570 ng/ml (Kannan et al. 2001). PFCs have also been identified in the eggs of wildlife species (fish and birds) (Kannan et al. 2005;Verreault et al. 2007). The detection of PFCs in wildlife

eggs suggests oviparous transfer and may have implications for early life stage toxic effects.

Two hypotheses have been proposed to explain the global distribution of PFCs. The first is based on the idea that PFCs are transported via oceanic currents. Ocean water transport is considered the main means by which PFCs reach remote regions (especially the Arctic), based on the physical-chemical properties of PFCs and the large proportion of emissions released into surface waters (Paul et al. 2009;Prevedouros et al. 2006). The second hypothesis involves the atmospheric transport of volatile precursors. It is suggested that volatile PFC precursors undergo long range transport before being oxidized by hydroxyl radicals in the atmosphere to form PFCs (Young et al. 2007). For example, ammonium perfluorooctanoate (APFO), a processing aid in the production of fluoropolymers, is released from manufacturing facilities, degrades in the atmosphere and undergoes sublimation and volatilization to form gaseous PFOA (Barton et al. 2007).

1.1.4. Environmental concerns

PFCs have generated considerable scientific, regulatory, and public interest on an international scale following their global detection and persistence in humans and wildlife (Giesy and Kannan 2001). Environmental concerns surrounding these contaminants include their environmental persistence, bioaccumulation and biomagnification potential, toxic effects in laboratory animals, and implications to human health.

1.1.4.1. Environmental persistence

The chemical structure and widespread use of PFCs have led to their environmental persistence. Higher PFC concentrations are detected in blood and liver tissue of animals occupying high trophic positions in the food chain. These contaminants have relatively long half lives and depending on the specific compound, it can take up to 3-8 years to eliminate them from human systems (Hekster et al. 2003). PFCs are resistant

to degradation by hydrolysis, photolysis, strong acids, alkalis, and oxidizing agents (Lau et al. 2007) which makes it important to determine the risks of continued exposure to these contaminants.

1.1.4.2. Bioaccumulation and biomagnification potential

A review by Conder et al. (2008) reported that PFC bioaccumulation was directly related to fluorinated carbon chain length and generally increased with longer chain lengths. Longer-chained PFCs (> 7 carbons) bind more tightly to proteins which may explain their increased bioaccumulation potential compared to shorter-chained PFCs (Jones et al. 2003). Furthermore, PFSAs typically have greater bioaccumulation than PFCAs (Conder et al. 2008).

Biomagnification factor (BMF) values are expressed as the concentration of a chemical within an organism divided by the concentration of the chemical in its food (Conder et al. 2008). BMF values greater than 1 suggest biomagnification potential. Similar to previous studies that examined the bioaccumulation of PFCs, it was determined that PFCs with less than seven fluorinated carbons did not biomagnify. Based on all published food web studies, PFOS was the only compound consistently identified as exhibiting the potential for biomagnification (Bossi et al. 2005;Kannan et al. 2005;Martin et al. 2004). In an Arctic food web study, BMF values for PFOS were as high as 9.0 (Tomy et al. 2004) and in the Great Lakes region, a BMF of 10-20 was reported in mink or bald eagles relative to their prey items (Kannan et al. 2005).

1.1.4.3. Toxic effects

PFC exposure has been associated with many detrimental effects in mammalian species including, higher incidences of mortality, reduced body weight, hepatomegaly and hepatotoxicity (Thibodeaux et al. 2003). Exposure to PFCs produce adverse reproductive outcomes in offspring including reduced fetal weight, cleft palate, anasarca (edema), delayed eye opening, delayed ossification and cardiac abnormalities (Lau et al.

2003;Thibodeaux et al. 2003). In avian studies, reduced hatching has been demonstrated following *in ovo* exposures to several PFCs (Molina et al. 2006;O'Brien et al. 2009;Pinkas et al. 2009;Yanai et al. 2008). Furthermore, higher incidences of brain asymmetry and physical deformities were observed following PFC treatment, which may suggest poor brain development (Peden-Adams et al. 2008;Yanai et al. 2008).

Recently, studies have investigated the potential for PFCs to affect processes involved in normal brain function and disturb the endocrine system. PFC exposure was reported to alter neuronal development and neural cell differentiation, and influence pre- and post- natal development (Slotkin et al. 2008;Yanai et al. 2008). Behavioural effects have also been observed in PFC-treated avian and mammalian species (Butenhoff et al. 2008;Fuentes et al. 2007;Pinkas et al. 2009;Yanai et al. 2008). The expression of hormone-responsive genes and circulating hormone levels (e.g. thyroid hormone, estrogen, and testosterone) in several mammalian and aquatic species have been altered following PFC treatment (Beigel et al. 2008;Lau et al. 2004;Liu et al. 2007;Thibodeaux et al. 2003).

1.1.4.4. Implications to human health

Although high PFC concentrations are typically measured in occupationally exposed workers (Olsen et al. 2003), overall, epidemiological studies have not revealed any associations between occupational exposure and adverse health effects. Few studies have examined the health effects of PFCs in humans. It was suggested that bladder and prostate cancer may be related to PFOS and PFOA exposure, respectively, however, more research is required based on the low number of deaths and potential confounders in the study (e.g. age, exposure to other contaminants, etc.). In general, there were no statistical differences in cancer mortality in workers employed at various fluorochemical manufacturing sites (Alexander et al. 2003;Gilliland and Mandel 1993).

The exposure of infants and children to PFCs is another concern. Given the fact that PFCs have been detected in the breast milk and umbilical cord blood of mothers,

there is a concern that pre- and post-natal exposure in children may affect development or health effects later in life. There have been conflicting results regarding the effects of maternal plasma PFC concentrations on infant weights which may be related to differences in the experimental designs of the studies (Fei et al. 2008;So et al. 2006). More work is necessary in order to determine the potential health risks in newborns exposed to PFCs via maternal transfer.

1.2. Thyroid hormones

Hormone-mediated events are critical in central nervous system development and function, especially those controlled by THs (thyroid hormones). PFC exposure has been reported to alter TH levels and the expression of TH-responsive genes in avian and mammalian species (Lau et al. 2003;Thibodeaux et al. 2003;Wang et al. 2010;Yu et al. 2009). For this reason, the effects of PFCs on the TH pathway were explored in more detail in avian species. Avian species provide a good means for assessing wildlife health and have been used to monitor environmental contamination of natural populations. Birds are abundant, highly visible, and can be studied in both the laboratory and the natural environment (Hill and Hoffman, 1984). Avian species are also well positioned from an evolutionary standpoint, providing an intermediate perspective between mammals (e.g. humans) and lower vertebrates (e.g.fish).

1.2.1. Thyroid hormones in birds

THs influence development, thermogenesis/ thermoregulation, eggshell formation, hatching, molting, reproduction, and behaviour in birds (reviewed in McNabb, 2007). THs stimulate and coordinate the growth, differentiation and maturation of most tissues including bone, heart, fat, liver, brain, gut, lung, and muscles (reviewed in McNabb, 2007). Hypothyroidism or hyperthyroidism (TH deficiency or overproduction, respectively) can result in irreversible biochemical, histological, and behavioural alterations. For example, hypothyroidism in the chicken embryo was reported to impair brain development by altering neurite growth and retraction (Bouvet et al., 1987).

Structural alterations in the brain may have functional consequences associated with delayed behavioural responses.

1.2.2. Hypothalamic-Pituitary-Thyroid (HPT) axis

TH synthesis is regulated by a negative-feedback system that involves the hypothalamus, pituitary, and thyroid gland (also known as the HPT axis) (Figure 1.1). In general, the functions of the HPT axis in avian species are similar to vertebrates. Thyrotropin-releasing hormone (TRH), a tripeptide synthesized in the hypothalamus, is transported to the pituitary where it binds to TRH receptors which induce the secretion of thyroid-stimulating hormone (TSH) (reviewed in McNabb, 2007). TSH, which binds to TSH receptors, then stimulates the synthesis and release of two THs, thyroxine (T4) and triiodothyronine (T3), from the thyroid gland. T4 is the major hormone that is secreted by the thyroid gland; approximately 99% is secreted as T4 (Darras et al. 2006;McNabb et al. 1984). The majority of T3, the more active hormone, is produced via 5'-deiodination of the outer ring of T4 by deiodinases in target tissues. The HPT axis is a negative feedback system; increased T3 and T4 levels signal the hypothalamus and pituitary to stop TRH and TSH production, respectively (reviewed in Yen et al., 2001; McNabb, 2007).

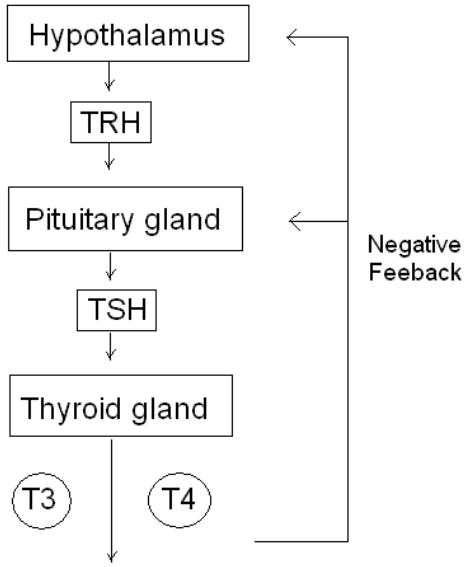


Figure 1.1. Schematic of the hypothalamic-pituitary-thyroid (HPT) axis.

1.2.3. Transport and regulation of thyroid hormones

In addition to the HPT axis, iodothyronine deiodinases also contribute to TH production. The predominance of T4 in the avian thyroid gland and the higher ratio of T3/T4 in circulation suggest that most T3 is produced extrathyroidally (reviewed in McNabb, 2007). In extrathyroidal tissues, iodothyronine deiodinases are responsible for the local synthesis and clearance of THs. Iodothyronine deiodinases activate and inactivate T4 and T3 by removing iodide from the outer- or inner- ring of the molecule (Darras et al. 2008). There are three important deiodination pathways that are involved in the local synthesis and clearance of THs (Figure 1.2). Type I iodothyronine deiodinase (D1), found in the liver, kidney, and small intestine of chicken, is primarily involved in converting T4 to T3 via outer ring deiodination (ORD) and inactivating T4 to reverse T3 (rT3) via inner ring deiodination (IRD). Type II iodothyronine deiodinase (D2), found in the brain, primarily converts T4 to its active T3 form via ORD. Type III iodothyronine deiodinase (D3), mainly found in the liver, inactivates T4 to rT3 and also T3 to 3,3'-diiodothyronine (T2) via IRD. Changes in iodothyronine deiodinase activities alter circulating TH concentrations during different developmental stages and physiological states. Furthermore, iodothyronine deiodinases respond to changes in circulating TH levels.

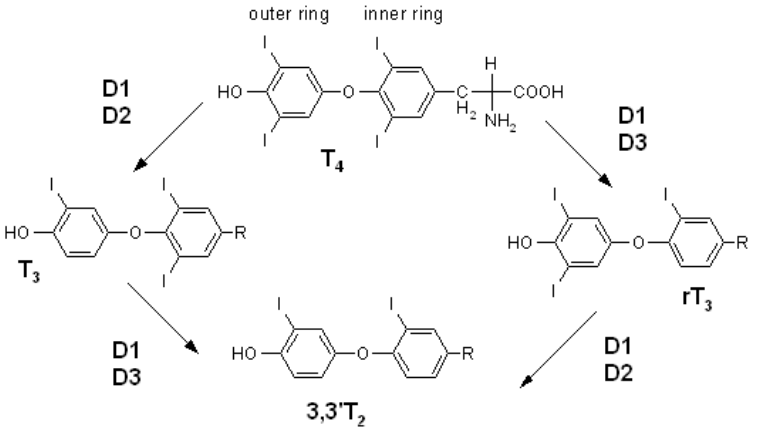


Figure 1.2. Deiodination pathways involved in TH synthesis and clearance

TH-binding proteins play an important role in transporting TH from the blood to different target tissues where it can be taken up by the cell. In avian species, the main TH-binding proteins in blood that modulate hormone availability are transthyretin (TTR) and albumin (reviewed in McNabb, 2007). It was reported that approximately 17-32% is bound to TTR, 66-75% to albumin, and the remainder to other plasma proteins (reviewed in McNabb, 2007). Once TH makes its way into target tissues via TH-binding proteins, TH transporters help facilitate its movement into the cell. TH needs to enter the cell in order to bind to receptors and activate TH-dependent processes. Two important TH transporters in the brain include monocarboxylate transporter 8 (MCT8) and organic anion transporting polypeptide 1C1 (Oatp1c1). MCT8 is involved in T3 transport into the brain and Oatp1c1 is thought to be a predominant transporter at the blood-brain barrier (Horn and Heuer 2010).

1.2.4. Thyroid hormone action

TH action is mediated by nuclear receptors that initiate the transcriptional regulation of target genes (Figure 1.3). There is considerable evidence that T3 is responsible for most TH action in birds. Although T4 and T3 are essentially equipotent in birds, T3 has a greater affinity for thyroid hormone receptors (TRs) (reviewed in McNabb, 2007). As mentioned earlier, in the cell, after T3 is produced via deiodination of T4, T3 is translocated through the cytoplasm to the nucleus where it binds to TRs. TRs heterodimerize with retinoid X receptors (RXRs) and the TR-RXR complex then binds to thyroid hormone response elements (TREs) in the promoter region of TH-regulated genes (Lazar et al., 1993; review Yen et al., 2001). The TR-RXR heterodimerization is important in stabilizing the binding between TRs and TREs. Activation results in a positive or negative regulation of the transcription of target genes followed by the regulation of protein synthesis that ultimately mediates TH effects (Lazar 1993).

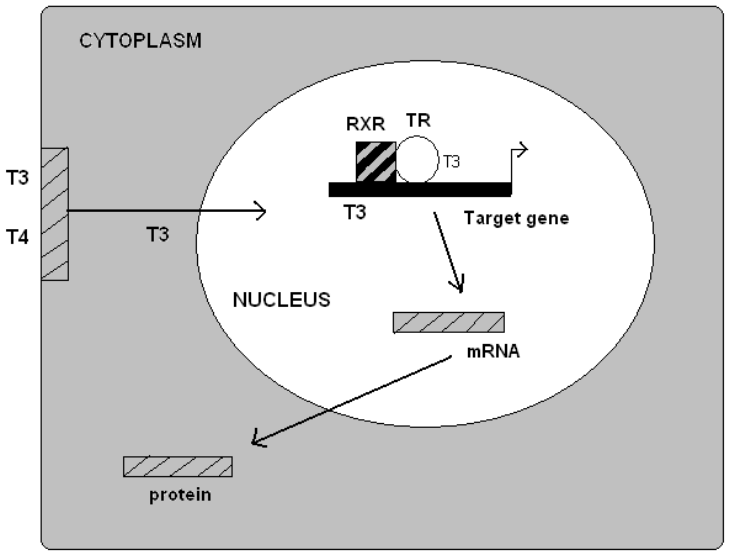


Figure 1.3. Schematic diagram of thyroid hormone action (adapted from Yen et al., 2001)

In the presence of T3, TRs can interact with several transcription factors to stimulate or repress transcription (reviewed in Yen et al., 2001). While the T3-TR complex can bind to TREs to initiate the transcription of target genes, it has been reported that unliganded TRs can bind TREs and modulate transcription, as well (Figure 1.4) (Darras et al. 2008). TR, in its free state, binds to the TRE and can recruit co-repressor proteins that repress gene transcription. It has also been reported that unliganded TR can activate the transcription of genes that are negatively regulated by T3 (Munoz and Bernal 1997). The exact mechanisms of ligand-independent activation have yet to be determined and further research is necessary.

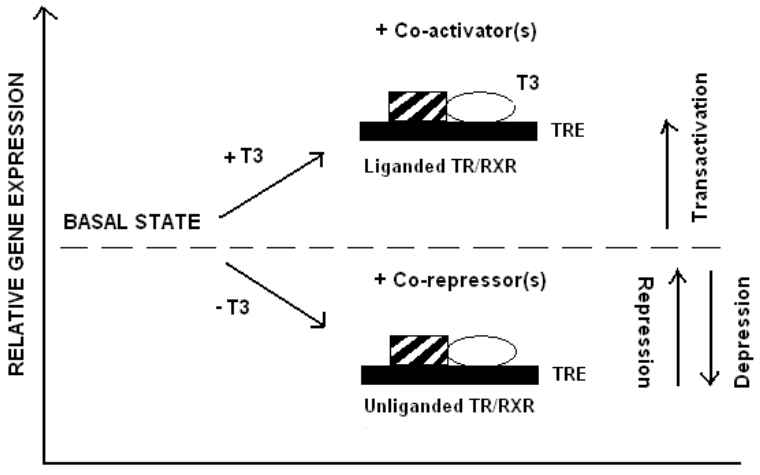


Figure 1.4. Schematic diagram of transcriptional activation and repression by thyroid hormone receptor in the presence and absence of triiodothyronine (T3) (adapted from Yen et al., 2001)

1.2.5. Thyroid hormone-responsive genes

As mentioned in the previous section, TH exerts its actions through nuclear receptors. The TH-TR binding modulates the transcription of downstream genes which are critical for central nervous system organization and development. This section will focus on several important TH-responsive genes that are involved in maintaining TH homeostasis and normal brain development.

1.2.5.1. Iodothyronine deiodinases

As mentioned previously, iodothyronine deiodinases are important in regulating intracellular TH levels in peripheral tissues and maintaining TH homeostasis. Deiodination is regulated by several endocrine axes, including THs, growth hormones and glucocorticoids. External factors such as nutrition, temperature, light and environmental toxicants can also influence deiodination (Darras et al. 2006). The brain is a tightly regulated organ that is protected against rapid changes of TH availability. D2 and D3 activity has been detected in the brain which suggests that these two iodothyronine deiodinases play a role in maintaining TH homeostasis in the brain.

Although D2 is detected in the pituitary, liver, and brown adipose tissues, it is the most highly expressed iodothyronine deiodinase in the brain. D2 is responsible for converting T4 to T3 and appears to be the main source of intracellular T3 in these tissues (Burmeister et al. 1997). In the avian brain, D2 activity was detected as early as day 7, before the onset of thyroid gland function, and increased to day 13 (Gereben et al. 2004). Similarly, D2 mRNA increased significantly from day 10 to day 17 (Gereben et al. 2004). The increase in D2 activity and mRNA expression corresponds with high T3 levels, increasing TRs, and a peak in neuroblastic proliferation in the brain (Valverde et al. 1993).

D3, on the other hand, is involved in T3 clearance, converting T3 to its inactive form, rT3 (Hernandez 2005). D3 is the most widely expressed iodothyronine deiodinase.

Although it is mainly located in the liver of birds, it is also detected in the kidney, small intestine, skin, gonads, thyroid, lung, heart, spleen, skeletal muscle, and brain (Darras et al. 2006). D3 protein has been detected in Purkinje cells in the cerebellum in the chicken (Verhoelst et al. 2002). Similarly, D3 mRNA has also been detected in cerebral cortex and hypothalamus in the mammalian brain (Hernandez 2005). Low D3 activity is detected at mid-incubation (day 11) and subsequently reaches maximal values on day 17 (Valverde et al. 1993). Low D3 activity at mid-incubation highlights the importance of high D2 activity during neuroblastic proliferation which takes place during this time period. From day 18-20, D3 activity is reduced by 40% in the chicken brain (Valverde et al. 1993).

These ontogenic expression studies highlight the importance of the interactions between D2 and D3 in regulating the availability of active hormone in the brain. As mentioned, external factors such as exposure to environmental contaminants can alter iodothyronine deiodinase activity and expression. For example, it was reported that chicken embryos exposed to 3,3',4,4'-tetrachlorobiphenyl (PCB 77) had reduced T4 and T3 levels in the brain (Beck et al. 2006). Elevated D2 and reduced D3 activity were observed in the cerebellum indicating a compensatory mechanism that protects the brain from TH deficiency (Beck et al. 2006).

1.2.5.2. Transport protein

TTR is a major TH-binding protein located in extracellular fluids that mediates TH transport into target tissues (Chang et al. 1999). TTR is synthesized by the choroid plexus and transports T4 from the blood into the brain and throughout the central nervous system (review Schreiber 2002; review McNabb, 2007). TTR is involved in maintaining extrathyroidal hormone stores, modulating TH supply to tissues, and regulating hormone distribution within tissues (review McNabb, 2007). Given that albumin concentrations are lower in the cerebrospinal fluid than in the blood, TTR is the major TH-binding protein in the extracellular space of avian brains (Chang et al. 1999).

In avian species, TTR has a higher affinity for T3 than for T4 (Chang et al. 1999). Generally, TTR synthesis is correlated to an increased demand for TH distribution. The TTR gene is expressed in the extraembryonic membranes of chicken embryos on day 2, in the liver on day 3, and in the choroid plexus on day 5 (Southwell et al. 1991). TTR mRNA levels in the choroid plexus increase from day 16 which coincides with major brain growth. It is suggested that the secretion of TTR from the choroid plexus at day 9 provides a means of transporting TH into the brain which coincides with the established timing of the blood-cerebral spinal fluid (Southwell et al. 1991). Exposure to several environmental contaminants was reported to alter TTR expression. It was previously determined that exposure to a commercial polybrominated diphenyl ether (PBDE) flame retardant (DE-71) in chicken embryonic neuronal (CEN) cells (at mid-incubation) decreased TTR mRNA which could negatively affect TH-dependent processes (Crump et al. 2008a).

1.2.5.3. Signal transduction

THs are essential for normal brain development and are involved in many important processes associated with brain differentiation, synaptogenesis, and neuronal migration. It has been postulated that the neurological and behavioural symptoms associated with hypothyroidism may be related to disturbances in synaptic plasticity in several brain regions (Vallortigara et al. 2008). RC3/neurogranin is a brain-specific gene involved in synaptic plasticity and learning and, based on mammalian studies, has been identified as TH-dependent (Iniguez et al. 1993). TH influences RC3 mRNA expression in several brain regions in rodents, primarily the cerebral cortex, striatum, and hippocampus (Iniguez et al. 1993). Hypothyroid rodents exhibited reduced RC3 expression; however a single T3 administration restored RC3 expression (Guadano-Ferraz et al. 1997;Iniguez et al. 1993;Vallortigara et al. 2008). Although the RC3 gene contains a functional TRE (Morte et al. 1997), it is currently unclear whether TH influences transcriptional changes in RC3 expression directly or indirectly.

RC3 plays a role in regulating Ca^{2+} and calmodulin (CaM) levels in neurons which are implicated in postsynaptic signal transduction pathways (Gerendasy 1999). RC3 is also involved in synapse formation (Gerendasy 1999). RC3 is a CaM-binding protein kinase C (PKC) substrate located in dendrites, dendritic spines, and cell bodies (Gerendasy 1999). The phosphorylation of PKC substrates (i.e. RC3) initiates downstream effects that lead to long term potentiation (LTP) (Gerendasy 1999). LTP is characterized by a rapid and persistent synaptic enhancement following brief trains of high frequency stimulation (Iniguez et al. 1993;Iniguez et al. 1996). Changes in RC3 expression could have functional consequences in synaptic plasticity, associative learning and memory (Iniguez et al. 1993;Iniguez et al. 1996).

RC3 expression has been demonstrated to alter neural plasticity in songbirds (Clayton et al. 2009). RC3 is abundantly expressed in the telencephalon and in Purkinje cells in the cerebellum in canaries and zebra finches. RC3 mRNA levels were high prior to post hatch day 25. An adult-like pattern of reduced RC3 expression emerged between post-hatch day 25 and 35 in the song nuclei of songbirds which may suggest more tightly regulated synaptic plasticity. Interestingly, research conducted on RC3 knockout mice reported anxiety-like tendencies and deficits in spatial learning based on poor performances on several behavioural assessments (Miyakawa et al. 2001;Pak et al. 2000). RC3 is an upstream modulator for calcium; therefore cognitive and behavioural deficits may be due to alterations in regulating neuronal Ca^{2+} and CaM levels which affect the activation of many Ca^{2+}/CaM dependent enzymes (Pak et al. 2000).

1.2.5.4. Transcription factor protein

Octamer transcription factor-1 (Oct-1) is a member of a homeobox gene subfamily that contains two DNA-binding protein-encoding domains: POU and POU homeodomain (POUHD) (Petryniak et al. 1990). These domains have been highly conserved during vertebrate evolution due to their DNA binding and transcriptional regulatory properties (Petryniak et al. 1990). Oct-l is involved in regulating the expression of cell cycle-specific and constitutive genes and is also necessary for cellular

DNA replication (Lakin et al. 1995). Oct-1 binds a number of transcription factors in a wide variety of cellular genes including small nuclear RNAs (snRNAs), histone H2B and immunoglobulins and regulates their expression (Lakin et al. 1995). In the chicken, Oct-1 is widely expressed in several tissues (e.g. blood, cerebrum, ovary, thymus, spleen, kidney, and lungs) (Heltemes et al. 1999).

Oct-1 mRNA was detected within the brain and outside the nervous system and is predominantly located in the ventricular zone of the cortex (Dowling et al. 2000). Oct-1 is considered a TH-responsive gene although its promoter region does not contain a TRE. It was previously determined that a single T4 administration increased Oct-1 mRNA in the cortex of rat pups exposed at gestation day 16 (athyroid state) (Dowling et al. 2000). Furthermore, Oct-1 expression was regulated by T3 in adult rats demonstrating continuous expression and retained responsiveness from fetal life to adulthood (Dowling et al. 2000). Oct-1 positively regulates the TR-β1 promoter in transfection studies using COS-1 cells (Nagasawa et al. 1997). However, conflicting studies determined that Oct-1 repressed the T3-dependent transcriptional activity of the TR-RXR heterodimer (Kakizawa et al. 1999). The POUHD in Oct-1 interacts with RXR and reduces the binding of TR-RXR heterodimers to TRE (Kakizawa et al. 1999). Follow-up studies reveal that Oct-1 interacts with a silencing mediator for retinoid and TR (SMRT) via direct protein-protein interactions (Kakizawa et al. 2001). More work is required to examine the role of TH and TRs on the transcriptional activity of Oct-1.

1.2.5.5. Myelination

THs are important in myelination which facilitates the conduction of nerve impulses. Myelin is a multicellular compacted membrane that surrounds and electrically insulates the axons. Myelin basic protein (MBP) is one of the major proteins in myelin and is involved in the process of myelination. MBP content increases during chicken brain development from day 16 to post-hatch (Shinomiya et al. 1982) and most of the chicken brain is myelinated between day 18 to post-hatch day 21 (Hartman et al. 1979). Few studies have examined MBP expression in avian species.

In mammals, it was reported that MBP expression was reduced in thyroidectomized rats, but was restored to normal levels following T4 administration (Farsetti et al. 1991). Additionally, euthyroid rodents have a 2-fold higher rate of MBP gene transcription than hypothyroid rodents (Farsetti et al. 1991). Treatment with xenobiotics was reported to alter MBP expression. For example, exposure to a commercial mixture of polychlorinated biphenyls (PCBs), Aroclor 1254 (A1254), elevated MBP expression in the brain tissues of rats exposed via gestational exposure (Zoeller et al. 2000). MBP contains a TRE in its promoter region which binds to TRs (Farsetti et al. 1991). Previous *in vitro* binding studies demonstrated that the presence of both TH and TRs are required to positively regulate the expression of MBP and the TRE is necessary to confer TH responsiveness (Farsetti et al. 1991).

1.3. PFC effects on thyroid hormone levels

Although no studies have assessed the effects of PFCs on thyroid hormone parameters in birds, based on mammalian studies there is evidence that these compounds influence TH homeostasis. Yu et al. (2008) observed reduced serum total T4 levels and increased serum total T3 in rodents following PFOS exposure *in vivo*. Altered TH levels may be related to changes in the mRNA expression of genes involved in TH biosynthesis and metabolism (i.e. increased hepatic T4 gluconoridation via UGT1A1 and increased thyroidal conversion of T4 to T3 via D1). Pre-natal (*in utero*) and post-natal (lactational) exposure to PFOS induced hypothyroxinemia (T4 deficiency) to a similar extent (Yu et al. 2009). Other studies have reported that *in utero* PFOS exposure decreased T4 and T3 levels in the pregnant rats, and caused T4 depletions in neonatal pups (Lau et al. 2003;Thibodeaux et al. 2003). Higher incidences of neonatal mortality were observed in addition to growth lags and slight delays in eye opening in surviving pups.

1.4. PFCs and its impact on the brain

1.4.1. PFC concentrations in the brain tissues of wildlife species

Recent monitoring studies have detected PFCs in the brain tissues of various wildlife species. These important discoveries provide evidence that PFCs are able to cross the blood brain barrier (Van de Vijver et al. 2007;Verreault et al. 2005). Several PFCs were detected in the brain tissues of harbour seals in the German Bright (Ahrens et al. 2008). PFOS was detected in the brains of sea otters from the west coast of the United States (<35ng/g ww) and harbor porpoises from the Black Sea (24 ng/g ww) (Kannan et al. 2001;Van de Vijver et al. 2007). In avian species, PFOS was measured in the brain tissues of glaucous gulls in the Norwegian Artic (above detection limits) and pelicans from Colombia (3.5ng/g ww) (Olivero-Verbel et al. 2006;Verreault et al. 2005).

In laboratory settings, PFCs have also been reported to accumulate in the brain tissues of mammalian species (Austin et al. 2003;Chang et al. 2009;Cui et al. 2009;Liu et al. 2010b;Sato et al. 2009). PFOS and PFOA concentrations were detected in the brains of rodents following oral administration (Cui et al. 2009). Similarly, adult female rats injected intraperitoneally with PFOS for 2 weeks accumulated PFOS concentrations in the brain (Austin et al. 2003). Another study determined a correlation between the daily doses administered to pregnant rats and PFOS levels in the brains of their offspring. At gestation day (GD) 20, PFOS concentrations in the fetal brain were 10 times higher than in the maternal brain with levels decreasing as pups got older (Chang et al. 2009). The blood brain barrier is not well developed at GD 20 which could explain why PFOS entered the brain more readily.

1.4.2. PFC effects on neurodevelopment

There are concerns that PFCs may be environmental neurotoxins. Recent studies have investigated the potential for PFCs to disturb the endocrine system, which is

regulated by the brain. It is suggested that the brain may be a possible route by which PFCs affect central nervous system and endocrine functions (Austin et al. 2003).

1.4.2.1. Cell lines

Perfluorodecanoic acid (PFDA) and PFOS stimulated cellular proliferation in a human glioblastoma (brain tumor) cell line, T98G cells, at 500 and 1000 nM, respectively (Merritt and Foran 2007). Another study demonstrated a wide range of effects of PFCs on differentiating and undifferentiating PC12 cells, a neuronotypic line used to characterize neurotoxicity (Slotkin et al. 2008). Each PFC displayed different outcomes regarding neural cell differentiation (i.e. different shifts in neurotransmitter phenotypes). For example, PFOS promoted the differentiation of the acetylcholine neurotransmitter phenotype at the expense of the dopamine phenotype, PFBS suppressed differentiation of both, and PFOA had no effect (Slotkin et al. 2008). These findings reveal that individual PFCs exhibit different mechanisms of action.

1.4.2.2. Avian species

The reduction in hatching success of chicken embryos following PFC exposure (Molina et al. 2006;O'Brien et al. 2009;Pinkas et al. 2009;Yanai et al. 2008) may suggest interference with brain development since hatchability is influenced by motor function. Accompanying physical deformities such as splayed legs may also reflect an interference with calcium metabolism (Yanai et al. 2008). Furthermore, increased brain asymmetry was observed in chicks following *in ovo* exposure to PFOS which may be related to altered learning or behavioural parameters (Peden-Adams et al. 2008). *In ovo* exposure to PFOS and PFOA was reported to interfere with imprinting behaviour by altering cytosolic PKC concentrations involved in transducing cholinergic input involved in learning and memory (Pinkas et al. 2009).

1.4.2.3. Mammalian species

1.4.2.3.1 Central nervous system (neuroendocrine effects)

PFOS and PFOA have been reported to influence the neuroendocrine system by altering several parameters in the central nervous system (Asakawa et al. 2007;Austin et al. 2003). Recently, Wang et al. (2010) conducted a microarray study to evaluate the transcriptional effects of prenatal and neonatal PFOS exposure in the developing rat brain. Prenatal exposure was determined to be more effective at altering gene expression. PFOS-treated pups had reduced TTR expression at postnatal day (PND) 1, which was further reduced with age. Furthermore, genes involved in neuroactive-ligand receptor interaction, calcium signaling pathways, cell communication, long-term potentiation/depression, cell cycle, and peroxisome proliferation-activated receptor (PPAR) signaling were significantly altered by PFOS treatment (Wang et al. 2010).

Other studies have reported that PFOS altered estrous cyclicity, serum corticosterone and leptin levels, and norepinephrine concentrations in the hypothalamus in adult female rats following a 2-week exposure (Austin et al. 2003). Asakawa et al. (2007) demonstrated that exposure to PFOS and PFOA influenced feeding behaviour in rodents by suppressing gastroduodenal motor activity via the activation of genes in the hypothalamus. These studies provide evidence that PFC exposure may interfere with the neuroendocrine system by altering the reproductive and stress axis and interfere with food intake in mammals.

1.4.2.3.2. Motor and behavioural modifications

Exposures to PFOS and PFOA have been shown to influence behaviour and motor activity in rodents. Gestational and lactational exposure to PFOS (1 mg/kg) in rats resulted in male offspring with increased motor activity and reduced habituation at PND 17 (Butenhoff et al. 2008). Another study demonstrated slight effects in the open-field test and water maze task, where PFOS-treated mice showed signs of anxiety and reduced

motor activity (Fuentes et al. 2007). Slight effects included spending less time in the center in an open-field test (sign of anxiolytic-like effects) and a reduced rate of vertical activity in the water maze task (Fuentes et al. 2007). Johansson et al. (2008) also reported neurobehavioural defects in adult mice administered PFOS and PFOA. Mice neonatally exposed to PFOS and PFOA displayed deranged spontaneous behaviour (reduced/ lack of habituation and increased hyperactivity) which worsened with age (Johansson et al. 2008). A follow-up study determined that PFOS and PFOA altered the levels of proteins involved in neuronal growth and synaptogenesis in the brain tissues of neonatal mice (Johansson et al. 2009). They induced the levels of Ca^{2+}/CaM-dependent protein kinase II (CAMKII), growth associated protein-43 (GAP-43), synaptophysin, and tau- proteins important for normal brain development.

1.4.2.3.3. Signaling pathways

PFOS has been reported to interfere with signaling pathways in the mammalian brain. The Ca^{2+} signaling pathways are involved in activating many biochemical reactions and regulating a variety of neuronal processes (Liu et al. 2010b;Liu et al. 2010a). PFOS induced the expression of Ca^{2+}- dependent molecules that are involved in calcium signaling (e.g. CAMKIIα, cAMP-response element binding protein (pCREB), c-fos, and c-jun) (Liu et al. 2010b). Similarly, studies examining the effects of gestational and lactation exposure to PFOS determined that perinatal exposures during critical periods of development can alter the expression of calcium-dependent signaling molecules (Liu et al. 2010a). Through the enhancement of Ca^{2+} channels, PFOS exhibited both acute excitotoxic effects on synaptic function and chronically inhibited synaptogenesis in the brain (Liao et al. 2008). Furthermore, PFOS treatment increased Ca^{2+} in neurons and inhibited neurite growth (Harada et al. 2006;Liao et al. 2008).

1.4.2.4. Other organisms

PFOS decreases behavioural performance and fitness in freshwater larvae exposed for 1-4 months (Van Gossum et al. 2008). Larvae were less active, less capable

to escape a simulated predator attack and less efficient at foraging. Increased PFOS exposure induced apoptosis in the brain, eye, and tail region of zebrafish embryos. PFOS-treated zebrafish embryos had higher swimming rates after 4 days of exposure compared to the control (Huang et al. 2010). PFOS exposure also altered spontaneous movement and the expression of genes involved in the HPT axis in zebrafish larvae embryos (Shi et al. 2009).

1.5. Thesis overview

1.5.1. Rationale

Studies investigating the toxicological effects of PFCs are necessary in order to fully elucidate the mechanism(s) of action and severity of the effects of PFCs in humans and wildlife. PFC exposure has been associated with many detrimental effects in mammalian species including, higher incidences of mortality, reduced body weight, and increased liver weights (Lau et al. 2007). PFCs display hepatotoxic effects in various species, however less is known about their effects in the brain. Studies have also been very limited in assessing the effects of PFCs in avian species. Given the complete phase out of PFOS and PFOA, it is important to assess the effects of the new and emerging short- and long- chained PFCs. This study focused specifically on the effects of PFCs on the expression of several genes involved in the TH pathways *in vitro*.

1.5.2. Hypotheses and objectives

My research is the first to assess the effects of PFCs on cultured avian neuronal cells. The hypotheses pertaining to the objectives of this study were:

1) PFC exposure will alter the transcription of several TH-responsive genes.
2) PFC exposure will result in similar transcriptional responses in chicken and herring gull neuronal cells.
3) Long-chained PFCs will be more transcriptionally active than short-chained PFCs.

The main objectives of this work were:

1) To assess the effects of PFCs on mRNA expression of TH- responsive genes in avian neuronal cells using a validated *in vitro* screening technique.
2) To compare the effects of PFCs in two avian species; a laboratory model, the chicken, and a wild avian species, the herring gull.
3) To examine the effects of several new and emerging short- and long- chained PFCs (<8 carbons and >8 carbons, respectively).

Chapter 2

Effects of perfluoroalkyl compounds (PFCs) on the mRNA expression levels of thyroid hormone-responsive genes in primary cultures of chicken neuronal cells

2.1. Introduction

Following the global detection of perfluoroalkyl compounds (PFCs) in wildlife species, researchers have assessed the potential toxic, physiological and molecular effects of these contaminants by conducting laboratory studies. As a result, the effects of PFCs are well characterized in mammals; however, more research is required to fully characterize the impact of these contaminants on avian species.

As mentioned previously, PFCs have been detected in a wide variety of wild avian species; examples include bald eagles, albatrosses, cormorants, and herring gulls, which highlights the importance of studying the effects of these compounds in birds (Gebbink et al. 2009;Houde et al. 2006;Verreault et al. 2007). An advantage to studying birds is that they are well positioned from an evolutionary standpoint; they provide an intermediate perspective between mammals (e.g. humans) and lower vertebrates (e.g. fish). Another advantage is that they can be studied in both the laboratory and the natural environment. The domestic chicken (*Gallus domesticus*) has historically been used as a model organism to study embryonic development. It is easy to examine the progression of embryonic development since eggs can be opened at different stages. Another advantage is that chicken eggs are relatively inexpensive and are available in virtually unlimited supply year round. The chicken also serves as a useful avian model because its genome is fully sequenced (Jensen and Leffers 2008), which makes associating structure and function much easier than other avian species.

Egg injection techniques provide a means to assess the toxic effects of contaminants on embryonic development in birds. Previous chicken egg injection studies have examined developmental toxicity and various biochemical responses following PFC

exposure. A perfluorooctanoic acid (PFOA) egg injection study revealed reduced hatching and higher incidences of physical deformities (e.g. splayed legs and abnormal pigmentation) in the PFOA-treated groups (Yanai et al. 2008). Similarly, a perfluorooctane sulfonate (PFOS) chicken egg injection study reported reduced hatchability at all treatment doses (0.1, 1, 10, or 20 μg PFOS/g egg) (Molina et al. 2006). O'Brien et al. (2009) observed a dose-dependent decrease in embryo pippability following exposure to PFOS. The highest PFOS treatment (100 μg/g) significantly lowered pippability from 89% in the DMSO vehicle group to 44%. Another *in ovo* study suggested that PFOS and PFOA interfered with posthatch cognitive behaviour by altering cytosolic protein kinase C (PKC) concentrations, which are involved in controlling imprinting behaviour (Pinkas et al. 2009).

Some of the results from *in ovo* studies highlight a need to further study the effects of PFCs in the avian brain. Reduced hatching and pippability may be consequences of poor motor skills associated with altered brain development (Molina et al. 2006;O'Brien et al. 2009;Yanai et al. 2008). Immune alterations, brain asymmetry, and cognitive behavioural changes have been observed following *in ovo* PFC treatment (Peden-Adams et al. 2008;Pinkas et al. 2009). Furthermore, studies with mammalian models provide evidence that PFCs alter thyroid hormone (TH) levels and interfere with brain development (Johansson et al. 2008;Johansson et al. 2009;Lau et al. 2003;Thibodeaux et al. 2003;Yu et al. 2009).

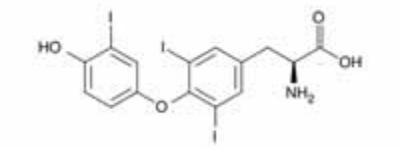
Although *in ovo* studies are important in assessing various measures of toxicity, the molecular mechanisms of PFCs in avian species have yet to be elucidated. The lack of avian studies highlights the importance of conducting preliminary *in vitro* studies. *In vitro* techniques provide a relatively quick screen of effects while utilizing fewer animals. To date, the only *in vitro* studies that have assessed the effects of PFCs in avian species were conducted using chicken embryonic hepatocyte (CEH) cultures (Cwinn et al. 2008; Hickey et al. 2009).

The research presented in this Chapter is the first to investigate the impact of PFCs on cultured avian neuronal cells. The main objective of this study was to utilize an *in vitro* screening method to determine the effects of PFC exposure on the mRNA expression of TH-responsive genes in primary cultures of chicken embryonic neuronal (CEN) cells. Several short- and long-chained PFCs (<8 carbons and ≥8 carbons, respectively) were assessed to determine if differences in transcriptional responses were related to PFC chain length.

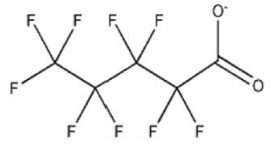
2.2. Materials and methods

2.2.1. Chemicals

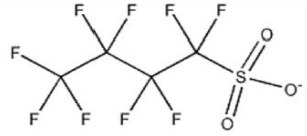
All PFCs were obtained from Wellington Laboratories (Guelph, ON, Canada). The PFCs were: perfluorobutanoic acid (PFBA) (Lot: PFBAGA06; molecular weight (MW): 236.02 g/mol), perfluorobutanesulfonate (PFBS) (Lot: 24318BB; MW: 338.19g/mol), perfluorohexanoic acid (PFHxA) (Lot: PFHxAGA06; MW: 336.04g/mol), perfluorohexanesulfonate (PFHxS) (Lot: LPFHxSAM05; MW: 422.10g/mol), perfluoroheptanoic acid (PFHpA) (Lot: NaPFHpARM07; MW: 386.04g/mol), perfluoroheptanesulfonate (PFHpS) (Lot: PFHpSM06; MW: 472.10g/mol), perfluorooctanoic acid (PFOA) (Lot: PFOAGA05; MW: 414.07g/mol), perfluorooctanesulfonate (PFOS) (Lot: TPFOS0907; MW: 538.22g/mol), perfluorononanoic acid (PFNA) (Lot: 01479; MW: 464.08g/mol), perfluoroundecanoic acid (PFuDA) (Lot: PFuDARM07; MW: 564.09g/mol), and perfluorododecanoic acid (PFDoA) (Lot: PFDoANR07; MW: 614.10g/mol) (Figure 2.1). Triiodothyronine (T3) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO).



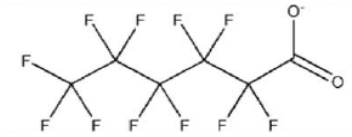
Triiodothyronine (T3) - Positive control



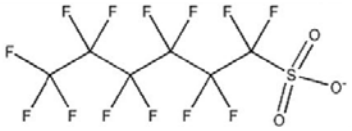
Perfluorobutanoicacid (PFBA)
($C_4F_9CO^-$)



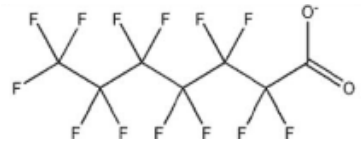
Perfluorobutanesulfonate (PFBS)
($C_4F_9SO_3^-$)



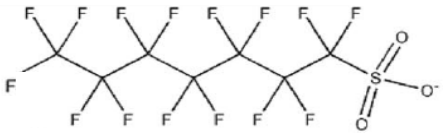
Perfluorohexanoic acid (PFHxA)
($C_5F_{11}COO^-$)



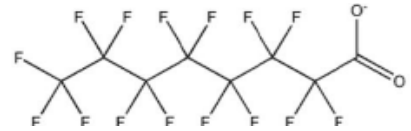
Perfluorohexanesulfonate (PFHxS)
($C_6F_{13}SO_3^-$)



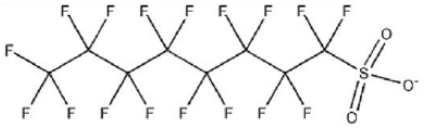
Perfluoroheptanoic acid (PFHpA)
($C_6F_{13}COO^-$)



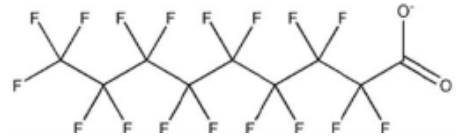
Perfluoroheptanesulfonate (PFHpS)
($C_7F_{15}SO_3^-$)



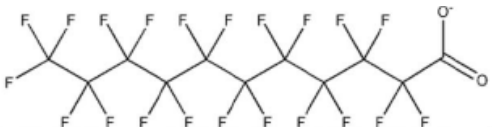
Perfluorooctanoic acid (PFOA)
($C_7F_{15}COO^-$)



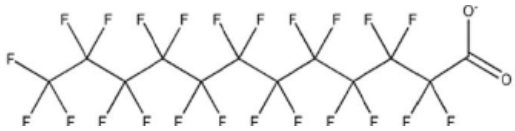
Perfluorooctanesulfonate (PFOS)
($C_8F_{17}SO_3^-$)



Perfluorononanoic acid (PFNA)
($C_8F_{17}COO^-$)



Perfluoroundecanoic acid (PFuDA)
($C_{10}F_{21}COO^-$)



Perfluorododecanoic acid (PFDoA)
($C_{11}F_{23}COO^-$)

Figure 2.1. Molecular structure of the compounds used to treat CEN cells in this study (adapted from Conder et al., 2007)

2.2.2. Source of eggs and incubation conditions

Fertilized, unincubated white leghorn chicken (*G. gallus domesticus)* eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, Canada). Eggs were incubated at 37°C and 60% relative humidity in Curfew Model RX250 incubators (Althorne, Essex, UK) for 11 days. This corresponds to mid-incubation for chickens as the time to hatch is 20-21 days (Hamilton and Hamburger, 1951). Twenty-four viable embryos were euthanized at mid-incubation by decapitation. All procedures were carried out using methods approved by the Animal Care Committee of the National Wildlife Research Centre.

2.2.3. Preparation and dosing of cultured neuronal cells

Primary cell cultures were prepared from the cerebral cortices of day 11 chicken embryos. The goal of this study was to culture cells comprising predominantly neuronal cells. Based on previous immunocytochemistry studies, it was determined that there was an abundance of neuronal cells at the mid-incubation stage of embryonic development (day 11) (Crump et al. 2008a). It was important to culture neurons before the *in vitro* system was completely taken over by the growth of other cell types (e.g. glial cells). Briefly, cerebral cortices were dissected from chicken embryos and pooled in a Petri dish containing ~40mL of fresh neuronal isolation medium (2.5mM HEPES, 128.5mM NaCl, 5.4mM KCl, 5.5mM D-glucose, 51.8mM Sucrose, and 0.1% BSA) and minced into small pieces using a scalpel. The mixture was incubated at room temperature for ~120 minutes and further dissociated by pipetting up and down against the glass bottom. The dissociated cells were vacuum-filtered through a series of nylon sieves of decreasing pore sizes (~200, 120, 45, and 22μm). The filtrate was centrifuged for 13 minutes at 60 x g at 14°C. The resulting pellet was resuspended in isolation medium and vacuum-filtered a second time. After another centrifugation, the pellet was resuspended in Neurobasal medium containing 0.5mM L-glutamine, 25μM glutamate and 2% B27 supplement (Invitrogen, Burlington, ON, Canada). Cells were plated at a density of 3.5mg cells/ml in 48-well plates that were pre-coated with 0.0033 mg/mL poly-D-lysine (Sigma-Aldrich).

Neuronal cultures were incubated at 37°C and 5% CO_2 for 24 hours prior to chemical administration to ensure cells equilibrated to their environment (the medium). Each PFC compound was administered at 6 concentrations: 0.01μM, 0.1μM, 1μM, 3μM, 10μM, and 50μM. A DMSO vehicle and an untreated (UT) control were also included. T3 was included as a positive control to determine how the TH-responsive genes would respond to their natural agonist. T3 was administered at 4 concentrations: 0.03nM, 0.3nM, 3nM, and 30nM. Similarly, a DMSO vehicle group was included for T3-treated CEN cells. The volume of PFC and T3 added to each well was 1.75μL/well and 2.5μL/well, respectively. All reported concentrations were final, in-well concentrations. Four replicate wells were included for each treatment group. Exposure to each compound was repeated on two separate plates – one plate was used for RNA isolation and the other plate was used to assess cell viability. Therefore, CEN cells used for cell viability determination were not used for RNA isolation. Following chemical administration, cells were incubated for another 24 hours at 37°C and 5% CO_2. After 24 hours, the medium was aspirated from all wells. Cell viability was assessed immediately after the 24 hour incubation whereas plates that were designated for gene expression analysis were immediately frozen at -80°C for subsequent RNA isolation.

2.2.4. Cell viability

Cell viability was determined for several perfluoroalkyl sulfonates (PFSAs) and perfluorocarboxylates (PFCAs). Each treatment group was assayed using three technical replicates (3 wells/ treatment group) and was compared to a DMSO vehicle, untreated (UT) control, and 100% ethanol (negative control). Cell viability was conducted using the Calcein- AM assay according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Calcein-AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. Upon its entry into the cell, it is hydrolyzed by intracellular esterases to produce calcein, a negatively charged, hydrophilic and strongly fluorescent compound that is well-retained in the cell cytoplasm (refer to Biotium protocol 30026). The Calcein-AM assay was performed 24 hours after dosing. A working solution was prepared by adding 3μL of Calcein-AM to 10mL of PBS-EDTA. The medium in each

well was aspirated and 200μL of the Calcein-AM solution was added to each well and left to incubate for 45 minutes. Fluorescence was measured by a fluorescence plate reader (Millipore, Billerica, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The fluorescence signal generated from the cell viability assay was proportional to the number of living cells. Statistically significant differences between experimental and DMSO vehicle control groups were determined using a one-way ANOVA followed by Bonferroni's t-test for multiple comparisons versus control. Changes were considered statistically different if $p<0.05$.

2.2.5. RNA isolation and complementary DNA synthesis

Total RNA was extracted using Qiagen RNeasy 96 kits (Qiagen, Mississauga, ON, Canada) as described by the manufacturer. The only modification was that the on-column DNase treatment was not performed in this experiment. Following the extraction of total RNA, a DNase treatment was performed using the Ambion DNA-free kit (Ambion, Austin, TX) and the DNase-treated RNA was quantified using the Nanodrop 2000 spectrometer (Thermo Scientific).

Complementary DNA (cDNA) was prepared using random hexamers and Superscript II reverse transcriptase (RT) (Invitrogen) according to the manufacturer's instructions. Reactions without reverse transcriptase (no-RT control) were included to verify the absence of genomic DNA. Aliquots of cDNA were prepared by diluting stocks 1:5 in diethyl pyrocarbonate-treated H_2O for real-time RT-PCR.

2.2.6. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

All real-time RT-PCR methods were performed using the Strategene Mx3000P or Mx3005P instruments (Strategene, La Jolla, CA). β-actin was used as a normalizer gene because its expression was found to be invariable in all treatment groups for which data are presented. The mRNA levels were quantified using TaqMan probes and Brilliant Quantitative-PCR Core Reagent kits (Stratagene). Taqman reactions (25μL) contained

1X Core RT-PCR buffer, 5mM $MgCl_2$, 0.8mM dNTPs, 0.08% vol/vol glycerol, 60nM ROX reference dye, 0.05U Surestart Taq DNA polymerase, forward and reverse primers and probes for β-actin and target genes (see Table 2.1 for final concentrations) and 5μL of 1:5 diluted cDNA. The thermal profile for all reactions was: 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, and 1 minute at 60°C.

Standard curves were generated for all candidate genes from a 1:2 dilution series of cDNA. Primer concentrations were optimized so that PCR reaction efficiencies for all target genes were within ± 10% the amplification efficiency of β-actin. No-RT and no-template controls (NTCs) were included in all assays to verify the absence of contaminating genomic DNA. Each treatment group consisted of four technical replicates (4 wells/ treatment group) and each sample was analyzed in duplicate by real-time RT-PCR.

2.2.7. Statistical analysis

Real-time RT-PCR data were analyzed using MxPro v3.00 software (Strategene). Relative quantity values were normalized to the internal control gene, β-actin, and fold changes were calculated relative to the DMSO-treated cells using the $2^{-\Delta\Delta Ct}$ equation (Livak and Schmittgen 2001). Statistically significant differences between experimental and DMSO vehicle control groups were determined using a one-way ANOVA followed by Bonferroni's t-test for multiple comparisons versus control. Changes were considered statistically different if $p<0.05$.

Table 2.1. – Genes that were studied using real-time RT-PCR analysis in CEN cells, including information on the nucleotide sequences of the primers and probes, final concentrations and accession numbers

Gene	Sequence (5'-3')	Final concentrations (nM)	Accession Number
β-actin	F: AAATTGTGCGTGACATCAAGGA R: GAGGCAGCTGTGGCCATCT P: TGCTACGTCGCACTGGATTTCGAGC	50 50 200	NM_205518.1
Thyroid receptor alpha (TR-α)	F: ATCTGCGTGGATAAGATAGAG R: GAATGTTGTGTTTGCGGTAGTTG P: GGAGACGTACCTGCTGGCGTTCGA	900 300 200	NM_205313.1
Thyroid receptor beta (TR-β)	F: TGAAAGTGACAGATCTGCGAATG R: AGGAACAATGGAGGGAAGAGTTCT P: CTGCCATGCCAGCCGCTTCCTGC	900 300 200	NM_205447.1
Type II iodothyronine 5' -deiodinase (D2)	F: GTGTTGCAGCACCTGGTAG R: TGTTTCTCGGCTATTTAAGCACTG P: CAGACCTCCCGCTCCCGTGTCAGT	900 300 200	NM_204114.2
Type III iodothyronine 5' -deiodinase (D3)	F: CCTCCTCTTTCCCCGCTTCC R: GTGGGCATCGTCAGCATCTTC P: CCGCTGTGATGCTCTGGCTCCTGG	900 300 200	NM_001122648.1
Transthyretin (TTR)	F: TTCTTGTTTTCTTAGCTGGACTGG R: CATGAGAGGGCATTTGGAATCAAC P: CCGAAGCTGCACCACTGGTCTCCC	900 900 200	NM_205335.2
Neurogranin (RC3)	F: ACGAAGCTGGATGAGGACATC R: GAAGCTGGCCTGGATCTTGG P: CCCCTGGACGACCCCGACGCC	900 900 200	XM_001232109.1
Octamer motif binding factor 1 (Oct-1)	F: CTGCTGGTGCCACCATCTCG R: AGGTTGAGATTCTGCTGCTGAAG P: CCTCTGCTGCAACGCCGATGACGC	900 900 200	NM_205472.1
Myelin basic protein (MBP)	F: CGGGGCTTTGGCAAGGATATAC R: GAAGAAGTGGACTACAGGGTTGTC P: CGCAGCCAGGGCCAGCCATGTAG	900 900 200	NM_205280.1

Note: F; forward primers, R; reverse primers, and P; probe.

2.3. Results

2.3.1. Cell viability

There were no significant changes in cell viability in T3-treated CEN cells up to the maximum concentration of 30nM (Figure 2.2). Ethanol-killed cells (negative control), as expected, exhibited a dramatic decrease in viability. Cell viability was determined for all PFCs with the results shown in Figure 2.3. Among all of the PFCs studied, a significant decrease in cell viability occurred following treatment with PFHxA at 30µM and 50µM. There were no significant changes in cell viability following treatment with the other PFCs examined. Only those treatment groups that had no significant effect on cell viability were assessed for mRNA expression. Consequently, 10µM was the highest PFC concentration used for subsequent mRNA expression analysis.

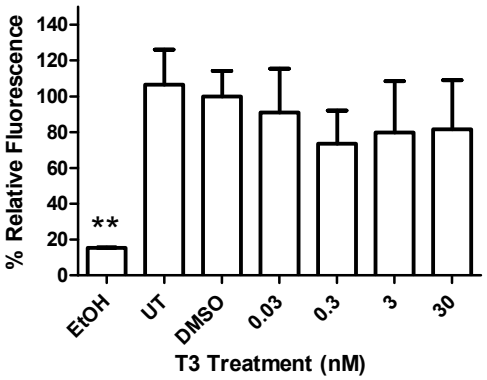


Figure 2.2. Calcein-Am cell viability assessment of CEN cells following T3 treatment. The percentage (%) fluorescence was calculated relative to the DMSO treatment. Standard deviation was calculated based on three replicate wells per treatment (** $p<0.001$). EtOH; 100% ethanol, UT; untreated.

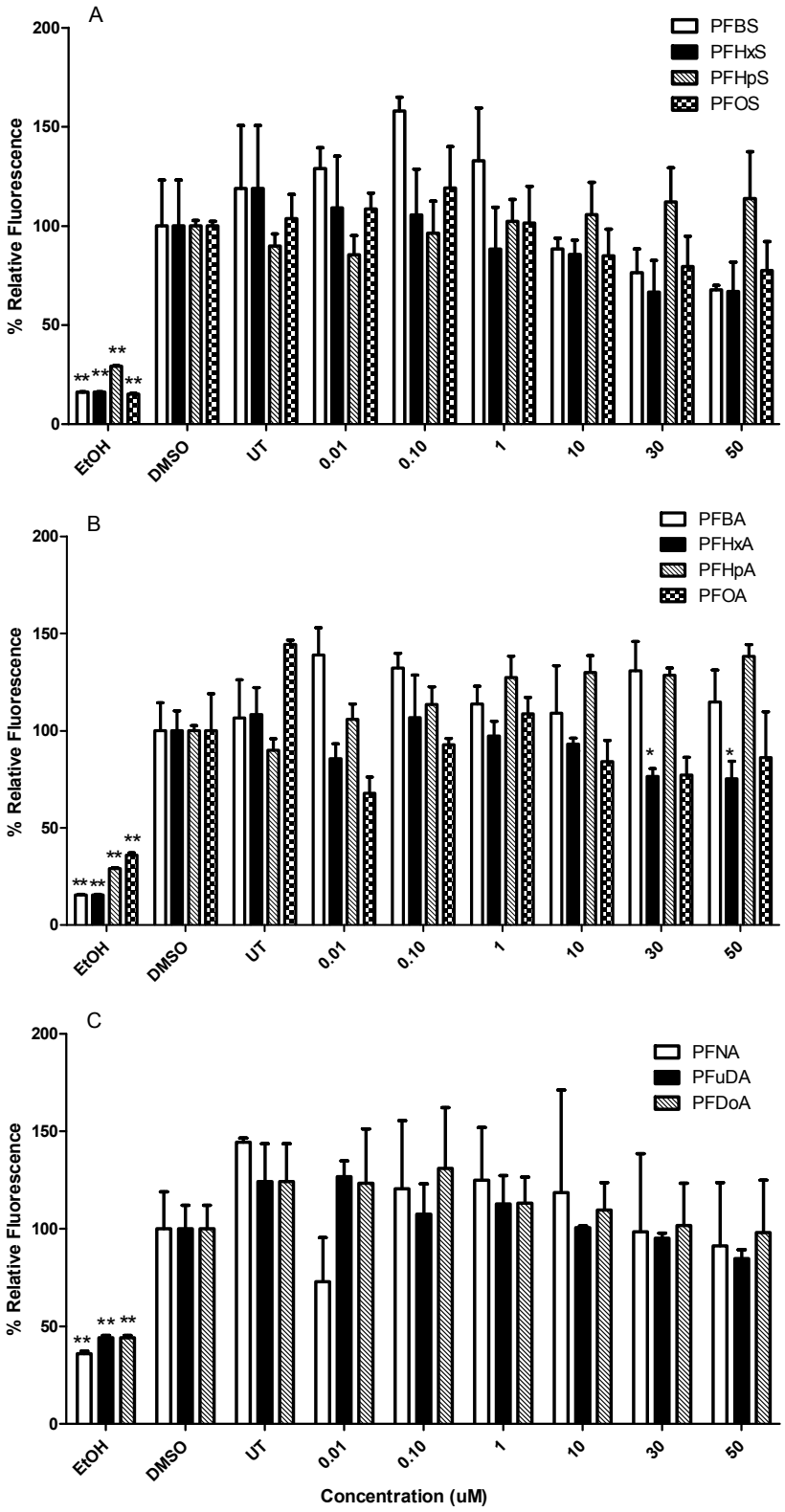


Figure 2.3. Calcein-Am cell viability assessment of CEN cells following treatment with several PFSAs (A), short- chained PFCAs (B) and long-chained PFCAs (C). The percentage (%) fluorescence was calculated relative to the DMSO treatment. Standard deviation was calculated based on three replicate wells per treatment (** $p<0.001$). EtOH; 100% ethanol, UT; untreated.

2.3.2. mRNA expression

2.3.2.1. T3 – positive control

Following T3 exposure, there was an up-regulation of thyroid hormone receptor-alpha and beta (TR-α and TR-β, respectively) expression (Figure 2.4), which indicated that primary cultures of chicken neuronal cells responded to treatment with their natural ligand.

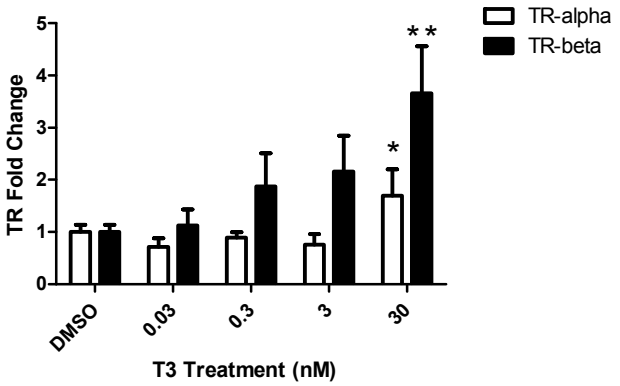


Figure 2.4. The relative expression of TR-α and TR-β mRNA in CEN cells following T3 treatment. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 3-4 wells/treatment; * $p<0.05$ and ** $p<0.001$ comparing DMSO- and T3-treated groups)

T3 treatment significantly increased D2 mRNA levels and decreased TTR expression in CEN cells (Figure 2.5A,B). RC3 expression also dramatically increased to approximately 14- and 30-fold following T3 administration (3nM and 30nM, respectively) (Figure 2.5C). There were no significant changes in the other TH-responsive genes examined (D3, Oct-1, and MBP; results not shown).

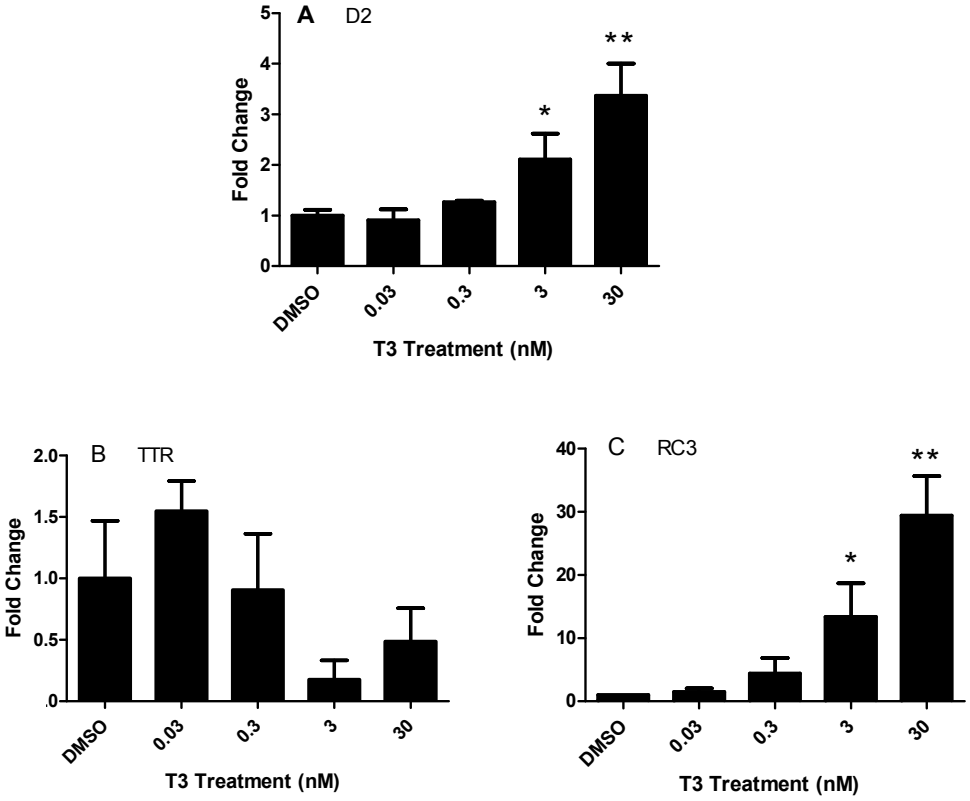


Figure 2.5. The relative expression of D2 (A), TTR (B), and RC3 (C) mRNA in CEN cells following T3 treatment. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 2-4 wells/treatment; * $p<0.05$ and **$p<0.001$ comparing DMSO- and T3-treated groups)

2.3.2.2. PFCs

The fold changes for the TH-responsive genes are summarized in Table 2.2. Only PFCs that caused significant concentration-dependent effects on the TH-responsive genes are presented in more detail in Figure 2.6.

Table 2.2. Mean fold change ± standard deviation (SD) of target gene mRNA expression following exposure of CEN cells to 11 PFCs. (SD was calculated from 3-4 replicate wells on a single 48-well plate)

	TH availability				TH transport		Signal transduction		Transcription factor activity		Myelination	
	D2		D3		TTR		RC3		Oct1		MBP	
PFC	3μM	10μM	3μM	10μM	3μM	10μM	3μM	10μM	3μM	10μM	3μM	10μM
PFBA	0.76 ± 0.09	0.76 ± 0.14	0.82 ± 0.11	0.59 ± 0.15	0.76 ± 0.10	0.53 ± 0.50	0.77 ± 0.20	0.59 ± 0.08	0.97 ± 0.04	1.03 ± 0.22	1.11 ± 0.28	0.94 ± 0.03
PFBS	1.03 ± 0.10	1.04 ± 0.11	2.34 ± 0.72	3.10 ± 0.41	0.58 ± 0.67	0.74 ± 0.27	1.35 ± 0.18	2.02 ± 0.27	1.08 ± 0.11	1.10 ± 0.02	0.78 ± 0.08	0.78 ± 0.01
PFHxA	1.08 ± 0.16	2.33 ± 0.53	3.03 ± 0.20	1.38 ± 0.17	1.37 ± 0.62	3.67 ± 4.33	0.99 ± 0.06	1.49 ± 0.18	0.75 ± 0.08	0.84 ± 0.09	2.01 ± 0.75	3.39 ± 0.36
PFHxS	1.71 ± 0.46	1.81 ± 0.16	5.34 ± 0.95	4.23 ± 0.95	0.22 ± 0.09	0.34 ± 0.23	6.58 ± 3.63	10.97 ± 2.24	1.52 ± 0.03	1.42 ± 0.15	0.77 ± 0.21	1.71 ± 0.36
PFHpA	1.02 ± 0.08	3.34 ± 0.08	0.66 ± 0.14	0.58 ± 0.07	0.69 ± 0.27	5.10 ± 1.73	0.51 ± 0.26	0.55 ± 0.10	0.86 ± 0.26	1.03 ± 0.06	0.88 ± 0.22	0.92 ± 0.19
PFHpS	1.39 ± 0.18	1.24 ± 0.21	0.95 ± 0.18	0.65 ± 0.21	0.64 ± 0.19	0.41 ± 0.16	0.57 ± 0.07	0.71 ± 0.17	0.79 ± 0.13	1.01 ± 0.19	1.10 ± 0.10	1.04 ± 0.16
PFOS	0.86 ± 0.11	1.00 ± 0.08	0.95 ± 0.04	0.97 ± 0.24	1.10 ± 0.33	0.66 ± 0.26	1.17 ± 0.22	1.09 ± 0.32	1.02 ± 0.08	0.96 ± 0.12	0.78 ± 0.08	0.91 ± 0.10
PFOA	1.01 ± 0.14	1.35 ± 0.11	1.27 ± 0.10	1.37 ± 0.12	0.76 ± 0.18	0.91 ± 0.06	0.77 ± 0.10	0.91 ± 0.11	0.85 ± 0.16	0.73 ± 0.06	0.88 ± 0.05	0.88 ± 0.12
PFNA	1.81 ± 0.19	3.46 ± 0.40	0.98 ± 0.16	0.88 ± 0.12	0.82 ± 0.33	0.91 ± 0.15	0.69 ± 0.27	1.10 ± 0.26	0.68 ± 0.08	0.80 ± 0.21	0.80 ± 0.33	0.85 ± 0.35
PFuDA	1.00 ± 0.12	1.10 ± 0.26	1.28 ± 0.29	0.71 ± 0.27	1.96 ± 1.21	1.11 ± 0.23	0.89 ± 0.08	0.84 ± 0.20	0.95 ± 0.11	0.94 ± 0.23	0.97 ± 0.15	1.07 ± 0.14
PFDoA	0.90 ± 0.24	1.73 ± 0.36	1.02 ± 0.22	0.77 ± 0.19	1.50 ± 0.10	1.24 ± 0.51	0.81 ± 0.19	1.14 ± 0.11	0.90 ± 0.11	1.18 ± 0.09	0.93 ± 0.11	1.15 ± 0.04

Note: Dark grey values indicate <0.5-fold changes ($p<0.05$), white values indicate 0.51- to 2-fold changes, and light grey values indicate >2-fold changes ($p<0.05$).

TH availability

There was an increase in D2 mRNA levels following exposure to PFHxA, PFHpA, and PFNA (Figure 2.6A and Table 2.2). This increase was similar to the trend observed in T3-treated CEN cells, albeit at 1000-fold lower concentration of exposure (Figure 2.5A). The complete concentration-dependent effects for PFNA were not included in Figure 2.6A due to an insufficient supply of samples. D3 expression increased 2- to 5-fold following exposure to several PFCs (PFBS, PFHxA, and PFHxS) (Figure 2.6B).

TH transport

PFHxS treatment significantly decreased TTR expression by as much as 10-fold at 1μM (Figure 2.6C). The decreasing trend following PFHxS treatment was similar to that observed following T3 treatment. On the other hand, PFHpA exposure increased TTR mRNA levels to 4-fold at 10μM (Table 2.2). The complete concentration-dependent effects for PFHpA were not incorporated in Figure 2.6C because the results for the lower concentrations were too variable to include.

Signal transduction

PFBS and PFHxS treatment increased RC3 expression, which was a similar trend observed in T3-treated cells, although to a lesser extent. There was a slight 2-fold up-regulation of RC3 expression following PFBS treatment at 10μM and a 6- to 11-fold up-regulation following PFHxS exposure (3μM and 10μM, respectively) (Figure 2.6D).

Transcription factor activity

There were no changes to Oct-1 mRNA levels following treatment with any of the PFCs examined which was similar to the response observed in T3-treated CEN cells.

Myelination

MBP was induced at 3µM and 10µM following PFHxA treatment relative to the control (Figure 2.6E). There were no significant changes in MBP expression following T3 exposure or treatment with the other PFCs.

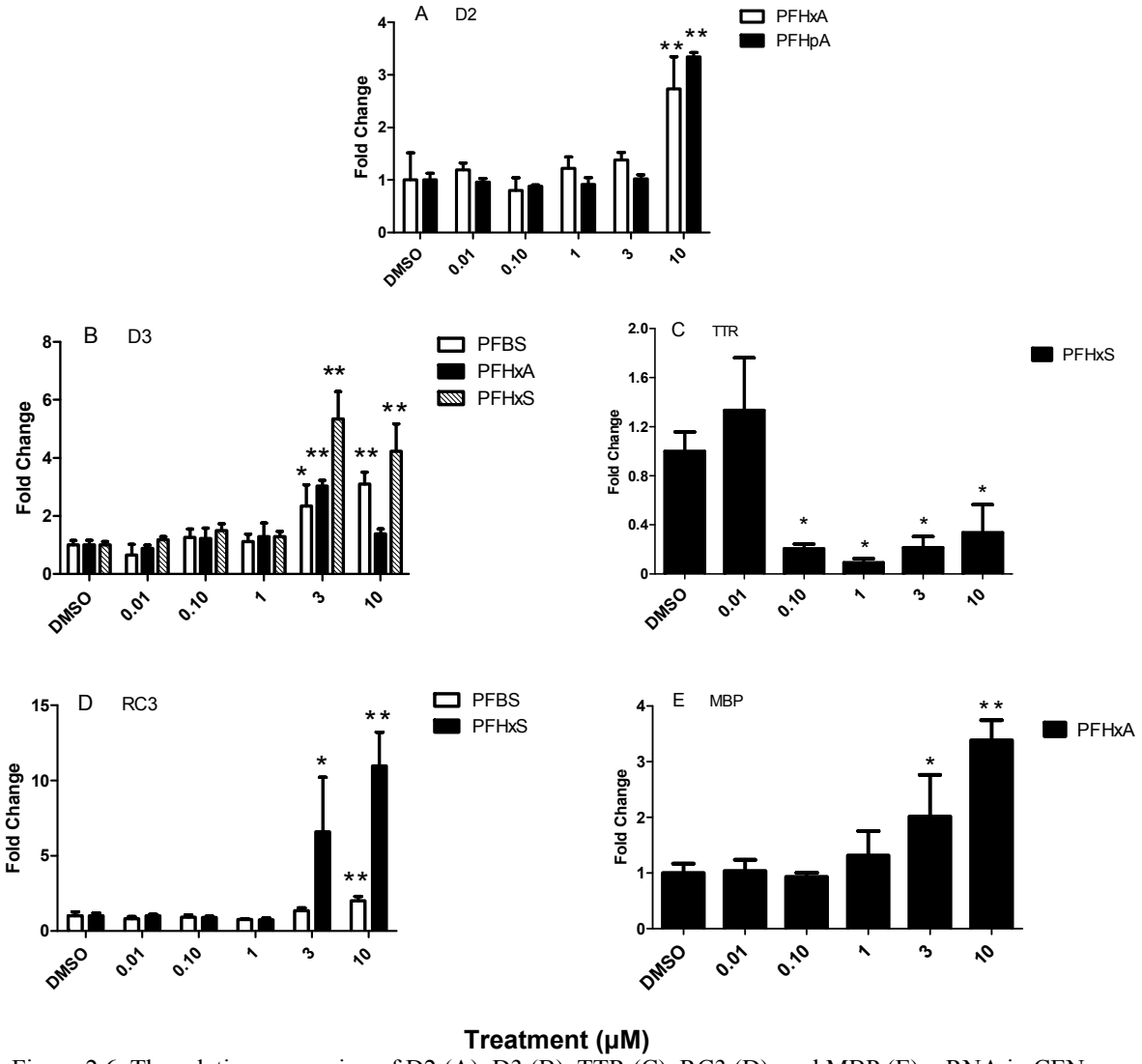


Figure 2.6. The relative expression of D2 (A), D3 (B), TTR (C), RC3 (D), and MBP (E) mRNA in CEN cells following treatment with several PFCs. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 3-4 wells/treatment; * $p<0.05$ and ** $p<0.001$ comparing DMSO- and PFC- treated groups)

PFC trends

Of the 11 PFCs analyzed, five caused significant gene expression changes in the candidate genes that were examined (Table 2.2). The shorter-chained PFCs (< 8 carbons) altered gene expression to a greater extent compared to the longer-chained compounds. Of the short-chained PFCs, PFBS, PFHxA, PFHxS, and PFHpA altered the expression of at least two or more TH-responsive genes in a concentration-dependent manner (Figure 2.6). With the exception of PFNA, there were no significant gene expression changes in the long-chained PFCs.

2.4. Discussion

This study has identified specific molecular endpoints that are responsive to PFCs in CEN cells and may provide some insights into the possible mechanisms of action of PFCs in the avian brain. It was determined that PFCs of different chain lengths elicited different transcriptional responses in CEN cells. Only those treatment groups that had no significant effect on cell viability were assessed for mRNA expression which ensured that transcriptional changes were related to mRNA expression and not cell viability. PFHxA was the only compound that significantly decreased cell viability at concentrations ≥30μM and there was no decreased cell viability at any of the T3 concentrations used. Therefore, the highest PFC and T3 concentrations used for mRNA expression analysis were 10μM and 30nM, respectively.

A positive control was included in this experiment to determine how TH-responsive genes would respond to the natural T3 agonist in CEN cells. Following T3 exposure, there was an up-regulation of TR-α and TR-β expression which indicated that primary cultures of avian neuronal cells responded to treatment with the natural ligand. Although non-nuclear actions of THs exist, the majority of T3 actions are mediated via nuclear TRs (McNabb 2007). Increased TR expression could activate TH-responsive genes since TRs bind with thyroid hormone response elements (TREs) in the promoter regions of target genes to initiate transcription (McNabb 2007).

During chicken development, increased T3 levels correspond with TR induction which reflects a coordinated developmental program (Oppenheimer and Schwartz 1997). At mid-incubation, maximal concentrations of TRs and high T3 levels are detected in the avian brain (Oppenheimer and Schwartz 1997). This was the developmental stage assessed in this study and as mentioned earlier, exogenous T3 administration induced TR expression in CEN cells. Similar to the observations in avian species, these results have also been reported in amphibian experiments. It was determined that T3 was correlated with metamorphic progression in frogs. During premetamorphosis, rising levels of endogenous T3 correspond with increased TR mRNA. The receptor, upon binding to its ligand, T3, can up-regulate its own expression in a phenomenon termed autoregulation. Exogenous T3 administration during premetamorphosis up-regulated TR-α and TR-β mRNA in whole tadpoles up to 2- and 20-fold, respectively (Yaoita and Brown 1990). Similarly, T3 administration induced TR-β mRNA levels up to 5-fold in the tadpole brain (Hogan et al. 2007). Therefore, exposing neuronal cells to the natural ligand in this study provided a good indication of responsiveness. The importance of exposure to the natural ligand was to establish a baseline for TH pathway activation, a reference in which to compare PFCs effects.

It was determined that treatment with PFHxA, PFHpA, PFNA, and T3 induced D2 mRNA levels in CEN cells. D2 converts T4 to its active form, T3, via 5' outer ring deiodination (ORD) (Bianco and Kim 2006). Increased D2 mRNA levels following T3 administration could have been attributed to the developmental time period during which the neuronal cells were harvested. In this study, T3 administration up-regulated D2 mRNA in neurons obtained from day 11 embryos - the typical developmental period at which both TH levels and D2 mRNA levels are high (McNabb 2007). D2 mRNA is expressed in brain tissue of chicken embryos as early as day 8 and increases up to 14-fold from day 10 to day 17 (Gereben et al. 2004). Elevated D2 mRNA levels, despite high TH concentrations during development, may be a mechanism to increases T3 bioavailability in the chick embryo, allowing it to prepare for pipping, hatching, and growth at later stages (McNabb 2007). The similar patterns of D2 induction following T3, PFHxA,

PFHpA, and PFNA treatment may suggest that these compounds share similar modes of action.

Several PFCs altered D3 mRNA levels. D3 catalyzes inner ring 5-deiodination (IRD), which degrades both T4 and T3 to their inactive derivatives; reverse T3 (rT3) and 3,3'-diiodothyronine (T2), respectively (Hernandez 2005). D3 is found primarily in the cerebral cortex (Hernandez 2005) which is the brain region where neuronal cells were harvested in this experiment. Exposure to PFHxA, PFHxS, and PFBS resulted in a 2- to 5-fold up-regulation in D3 expression. Elevated D3 mRNA levels may be due to excess TH (T3 or T4) levels. For example, increased D3 expression may indicate an over abundance of T3 which signals the system to eliminate excess T3 in order to maintain homeostasis. An up-regulation in D3 expression may occur as a means to protect the brain from elevated TH levels (Hernandez 2005).

In the present study, T3 administration did not increase D3 given that an excess of T3 was administered to CEN cells. This could be due to the developmental time period of the neuronal cells harvested in this study. D3 mRNA expression correlates with D3 activity and low D3 activity in the chicken brain is detected at mid-incubation (day 11) which highlight the importance of TH availability during this stage of development (Hernandez 2005;Valverde et al. 1993). Another possibility for the lack of D3 response could be due to the differences in the concentrations that were used; T3 was administered in the nanomolar range whereas PFCs were applied in the micromolar range. The highest concentrations used in the PFC and T3 treatments were 10μM and 30nM, respectively; thus the threshold for D3 induction may not have been reached with the concentrations used in this study. Hogan et al. (2007) previously reported elevated D3 mRNA levels following T3 administration (50nM) in amphibians.

There were considerable transcriptional changes in TTR expression following treatment with several PFCs. PFHxS treatment significantly decreased TTR expression by as much as 10-fold at 1μM. The significant gene expression effects were observed at concentrations as low as 0.1μM which was the lowest effective concentration for any of

the mRNA endpoints that were examined. T3 treatment also decreased TTR mRNA levels. A study previously conducted in our laboratory by Crump et al. (2008b) measured TTR expression following T3 administration in primary cultures of CEN cells and herring gull embryonic neuronal (HGEN) cells. In CEN cells, a 36 hour exposure to T3 (instead of 24 hours, as in the present study) decreased TTR expression (unpublished results). Similarly, TTR expression was decreased by 2- to 5-fold in T3-treated HGEN cells (Crump et al. 2008b).

TTR is a major TH-binding protein in birds that mediates TH transport into target tissues (Chang et al. 1999). TTR, located in extracellular fluids, is synthesized by the choroid plexus and transports T4 into the central nervous system (review Schreiber 2002; review McNabb, 2007). A reduction of TTR expression could result in decreased TH availability to target tissues which could affect TH-dependent processes. TTR transports T4 into target tissues where it is converted into its more active T3 form by deiodination. It would be expected that high T3 levels would decrease TTR expression as this would reduce the need to transfer T4 into target tissues to produce more T3. The down-regulation of TTR expression following T3 and PFHxS exposure could indicate the possibility of shared mechanisms of action between these two compounds. Alternatively, PFHpA exposure increased TTR mRNA levels to 4-fold at 10μM which would suggest increasing TH availability to target tissues.

Significant changes in RC3 expression were observed following treatment with the 4- and 6- carbon PFSAs. RC3 mRNA levels increased up to 2- and 11-fold following PFBS and PFHxS treatment, respectively. Similarly, T3 administration dramatically increased RC3 expression up to approximately 30-fold. Based on mammalian studies, it has been determined that TH alters RC3 expression in several brain regions; primarily the cerebral cortex, striatum, and hippocampus (Iniguez et al. 1993). A study by Guadano-Ferraz et al. (1997) observed an induction in RC3 transcription in several brain regions following a single T3 administration in hypothyroid rats. Exposure to environmental contaminants can also alter RC3 expression. It was reported that treatment with a commercial mixture of polychlorinated biphenyls (PCBs), Aroclor 1254 (A1254),

induced RC3 expression in the cortex of fetuses derived from A1254-treated dams (Gauger et al. 2004). The elevated transcriptional responses of RC3 following exposure to T3, PFCs, and other anthropogenic chemicals make it a useful marker to examine disturbances in TH homeostasis.

RC3 is a CaM-binding protein kinase C (PKC) substrate that is involved in the Ca^{2+} signal transduction system (Gerendasy 1999). Changes in RC3 expression could have functional consequences in synaptic plasticity, associative learning and memory (Iniguez et al. 1993;Iniguez et al. 1996). RC3 expression demonstrated a similar induction following treatment with PFBS and PFHxA compared to T3, although to a lesser extent. Given that the RC3 gene has been reported to contain a functional TRE (Morte et al. 1997), T3 administration may have led to increased TR binding to TREs resulting in increased RC3 expression. It is possible that PFBS and PFHxA may have shared mechanisms of action given the similar patterns in RC3 expression.

This study determined that PFC exposure did not significantly alter Oct-1 expression in CEN cells. Similarly, T3 administration had no effect on Oct-1 expression. Despite a lack of Oct-1 responsiveness to T3 or PFC treatment in this study, Oct-1 has demonstrated responsiveness following exposure to anthropogenic chemicals. Gauger et al. (2004) previously reported induced Oct-1 expression in the fetal rat brain following PCB exposure. Although its promoter region does not contain a TRE, Oct-1 is considered to be a TH-responsive gene for several reasons (refer to Introduction). There have been conflicting results regarding the mechanisms of action of Oct-1. In transient transfection studies, Nagasawa et al. (1997) determined that Oct-1 positively regulated the TR-β1 promoter. However, other transient transfection studies demonstrated that Oct-1 interacts with TR-RXR heterodimers and a silencing mediator for retinoid and TR (SMRT) to repress T3-dependent transcriptional activity (Kakizawa et al. 1999;Kakizawa et al. 2001). This study is the first to examine Oct-1 expression in CEN cells following PFC exposure, however further work is required to examine the role of TH and TRs on the transcriptional activity of Oct-1.

PFHxA treatment induced MBP expression at 3μM and 10μM relative to the control. MBP is essential in the process of nerve myelination which facilitates the conduction of nerve impulses. MBP contains a TRE in its promoter region which binds to TRs (Farsetti et al. 1991). Previous *in vitro* binding studies demonstrated that both TH and TRs are required to positively regulate the expression of MBP (Farsetti et al. 1991). In this study, T3 administration did not alter MBP expression, although previous rodent studies observed MBP induction following T3 treatment (Farsetti et al. 1991). The study by Farsetti et al. (1991) used isolated whole brain nuclei whereas our study specifically examined primary cultures of neuronal cells extracted from avian cerebral cortices. Another explanation for the lack of response in MBP following T3 exposure could be that the threshold concentration for induction may not have been reached with the T3 concentrations used. The inclusion of higher T3 concentrations (e.g. 50nM) may have elicited a change in MBP mRNA.

The results of this study demonstrate that PFCs differ substantially in their effects on mRNA expression in CEN cells. In general, the short-chained PFCs (< 8 carbons) elicited more significant gene expression changes compared to the long-chained PFCs (≥ 8 carbons) (Table 2.3). These results may have been due to differences in the bioavailability of the compounds. It has previously been determined that long-chained PFCs bind more strongly to protein than short-chained PFCs (Jones et al. 2003). Long-chained PFCs may bind to the protein in the media, the walls of the plastic wells, or to non-specific proteins on the outside of CEN cells. Therefore, short-chained PFCs may have entered neuronal cells more easily due to their lower binding affinities to protein.

Table 2.3. Summary of the overall significant gene expression changes following exposure to T3 and several PFCs

Compound		TH availability D2	TH availability D3	TH transport TTR	Signal transduction RC3	Transcription factor activity Oct-1	Myelination MBP
T3	---	↑	---	↓	↑	---	---
PFBS	C_4	---	↑	---	↑	---	---
PFHxA	C_6	↑	↑	---	---	---	↑
PFHxS	C_6	---	↑	↓	↑	---	---
PFHpA	C_7	↑	---	↑	---	---	---
PFNA	C_9	↑	---	---	---	---	---

In the present study, the 6-carbon PFHxA and PFHxS were the most transcriptionally active compounds, altering the expression of three TH-responsive genes. PFBS, PFHxS, and PFHxA may share similar modes of action with T3 given the similar patterns in transcriptional responses. For example, PFHxA treatment induced D2 and D3 expression; genes associated with the production and clearance of T3. Although T3 did not alter MBP expression, PFHxA treatment induced MBP mRNA levels which could provide another link to potential TR-mediated effects. PFHxS increased the mRNA levels of RC3, which could induce synaptic plasticity, and decreased TTR expression, which could reduce the transport of TH to target tissues decreasing TH availability. Lastly, PFBS increased the expression of two TH-responsive genes, D3 and RC3, which was also similar to T3 effects.

There is an abundance of information in the PFC literature on the effects of PFOS and PFOA in mammalian species. Few studies that have assessed the effects of these contaminants on avian species and even less is know about the potential impact of shorter- or longer-chained PFCs. Interestingly, there were no effects in TH-responsive genes following PFOS or PFOA treatment in this study. The short-chained PFCs, on the other hand, demonstrated significant transcriptional responses. Since these compounds are being manufactured as replacement alternatives to PFOS and PFOA, the production of short chained PFCs (< 8 carbons) is expected to rise.

PFC chain length effects have been demonstrated in other studies. Hickey et al. (2009) determined that long-chained PFCs induced the expression of liver fatty acid binding protein (L-FABP) while short-chained PFCs induced cytochrome P450 enzyme expression in CEH cultures. Liao et al. (2009) assessed the effects of PFC chain length in primary cultures of mammalian hippocampal neurons. They determined that the longer the chain length, the greater the adverse effects in cultured neurons (changes in synaptic transmission, calcium current, and inhibition of neurite growth). These results were different from our findings; discrepancies may be related to differences in model organisms (i.e. mammalian vs. avian species), brain regions (i.e. hippocampus vs.

cortex), experimental designs (i.e. chemical concentrations, cell composition, and number of days *in vitro*) and endpoints that were examined. Weiss et al. (2009) also reported PFC chain length effects in competing with T4 for binding to the mammalian TTR. The binding potency in decreasing order was PFHxS>PFOS≈PFOA>PFHpA>PFNA with binding potencies determined to be 12.5-50 times lower than T4 (Weiss et al. 2009) Studies that have assessed PFC chain length effects, in general, have reported that the degree of fluorination, carbon chain length, and functional group are key factors that influence PFC effects (e.g. binding potency, neural functions, etc.) (Liao et al. 2009;Upham et al. 1998;Weiss et al. 2009).

The present study demonstrated significant mRNA expression fold changes in the avian brain. The brain is a tightly regulated organ in which large fold change are typically rare, however, significant gene expression changes of up to 12-fold were demonstrated in PFC-treated neuronal cells. In addition, significant changes were observed at relatively low concentrations compared to previous cell culture studies treated with PFCs in our laboratory (Cwinn et al. 2008;Hickey et al. 2009). For example, it was determined that PFHxS treatment altered TTR expression at concentrations as low as 0.1μM; approximately two orders of magnitude greater than actual concentrations detected in the environment. These calculations of *in vitro* concentrations were based on a maximum cellular uptake rate of 100%. However, a study by Mundy et al. (2004) determined that only 15% of the applied 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) was associated with primary cultures of rat neocortical cells. The results of that study highlight the fact that chemicals administered to neuronal cells are not entirely taken up by the cells. It should also be noted that the environmental concentrations of short-chained PFCs (e.g. PFHxS) are expected to rise due to increased production. PFHxS has been measured in house dust samples, surface water, blood plasma of fish, and more recently, in herring gull eggs collected from the Great Lakes region (Gebbink et al. 2009;Kubwabo et al. 2005;Sinclair et al. 2006;Taniyasu et al. 2003). Because of the unknown cellular uptake rate and the increased production of short-chained PFCs, it is suggested that the concentrations used in this study are comparable to environmental concentrations.

Of the candidate genes that were examined in this study, D2, TTR and RC3 were identified as potential molecular markers for TH disturbances as they demonstrated significant gene expression changes in T3-treated CEN cells. These TH-responsive genes are valuable for their use in the *in vitro* screening method to examine the impact of PFCs in primary cultures of CEN cells. This study has contributed to our understanding of PFC effects on the TH pathway and has provided evidence that the brain may be a target organ for PFC effects in birds.

Chapter 3

Effects of perfluoroalkyl compounds (PFCs) on the mRNA expression of thyroid hormone-responsive genes in primary cultures of herring gull neuronal cells

3.1. Introduction

PFCs have been detected in seabirds, waterfowl, and terrestrial birds worldwide. These contaminants have been identified in plasma, tissues and eggs of wild avian populations including cormorants, guillemots and gulls (Bossi et al. 2005;Gebbink et al. 2009;Holmstrom and Berger 2008;Kannan et al. 2002;Verreault et al. 2005). Temporal trends have shown increases in PFC concentrations in wildlife species. For example, perfluorooctane sulfonate (PFOS), the predominant PFC measured in the environment, increased 2-fold in herring gull eggs in Northern Norway from 1983 to 2003 (21ng/g wet weight (ww) to 42ng/g ww, respectively) (Verreault et al. 2007). Several PFCs have also been detected at high concentrations in herring gull eggs from colonies spanning the Great Lakes of North America (∑PFCs: concentrations up to 600 ± 56 ng/g ww) (Gebbink et al. 2009).

Historically, hundreds of man-made chemicals have been detected in the Great Lakes of North America, and some of these chemicals have contributed to the decline of certain avian populations (Gilbertson et al. 1991). Consequently, the Great Lakes Monitoring Program was implemented to monitor the levels of toxic chemicals in this important ecosystem. It is important to monitor the changes in contaminant levels affecting wildlife and biota over time and to develop approaches and tools to determine the biological effects of exposure to these contaminants. Since the 1970s, the herring gull (*Larus argentatus*) has been used as an indicator species for environmental contamination in the Great Lakes in an attempt to assess the health status of the Great Lakes (Mineau, 1984; Hebert et al. 1999).

In addition to assessing the effects of PFCs in a model avian species (e.g., the work described in Chapter 2), priority should also be made to examine their impact on wild bird species. There are many advantages to using the herring gull as an indicator for environmental contamination. Herring gulls are top predators and permanent residents of the Great Lakes and display little movement during breeding season (Hebert et al., 1999). Herring gulls also nest in colonies which make it easy to locate and sample eggs. Additionally, the herring gull is a common species that is widely distributed around the Great Lakes and other regions of the world which allows for direct comparisons of contaminant levels to be made in geographically distinct areas (Hebert et al., 1999). The Great Lakes Herring Gull Monitoring Program, which involves the collection and chemical analysis of eggs from 15 colonies across all five lakes, has been important in identifying hundreds of contaminants over many decades. These include PFCs, which provides a degree of ecological relevance to the work with chicken embryonic neuronal (CEN) cells described in Chapter 2.

The objective of this study was to determine the *in vitro* effects of PFCs in a wild avian species, the herring gull. Specifically, the effects of several new and emerging short-chained PFCs (<8 carbons) on mRNA expression of thyroid hormone (TH)-responsive genes in primary cultures of herring gull embryonic neuronal (HGEN) cells were determined.

3.2. Materials and methods

3.2.1. Chemicals

All PFCs were obtained from Wellington Laboratories (Guelph, ON, Canada). The PFCs were: perfluorobutanoic acid (PFBA) (Lot: PFBAGA06; molecular weight (MW): 236.02 g/mol), perfluorobutanesulfonate (PFBS) (Lot: 24318BB; MW: 338.19g/mol), perfluorohexanoic acid (PFHxA) (Lot: PFHxAGA06; MW: 336.04g/mol), perfluorohexanesulfonate (PFHxS) (Lot: LPFHxSAM05; MW: 422.10g/mol), perfluoroheptanoic acid (PFHpA) (Lot: NaPFHpARM07; MW: 386.04g/mol), and perfluoroheptanesulfonate (PFHpS) (Lot: PFHpSM06; MW: 472.10g/mol).

Triiodothyronine (T3) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO).

3.2.2. Source of eggs and incubation conditions

Fifteen fertile, unincubated herring gull eggs were collected from Chantry Island, ON, Canada (44°29′22″N, 81°24′7″W) in April, 2009. Herring gull eggs were transported back to the laboratory in a padded cooler at ambient temperature and then incubated at 37°C and 60% relative humidity in Curfew Model RX250 incubators (Althorne, Essex, UK) for 14 days. This corresponds to mid-incubation for herring gulls as the time to hatch is 28-30 days. Fifteen viable embryos were euthanized at mid-incubation by decapitation. All procedures were carried out using methods approved by the Animal Care Committee of the National Wildlife Research Centre.

3.2.3. Preparation and dosing of cultured neuronal cells

The protocol to prepare and dose cultured HGEN cells was the same as the method used to culture CEN cells (Chapter 2) with some minor modifications (listed below).

Each PFC was administered at 5 concentrations: 0.01μM, 0.1μM, 1μM, 3μM, and 10μM. T3 was administered at 5 concentrations: 0.03nM, 0.3nM, 3nM, 30nM, and 300nM. Additionally, a DMSO vehicle group was included for both T3- and PFC-treated cells. Exposure to each compound was repeated on two separate plates – one plate was used for RNA isolation and the other plate was used to determine cell viability. Therefore, HGEN cells used for cell viability determination were not used for RNA isolation. Following chemical administration, PFC-treated cells were incubated for 24 hours at 37°C and 5% CO_2. T3-treated cells were incubated at 37°C and 5% CO_2 for 3, 6, 12, 18, 24, and 36 hours. After their respective time periods, the medium was aspirated from all wells and the plates were immediately frozen at -80°C for subsequent RNA isolation or assessed for cell viability.

3.2.4. Cell viability

Cell viability was determined for T3 treatment (at 3 and 24 hours) and was conducted using the Calcein-AM assay. Each treatment group was assayed in three to four technical replicates (3-4 wells/treatment group) and was compared to a DMSO vehicle, untreated (UT) control, and 100% ethanol (negative control). The Calcein-AM assay was performed according to the manufacturer's instructions (Molecular Probes, Eugene, OR) and was similar to the protocol used in Chapter 2.

3.2.5. RNA isolation and complementary DNA synthesis

The protocol used to isolate RNA and synthesize complementary DNA (cDNA) from HGEN cells was the same as the method described in Chapter 2.

3.2.6. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

Primer pairs and Taqman fluorogenic probes for real time RT-PCR were designed using partial herring gull mRNA sequences obtained by homology cloning for Type II iodothyronine deiodinase (D2), neurogranin (RC3), and octamer motif binding factor-1 (Oct-1) (accession numbers NM24114, GQ281790, GQ281789, respectively). The TR-alpha (TR-α) and TR-beta (TR-β) primers and probes were previously designed by Crump et al. (2008b). The primer and probe sequences are shown in Table 3.1. All real-time RT-PCR methods were performed using the Strategene Mx3000P or Mx3005P instruments (Strategene, La Jolla, CA) with the same protocol described in Chapter 2.

3.2.7. Statistical analysis

The statistical analyses in this study were the same as described in Chapter 2.

Table 3.1. – Genes that were examined using real-time RT-PCR analysis in HGEN cells, including information on the nucleotide sequences of the primers and probes, final concentrations and accession numbers

Gene	Sequence (5'-3')	Final concentrations (nM)	Accession Number
β-actin	F: TGGGTATGGAGTCCTGTGGTA R: CGGATATCCACATCGCACTT P: CCATGAAACCACCTTCAACTCCATCA	300 60 200	AY045724
Thyroid receptor alpha (TR-α)	F: ATCTGCGTGGAGAAGATCGAG R: GAATGTTGTGTTTGCGGTAGTTG P: TCGAAGGCCAGCAGGTACGTCTCC	900 300 200	AY914170
Thyroid receptor beta (TR-β)	F: TGAAAGTGACAGACCTGCGAATG R: AGGAACAATGGAGGGAAGAGTTCT P: CTGCCATGCCAGCCGCTTCCTGC	900 300 200	AY914171
Type II iodothyronine 5' -deiodinase (D2)	F: CGCATCGGTCTTCTTGGTTCC R: GCTGACTTTCTGTTGGTCTACATC P: CACCCGTCAGATGGCTGGGCTGCC	300 900 200	NM24114
Neurogranin (RC3)	F: TGGATGAGGACATCCTGGACATC R: TTGATCTTCTTGCGGGTCATATGG P: CTTGGACGATCCCGACGCCAACGC	900 900 200	GQ281790
Octamer motif binding factor 1 (Oct-1)	F: GCCGGAGGACAGATCACTGG R: GCTGCTGACTGGCTGAATGC P: CAGCCGCCAGCAACTGTGCCTGC	300 900 200	GQ281789

3.3. Results

3.3.1. Cell viability

There were no significant changes in cell viability in T3-treated HGEN cells up to 300nM (Figure 3.1), but there was a decrease in cell viability in ethanol-killed cells (negative control). It was previously determined that there were no significant changes in the cell viability of PFC-treated cells up to 10μM in primary cultures of CEN cells (Chapter 2). Based on those results and the limited number of neuronal cells in this cell culture, cell viability was not determined for PFC-treated HGEN cells. Consequently, the maximum concentrations of T3 and PFCs used for subsequent mRNA expression analysis in this study were 300nM and 10μM, respectively.

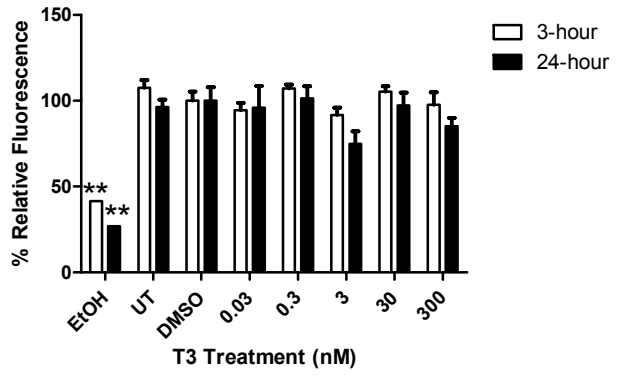


Figure 3.1. Calcein-Am cell viability assessment of HGEN cells following T3 treatment. The percentage (%) fluorescence was calculated relative to the DMSO treatment. Standard deviation was calculated based on three to four replicates per treatment (** $p<0.001$). EtOH; 100% ethanol, UT; untreated.

3.3.2. mRNA expression

3.3.2.1. T3 – positive control

T3 treatment for 24 and 36 hours induced TR-α and TR-β expression up to 2- and 4-fold, respectively (Figure 3.2.). Additionally, T3 treatment for 36 hours increased RC3 expression more than 2-fold (Figure 3.3.). There were no significant changes in the TH-responsive genes at any of the earlier time points (i.e. 3, 6, 12, or 18 hours).

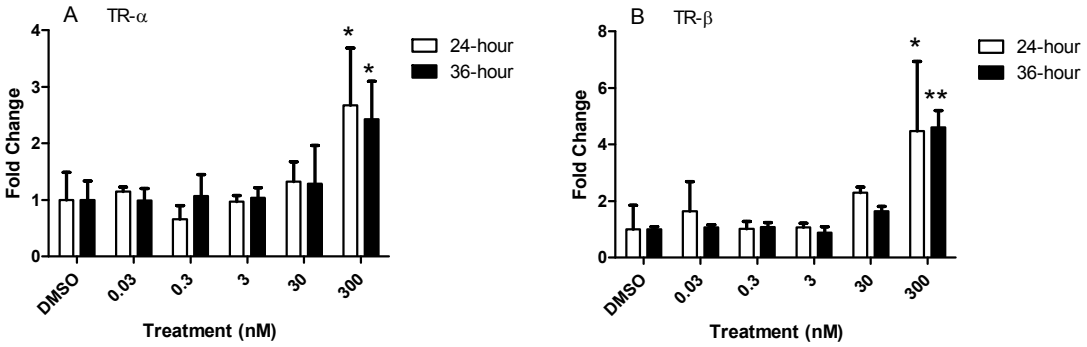


Figure 3.2. The relative expression of TR-α and TR-β mRNA in HGEN cells following T3 treatment for 24 and 36 hours. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 3-4 wells/treatment; * $p<0.05$ and **$p<0.001$ comparing DMSO- and T3- treated groups)

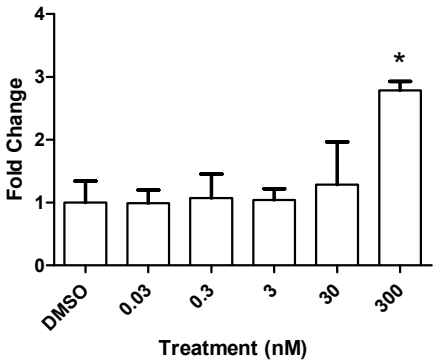


Figure 3.3. The relative expression of RC3 mRNA in HGEN cells following T3 treatment for 36 hours. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 3-4 wells/treatment; * $p<0.05$ comparing DMSO- and T3-treated groups)

3.3.2.2. PFCs

The fold changes for the TH-responsive genes are summarized in Table 3.2. Only PFCs that demonstrated significant concentration-dependent effects on the TH-responsive genes are presented in more detail in Figure 3.4.

Table 3.2. Mean fold change ± standard deviation (SD) of target gene mRNA expression in PFC-treated HGEN cells. (SD was calculated from 3-4 replicate wells on a single 48-well plate).

		TH availability		Signal transduction		Transcription factor activity	
		D2		RC3		Oct-1	
PFC		3uM	10uM	3uM	10uM	3uM	10uM
PFBA	C_4	0.91 ± 0.37	1.02 ± 0.19	0.97 ± 0.22	1.05 ± 0.14	0.89 ± 0.08	1.69 ± 0.35
PFBS	C_4	1.09 ± 0.01	1.39 ± 0.25	1.10 ± 0.15	0.85 ± 0.12	2.31 ± 1.03	6.47 ± 1.99
PFHxA	C_6	0.88 ± 0.07	0.98 ± 0.23	0.80 ± 0.11	1.01 ± 0.36	3.37 ± 0.35	4.96 ± 0.49
PFHxS	C_6	1.32 ± 0.14	1.41 ± 0.10	1.20 ± 0.11	1.12 ± 0.05	2.58 ± 0.29	2.32 ± 0.10
PFHpA	C_7	1.81 ± 0.51	2.00 ± 0.50	2.32 ± 0.78	5.74 ± 0.70	0.79 ± 0.15	0.97 ± 0.14
PFHpS	C_7	1.16 ± 0.31	0.98 ± 0.16	0.85 ± 0.13	0.97 ± 0.42	0.81 ± 0.28	0.81 ± 0.15

Note: White values indicate 0.51- to 2-fold changes and light grey values indicate >2-fold changes ($p<0.05$).

TH availability

There were no significant changes in D2 mRNA levels following treatment with any of the PFCs that were examined. Similarly, no significant effects were observed following T3 administration.

Signal transduction

There was an up-regulation in RC3 expression (Figure 3.4A) following PFHpA exposure which was similar to the pattern observed in T3-treated HGEN cells (Figure 3.3). There were no changes in RC3 expression following treatment with any of the other PFCs examined.

Transcription factor activity

There was an increase in Oct-1 mRNA levels following treatment with PFBS, PFHxA, and PFHxS (Figure 3.4B). There were no significant changes in Oct-1 expression in T3-treated cells. Additionally, no significant changes were observed in any of the other PFCs that were assessed.

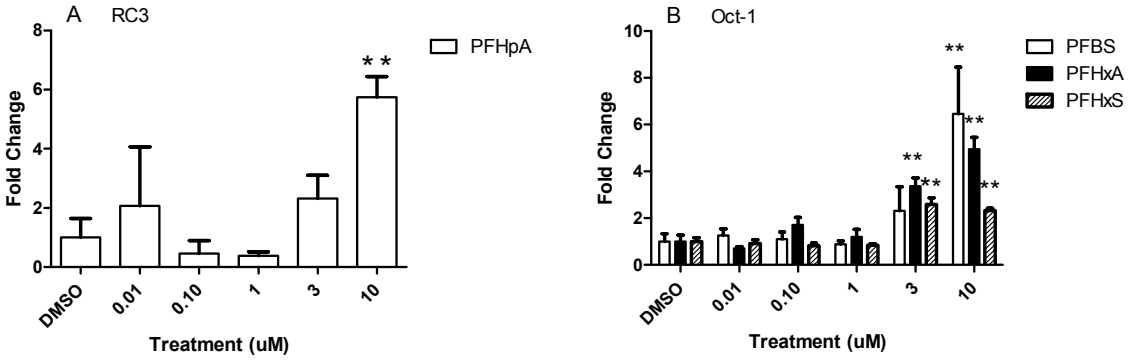


Figure 3.4. The relative mRNA expression of RC3 (A) and Oct-1 (B) in HGEN cells following treatment with several PFCs. Error bars represent the standard deviation of the mean and a one-way ANOVA was used to determine significant differences. (N= 3-4 wells/treatment; * $p<0.05$ and **$p<0.001$ comparing DMSO- and PFC- treated groups)

3.4. Discussion

This is the first study, to our knowledge, to assess the effects of PFCs on mRNA expression in cultured herring gull neuronal cells. This present study has demonstrated significant gene expression changes in primary neuronal cell cultures of a wild avian species following PFC exposure. Treatment with various short-chained PFCs induced the expression of several TH-responsive genes including RC3 and Oct-1 in HGEN cells.

Based on the cell viability results in Chapter 2, the highest PFC and T3 concentrations used for mRNA expression analysis were 10μM and 300nM, respectively. These concentrations ensured that transcriptional changes were related to mRNA expression and not cell viability. A 24 hour incubation period following chemical administration was determined as the minimum time period required to observe significant gene expression changes in HGEN cells. T3 treatment at 3, 6, 12, and 18 hours did not elicit any transcriptional responses in the TH-responsive genes examined. Given the dynamic nature of gene expression regulation, it was important to characterize gene expression changes over time. RNA half-lives can be highly variable and the responsive time point may be missed if only one time point is used. These results suggest that a 24 hour incubation period is necessary following chemical administration to ensure that the chemical equilibrates into its environment (the cells and medium).

Following T3 exposure (24 and 36 hours), there was an up-regulation of TR-α and TR-β expression, which indicated that primary cultures of HGEN cells responded to treatment with the natural ligand. Elevated T3 levels correspond with TR induction during chicken and amphibian development (Oppenheimer and Schwartz 1997;Yaoita and Brown 1990). The gene expression patterns following T3 administration provide a reference (or baseline) in which to compare PFC effects.

Although many significant changes were observed in TH-responsive genes following PFC exposure in CEN cells (Chapter 2), minor changes were displayed in PFC-treated HGEN cells. The mRNA levels of three TH-responsive genes (D2, Oct-1, and RC3) were examined in HGEN cells treated with the following short-chained PFCs:

PFBA, PFBS, PFHxA, PFHxS, PFHpA, and PFHpS. This study focused primarily on the effects of short-chained PFCs because it was determined that, based on the results in Chapter 2, short-chained PFCs were more transcriptionally active in CEN cells than long-chained PFCs.

T3- and PFHpA-treated HGEN cells induced RC3 expression up to 2- and 6-fold, respectively. A study by Zoeller et al (2005) reported increased RC3 expression following treatment with bisphenol A, another environmental contaminant, in mammals. RC3 has been implicated in post-synaptic signal transduction events involving Ca^{2+} and calmodulin (CaM) leading to synaptic plasticity (Guadano-Ferraz et al. 1997;Iniguez et al. 1996). Therefore, changes in RC3 expression may have functional consequences in synaptic plasticity, associative learning and memory (Guadano-Ferraz et al. 1997;Iniguez et al. 1993;Iniguez et al. 1996). RC3 contains a thyroid hormone response element (TRE) in its promoter region (Morte et al. 1997) and the transcriptional effects of T3 are mediated by TR. Hypothyroid mice demonstrated reduced TR-β and RC3 expression; however, RC3 expression was restored following T3 administration (Vallortigara et al. 2008). In this study, both T3 and PFHpA increased RC3 mRNA levels which could suggest that these two compounds have shared mechanisms of action in HGEN cells.

In this study, exposure to PFBS, PFHxA, and PFHxS induced Oct-1 expression. Oct-1, a DNA-binding protein, has been associated with DNA replication and the transcription of histone 2B genes, small nuclear RNAs (snRNAs), and immunoglobulins (Fletcher et al. 1987;Mittal et al. 1996;Sive and Roeder 1986;Verrijzer et al. 1990). Oct-1 may be important in regulating the growth of eukaryotic cells since reduced Oct-1 expression was associated with cell cycle arrest and morphological differentiation (Lakin et al. 1995). A study by Nagasawa et al. (1997) demonstrated that Oct-1 is expressed in the periventricular zone of the cortex which contains proliferating neurons that express only TR-β1 (Bradley et al. 1992). Once these neurons leave the ventricular zone and begin to differentiate, TR-β and Oct-1 expression declines (Bradley et al. 1992). Histone gene expression and DNA replication are not required in mature non-dividing neurons

which may explain the lack of Oct-1 mRNA present in these cell types (Lakin et al. 1995).

In contrast to the present study, previous mammalian studies reported increased Oct-1 expression following T3 administration. A single TH administration elevated Oct-1 mRNA within 12 hours in the fetal rat brain (Dowling et al. 2000). Other studies examined the expression of TH-responsive genes following exposure to polychlorinated biphenyls (PCBs), which are known to alter TH homeostasis. Gauger et al. (2004) observed induced Oct-1 expression in the offspring of dams treated with a commercial PCB mixture, Aroclor 1254 (A1254). Differences in species, developmental time periods and experimental designs (*in vitro* vs. *in vivo*) may account for the lack of induction in Oct-1 in this study. Based on differences in transcriptional responses, T3 and PFCs (PFBS, PFHxA, and PFHxS) may not necessarily share similar mechanisms in terms of regulating Oct-1 expression.

Exposure to several PFCs revealed variable transcriptional responses in primary cultures of herring gull and chicken neuronal cells (Table 3.3). Compared to the results in Chapter 2, none of the PFCs (or T3) altered D2 expression in HGEN cells. Another noticeable finding was the difference in Oct-1 induction. In the CEN study (Chapter 2), Oct-1 expression remained unchanged following PFC treatment, however, in HGEN cells, Oct-1 expression increased following treatment with several PFCs (PFBS, PFHxA, and PFHxS). It should also be noted that changes in RC3 expression were observed in both studies, although the PFCs that induced transcriptional responses and the magnitude of the fold changes following T3 administration differed between species. In this study, PFHpA increased RC3 expression, however in the CEN study (Chapter 2), PFBS and PFHxS treatment up-regulated RC3 expression. Additionally, T3 treatment (30nM) induced RC3 expression up to 14-fold in CEN cells (Chapter 2), whereas a higher T3 concentration was required to induce RC3 expression in HGEN cells (i.e. 3-fold change at 300nM).

Table 3.3. Summary of the overall significant gene expression changes following exposure to T3 and several PFCs in two avian species

Compound			TH availability D2	Signal transduction RC3	Transcription factor activity Oct-1
Chicken	T3	---	↑	↑	---
	PFBS	C_4	---	↑	---
	PFHxA	C_6	↑	---	---
	PFHxS	C_6	---	↑	---
	PFHpA	C_7	↑	---	---
Herring Gull	T3	---	---	↑	---
	PFBS	C_4	---	---	↑
	PFHxA	C_6	---	---	↑
	PFHxS	C_6	---	---	↑
	PFHpA	C_7	---	↑	---

This study provides further evidence that different species of birds respond differently when exposed to the same contaminants. Differences in species sensitivities to contaminants may explain the variable transcriptional responses observed following PFC exposure in the chicken and herring gull. The degree of species resistance to environmental contamination may be related to physiological (i.e. induction of xenobiotic-metabolizing enzymes), biological, and genetic factors.

Although no studies to date have examined the effects of PFCs in herring gulls, in some cases, it appears that herring gulls are less sensitive to chemical exposure than other avian species. For example, primary cultures of herring gull hepatocytes had lower sensitivity to cytochrome P4501A enzyme (CYP1A) induction (i.e. induced xenobiotic metabolism) following 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) exposure compared to the chicken (Herve et al., 2010). Fossi et al. (1995) also determined that herring gulls were more resistant to organochlorine compounds compared to other avian species (e.g. bald eagles and cormorants) due to their ability to modulate enzyme activities in response to chemical exposure (Fossi et al. 1995).

The herring gull eggs that were used in this study were collected in the field, where they are potentially exposed to hundreds of toxic chemicals. The chicken eggs, on

the other hand, were obtained from a managed breeding facility where the exposure to environmental contaminants is minimal. Differences in baseline contaminant levels may affect the way in which species respond to xenobiotic chemicals over time. For example, xenobiotics-driven selection has been observed in microbes, plants, and invertebrates (Klerks and Weis, 1987). Some evidence suggests that vertebrate populations are also evolving in response to contaminated environments. Killifish embryos and larvae from highly contaminated sites were determined to be less sensitive to contaminants than the control (Nacci et al. 2009). Additionally, the offspring of killifish embryos and larvae from contaminated sites were more resistant to toxicity than offspring from reference sites (Meyer et al., 2003).

This study was the first to assess the effects of PFCs in primary cultures of HGEN cells and determined that PFC exposure altered the expression of several genes in a wild avian species. More work should be conducted to determine whether similar results are found in other wild avian species and how changes in the transcriptional responses of TH-responsive genes affects these bird populations. It would be important to try and determine how subtle changes in gene expression in the brain may impact the whole organism. There is little toxicology research carried out in the brain and this technique permits an initial assessment of PFC effects on various avian species providing more insights into the mechanisms of action of these contaminants.

Chapter 4: General conclusions and future directions

4.1. General conclusions

A great deal of scientific information on PFCs has been collected over the last few decades regarding animal toxicity, chemical profiles, and global distribution. However, there are still gaps in the current literature regarding the mechanisms of action of PFCs, the effects of short- (<8 carbons) and long- chained (>8 carbons) PFCs, and the impact that these contaminants have on non-mammalian species (e.g. avian species). The main objectives of this study were to determine and compare the effects of PFCs in two avian species; an experimental model (chicken) and a wildlife species (herring gull). The data presented in this thesis are the first to demonstrate that exposure to PFCs alters mRNA expression in primary cultures of avian neuronal cells.

Several PFCs were shown to cause perturbations in the TH pathway in avian neuronal cells. Exposure to short-chained PFCs elicited variable transcriptional responses in primary cultures of herring gull and chicken neuronal cells (refer to Table 3.3 in Chapter 3), providing further evidence that different species of birds respond differently when exposed to the same contaminants. Other interesting findings were the lack of transcriptional responses in PFOS- and PFOA- treated CEN cells despite the fact that they were reported to cause a variety of adverse effects in laboratory animals (Johansson et al. 2008;Johansson et al. 2009;Lau et al. 2003;Thibodeaux et al. 2003;Yu et al. 2009). It is important to acknowledge that, although rodent studies provide a comparative data set in terms of possible mechanisms of action (e.g. TH pathway), comparisons should still be made cautiously.

The main findings from these experiments were that: 1) significant gene expression changes were detected in neuronal cells, 2) transcriptional changes occurred at relatively low PFC concentrations (as low as 0.1μM), 3) variable transcriptional responses were observed in different avian species, and 4) short-chained PFCs altered the expression of TH-responsive genes to a greater extent than long-chained PFCs.

4.2. Future directions

4.2.1. *In vitro* studies

The information gathered from *in vitro* assays is typically used to screen and prioritize environmental contaminants for further *in vivo* toxicity testing (Judson et al. 2010). The results from this study provide an opportunity to develop hypotheses and directions for follow-up research. Based on the results of this thesis, future studies should further examine the effects of the new and emerging short-chained PFCs, especially the 6-carbon sulfonate and carboxylate PFCs. This study has determined that the TH pathway may be a potential target of PFCs; therefore future work could look into examining additional parameters involved in this pathway such as the expression of key proteins or other biochemical responses. The suggestions for future work listed in this section could also be carried out in *in vivo*, or *in ovo* studies.

4.2.1.1. DNA microarray

Conducting a large-scale DNA microarray study would expand our understanding of molecular and biochemical pathways that are influenced by exposure to these compounds. It would facilitate the examination of a much larger suite of genes than those assessed in this study and help broaden the scope beyond the TH pathway to include multiple pathways. For example, PFC exposure has previously been shown to alter hormone levels (e.g. estrogen and testosterone) and the expression of hormone-responsive genes in mammalian and aquatic species. PFCs altered estradiol levels and the expression of estrogen-responsive genes in several fish species such as tilapia, rare minnows, and zebrafish (Du et al. 2009;Liu et al. 2007;Shi et al. 2008;Wei et al. 2007). PFC exposure also decreased serum testosterone levels and the expression of genes involved in cholesterol transport and steroidogenesis in rodents (Shi et al. 2007). In avian species, it was determined that the PPAR pathway may be activated in primary cultures of chicken hepatocytes; PFC exposure induced PPARα-dependent transcriptional activity (Cwinn et al. 2008;Hickey et al. 2009). A key advantage of using DNA microarrays is the

capacity for high throughput analysis of gene expression; the expression levels of thousands of genes can be determined simultaneously. A DNA microarray study could uncover important genes that are affected by PFC exposure in multiple different pathways.

4.2.1.2. Protein expression

Future research could assess the protein levels for transcripts that were found to be the most responsive in this study. In some cases, protein expression corresponds with mRNA expression patterns. However, in other instances this does not occur. RNA only reveals part of the story. Differences in the half life of RNA and protein, as well as post-transcriptional and/or post-translational modifications to the protein can lead to differences in RNA and protein levels (Hargrove and Schmidt 1989). It would be interesting to examine the protein expression patterns in neuronal cells following PFC exposure, to determine whether there is a correspondence with mRNA expression patterns.

4.2.1.3. Enzyme activity

This present study determined that PFC exposure altered the expression of several genes involved in functions associated with normal brain development (i.e. RC3). It would be interesting to examine the activity of enzymes involved in various brain functions such as synaptic transmission and synaptic plasticity following PFC exposure. Given that RC3 expression initiates downstream effects implicated in signal transduction pathways (Gerendasy 1999), the activity of Ca^{2+}/calmodulin (CaM)- dependent enzymes such as calmodulin-dependent protein kinase II (CAMKII) could be measured. Additionally, the activity of deiodinase enzymes could be examined to determine whether deiodinase activity corresponds to the mRNA expression results in this study.

4.2.2. *In ovo* egg injection studies

Conducting an egg injection study would provide a means of evaluating the developmental toxicity of PFC exposure. PFCs would be injected into the air cell of fertile chicken eggs prior to incubation. Subsequently, embryos would be incubated and euthanized at the pipping stage, after which various endpoints would be measured. Developmental toxicity endpoints include measurements of mortality, body weight, and histopathology. Other parameters that could also be included are measurements of TH levels and behavioural assessments, which will be discussed further in this section.

Future studies could measure thyroidal T4 content in avian species following PFC exposure. Thyroidal T4 content was determined to be a more sensitive index of decreased thyroid function than plasma TH measurements (McNabb et al. 2004). For example, studies that measured several TH parameters in perchlorate-treated bobwhite quails reported that measurements of circulating TH levels produced the most variable measurements and were the least sensitive indicator of altered thyroid function (McNabb et al. 2004). Behavioural assessments could also be performed following PFC exposure. Similar to the Pinkas et al. (2010) study, imprinting behaviour could be evaluated immediately after birth to determine if PFCs alter cognitive behaviours. Given that PFC exposure altered the expression of RC3 which is associated with learning and memory, neurobehavioural tests that examine motor skills, coordination, and learning could be performed. Burger and Gochfeld (2005) assessed neurobehavioural parameters in herring gulls exposed to lead by examined walking, begging, feeding, behavioural thermoregulation, individual recognition, and treadmill learning. It might also be worthwhile to examine behaviours related to hearing given that TH deficiencies were shown to permanently alter the development of hearing in mammals (McNabb 2007).

4.2.3. *In vivo* feeding studies

To date, only one study has examined the effect of a short-chained PFC (i.e. PFBS) on an avian species. Newsted et al. (2008) assessed the acute and chronic effects

of dietary PFBS exposure on the mallard duck and bobwhite quail. They did not observe significant effects on mortality, body weight, feed consumption, histopathology measures, or reproductive parameters following PFBS exposure (Newsted et al. 2008). Although no significant effects were observed in PFBS-treated birds, the acute and chronic effects of dietary exposure to other short-chained PFCs (e.g. PFHxA or PFHxS) could be examined. It may also be useful to include additional parameters such as measurements of TH levels or behavioural endpoints (mentioned above).

4.2.4. Other parameters

4.2.4.1. Mixture effects

Many contaminants are detected in human, animal, and environmental matrices - not just individual chemicals. Historically, toxicological research focused on single compounds. For example, in 1998, it was estimated that over 95% of the research conducted in toxicology was devoted to studies of single chemicals (Cassee et al. 1998). More attention should be aimed at determining the potential risks that mixtures of chemicals may pose. A study by Silva et al. (2002) demonstrated substantial mixture effects in a mixture of estrogenic chemicals at concentrations below their individual no observable effect concentration (NOEC). The notion that individual chemicals do not cause any harm because they are present at low, ineffective concentrations in humans and wildlife may be inaccurate when dealing with mixed exposures (Silva et al. 2002). Future studies could examine the impact of a mixture of PFCs on organisms. It would be interesting to examine the effects of a mixture of multiple PFCs (at environmentally-relevant exposure levels).

4.2.4.2. Long-term effects

Another future direction could be to examine longer term effects of PFC exposure *in vitro*. It was determined that the minimum incubation period required to observe significant gene expression changes in neuronal cells was 24 hours following chemical

administration (Chapter 3). It would be interesting to determine whether a longer PFC exposure (e.g. several days) would elicit different transcriptional responses. Cultured neuronal cells typically run out of nutrients after 4 days (refer to Invitrogen protocol 3946), therefore neuronal cells cultured for longer periods of time would require a replenishment of PFC treatment, medium, and supplements. Massarelli et al. (1976) reported that longer term exposure to ethanol (>7 days) in glioblast and neuroblast cell lines increased choline uptake while no change was observed following the shorter exposure time (<7 days). It was suggested that this may be related to changes in the structure of the plasma membrane, the activity of related enzymes and prolonged contact with ethanol (Massarelli et al. 1976). Therefore, a longer time period following PFC treatment could allow the cells more time to take up the chemical which could influence transcriptional responses.

4.2.5. Measurements of PFC concentrations

4.2.5.1. Neuronal cell uptake

The actual concentration of PFCs taken up by neuronal cells was not quantified in this study, which limits the interpretation of data in terms of comparing *in vitro* exposures to actual concentrations measured in wildlife. This limitation is not restricted to the present study as few studies have assessed the final concentration of compounds in cells after exposure. A study by Mundy et al. (2004) investigated the concentration- and time-dependent accumulation of 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) in primary cultures of rat neocortical cells. It was determined that after one hour only 15% of the applied PBDE-47 was associated with the cells whereas 55% remained in the medium and 30% was associated with the plastic culture dish (Mundy et al. 2004). A similar study has not been conducted with PFCs but it would be beneficial to measure the exact PFC concentration in avian neuronal cells in this *in vitro* assay to compare it to concentrations detected in the environment.

4.2.5.2. PFC concentrations in the brain tissue of wild avian species

Additionally, it would be useful to measure the exact concentration of PFCs in the brain tissue of wild avian species. Recent monitoring studies have detected PFOS in the brain of various wildlife species which provides evidence that this compound can cross the blood-brain barrier. Verreault et al (2005) detected PFOS in glaucous gull brain tissues in the Norwegian Artic at levels slightly above detection limits; however exact concentrations were not reported. PFOS was also measured in the brain tissues of pelicans from Colombia (3.5 ng/g ww) (Olivero-Verbel et al. 2006). PFOS is the only PFC that has been measured in the brain tissues of wild birds which creates a large data gap in terms of putting the results obtained in this study into a more environmentally-relevant context. Future studies should look into measuring the concentrations of other PFCs in wild avian species.

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