Gestational and Postnatal Exposure to a Contaminant Mixture: Effects on Estrogen Receptor Protein Expression in the Postpartum Maternal Brain

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Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the Master of Science in Interdisciplinary Health Sciences

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Abstract

Maternal behaviours are those that increase offspring survival. Estrogens affect maternal behaviour by activating Estrogen Receptors (ER) in the brain. Maternal brain plasticity was explored by characterizing the effects of exposure to a mixture of environmental pollutants on number of ERs. Following exposure to the toxicants during pregnancy and lactation, brains of female rats were collected, sectioned at 30 µm and immunohistochemistry for ERα performed. Immuno-positive cells in the mPOA, VTA and NAc were counted. A two way ANOVA revealed no main effect of Treatment on the number of immunopositive cells for all three brain regions. However, a significant difference between the High and Low Doses with the high dose reducing the number of ERα+ cells in the mPOA and VTA. Our work showcases the importance of studying the effects of multiple chemical co-exposures on the mother's brain, as maternal brain changes impact maternal behaviour consequently affecting offspring neurodevelopment.

Les comportement maternels vise à améliorer la survie de la progéniture. L'œstrogène agit sur les récepteurs ostrogéniques (ER) dans le cerveau pour influencer le comportement maternel. Notre étude explore les effets d'une exposition périnatale à un mélange de polluants environnementaux sur la plasticité du cerveau maternel du rat, en ce qui est des ERs. Suite à cette exposition périnatale, les cerveaux des mères ont été collectés et tranchés, suivi par une immunohistochimie pour ER. Le compte de cellules positives pour ER a été fait dans l'AMPO, l'ATV, et le NAc. Une ANOVA a démontré aucun effet de traitement; des comparaisons entre les doses élevées et basses ont révélées une différence significative dans l'AMPO, l'ATV. Notre étude démontre l'importance d'étudier les effets d'une co-exposition aux polluants sur le cerveau maternel puisque des effets sur le cerveau peuvent avoir un impact sur le comportement maternel, qui, par conséquent, influence le neurodéveloppement de la progéniture.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my thesis supervisor Dr. Anne TM Konkle for her continuous support and direction over the course of my master's research. I am truly thankful for her patience, motivation, immense knowledge and guidance through the time of research and writing of this thesis. I could not have imagined having a better supervisor and mentor!

To my committee members, Dr. Hélène Plamondon and Dr. Bénédicte Fontaine-Bisson, I am grateful for your guidance to steer me in the right the direction when needed and for being ever ready with advice. A special thanks goes out to my fellow lab members and the undergraduate students who contributed to this research. You are a talented group of individuals and I am very grateful to all of you. To my friends and my shabaloo, thank you for your constant support and kindness throughout my Master's. I hope and pray that I have made you proud!

I am grateful to parents and my sister who provided continuous encouragement throughout the process of researching, analysing and writing this thesis. This dissertation stands as a testament of your unconditional love and encouragement. Finally, I would like to thank God for giving me the strength, knowledge and opportunity to undertake this degree for without his blessings, this achievement would not have been possible.

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Abbreviations

BBB	Blood Brain Barrier
DA	Dopamine
DAB	3’-diaminobenzidine-tetrahydrochloride-dihydrate
E_2	Estradiol
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
fMRI	Functional magnetic resonance imaging Gamma-
GABA	Aminobutyric Acid
GD	Gestation day
HPA axis	Hypothalamic-pituitary-adrenal **axis**
MeHg	Methylmercury
mPOA	Medial Preoptic Area
NAc	Nucleus Accumbens
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyls
PND	Postnatal day
vBST	Ventral bed nucleus of the stria terminalis
VP	Ventral palladium
VTA	Ventral tegmental area

Chapter 1. Introduction

The environment in which a pregnant female is exposed can affect her and her offspring (Ashworth, George, Hogg, Lai, & Brunton, 2016). Of particular environmental concern are endocrine-disrupting chemicals. These are exogenous substances that interfere with the function of hormonal systems and cause deleterious effects ascribed to their interaction with steroid receptors, often times the estrogen receptors (ER) α and β (Gore & Dickerson, 2012). Although many researchers have explored the impact of pollutants on the neurological health of offspring (Elabbas et al., 2011; Gill et al., 2013; Kyriklaki et al., 2016; Pelletier et al., 2009), little research has sought to explore their effects on the mothers during pregnancy and post-partum. Given the mother's importance in caring for her offspring once they are born, any changes in brain plasticity during this important period may in turn influence the quality of care she provides to her offspring; quality of maternal care is an important component of offspring neurodevelopment.

1.1 Maternal Behaviour

Maternal behaviour, in nonhuman mammals, is defined as the collection of behaviours by the mother that can increase offspring survival and thus promote the independence of the offspring (Stack, Balakrishnan, Numan, & Numan, 2002).

Studies on the biopsychological bases of maternal behaviour in nonhuman mammals focus primarily on the laboratory rat; it is representative of altricial mammalian species because of the elaborate, stereotyped, and comprehensive nature of its maternal behaviour (Dollinger, Holloway, & Denenberg, 1980; Rosenblatt, 1980). According to Bridges (1996), the four major components of maternal behaviour

in rats are pup retrieval behaviour, nest building, nursing behaviour and pup licking/grooming. Retrieval behaviour involves the mother transporting the pup, in her mouth, around the nest. Nest building, induced by alteration in her perception of ambient temperature, insulates the pups during the mother's absence. Licking/grooming cleans the pups and when combined with other forms of body contact, it provides pups with tactile stimulation that can increase their growth. Finally, nursing behaviour requires the mother to expose her mammary region as to provide milk to the pups (Bridges, 1996). Maternal behaviour depends on a synchronized engagement of systems involved in sensation, perception, and cognition regarding input arriving through different modalities at different times; motivation and reward; learning and memory; emotion and stress; and motor output (Kristal, 2009). The coordination of all these systems is orchestrated by various hormones, neurotransmitters, sensory receptors, and neural circuits (Kristal, 2009).

Although the immediate initiation of maternal behaviour in the rat is under hormonal control, after its establishment the continuance of this behaviour is no longer solely hormonally mediated but is also under the action of neurotransmitters such as Gamma-Aminobutyric Acid (GABA) and dopamine (Numan et al., 2005). The medial preoptic area (mPOA) has been deemed as one of the key brain regions associated with maternal behaviour; with data suggesting that pregnancy may enhance neuronal growth and activity in this region (Kinsley, Silva, & Freeman, 2012). Under the action of the hormones at the end of pregnancy, signals from the mPOA activate dopaminergic neurons in the ventral tegmental area (VTA) that release dopamine into the Nucleus Accumbens (NAc; Numan 2005). Activation of the VTA dopaminergic neurons alters the amount of neurotransmitter content of mPOA neurons, or changes the expression of these neurotransmitters (Numan et al., 2010). Dopaminergic input to the NAc facilitates the dam's reactivity to biologically significant sensory stimuli, which are then relayed to the NAc and

adjoining ventral pallidum (VP) by afferents from the amygdala, hippocampus, and allocortical parts of the prefrontal cortex (Numan & Stolzenberg, 2009). The NAc is not only a key brain region of the mesocorticolimbic reward system, but is also involved in motivation behaviours important for survival, reproduction, and fitness (Dölen & Malenka, 2014). In the NAc, oxytocin serves as a social reward signal, with oxytocin receptors in this region playing an important role in mediating social cognition (Dölen & Malenka, 2014). Parvocellular neurons of the paraventricular nucleus of the hypothalamus send axon terminals to the NAc. Oxytocin released by these parvocellular neurons activates oxytocin receptors in the NAc as to induce plasticity at excitatory synapses (Dölen & Malenka, 2014). Therefore, blockage of oxytocin receptors in this area (via pharmacologic agents) impairs social reward processing and reduces plasticity at these excitatory synapses (Dölen & Malenka, 2014). Administration of oxytocin into the cerebral ventricles also activates brain areas involved in maternal behaviour and can elicit maternal behaviour in virgin rats; while blocking the action of oxytocin would have contrary effects (Pedersen, Ascher, Monroe, & Prange, 1982). Consequently, maternal exposure to environmental toxicants may lead to long-term changes in maternal behaviour, observed as a potential reduction in maternal activities such as pup retrieval, nest building, contact, nursing of offspring, pup licking/grooming as well as increased infanticide (Champagne & Curley, 2016), by affecting the plasticity of the brain systems that underlie them.

1.2 Neurobiology

With the myriad of neuro- and endocrine systems involved in the initiation and execution of maternal behaviour, it is challenging to determine which manipulations will affect the onset, quality, or maintenance of this behaviour (Kristal, 2009). Due to the complexity of maternal behaviour in rats, it is not surprising that many forebrain sites have been implicated in its mediation and regulation (Kristal, 2009). Hypothalamic structures, particularly the mPOA, are involved in the coordination of incoming chemical and neuronal signals while also monitoring and regulating the activity of other brain areas that

are important in the initiation of behaviours related to maternal care such as: motivation and reward, stress, pain and hypoalgesia, emotion, thermoregulation, feeding, drinking, motor output, and learning and memory (Kristal, 2009). The mPOA of the hypothalamus and adjoining ventral bed nucleus of the stria terminalis (vBST), are critical brain regions for the expression of maternal behaviour (Bridges & Ronsheim, 1990). Lesions to the mPOA/vBST disrupt maternal behaviour whereas the administration of estradiol (E_2) and prolactin in the mPOA promotes the onset of maternal behaviour, particularly among naive female rats (Numan & Young, 2016). Connections to the mesolimbic dopamine system, at the mPOA, control retrieval behaviour by increasing proliferation of midbrain dopamine neurons projecting from the VTA, as to increase the sensitivity of GABAergic inputs from the VP to the NAc and the release of dopamine into NAc (Numan et al., 2010; Numan & Stolzenberg, 2009). The mesolimbic dopamine system, at the mPOA, plays a role in the onset of parental retrieving behaviour in nulliparous female rats (Numan et al., 2010; Numan & Stolzenberg, 2009). The mPOA also coordinates incoming chemical and neural signals as well as monitors and regulates the activity of other hypothalamic nuclei and other brain structures implicated in maternal behaviour (Kristal, 2009).

1.2.1 Dopamine and Maternal Behaviour

Hormones prominent during pregnancy, such as prolactin, cholecystokinin and estrogen, have influence on the dopaminergic system (Castner, Xiao, & Becker, 1993). Research has shown that Dopamine (DA) is involved in both the onset and maintenance of maternal behaviour (Felicio, Florio, Sider, Cruz-Casallas, & Bridges, 1996). In fact, fMRI studies exposing human mothers to infant stimuli, showed brain activity patterns in regions that coincide with the mesocorticolimbic DA system (Barrett & Fleming, 2011). As part of the reward pathway, the mesocortical DA system is involved in motivational processes, such as reward and reinforcement (McCullough, Cousins, & Salamone, 1993; Stern & Keer, 1999). During maternal licking and grooming of rat pups, DA is released into the NAc (Champagne et al., 2004; Numan

et al., 2005). DA functions to depress NAc GABAergic output to the VP through an inhibitory process, which then allows VP outputs to promote behavioural reactivity (Mogenson, 1987).

There are two pharmacologically distinct DA receptor families, D1-like and D2-like (Seeman, 1980). D1 receptor agonists can act on either the mPOA or NAc to stimulate the onset of maternal behaviour; similar effects are also observed with the release of estradiol in the same area which suggests an overlap in underlying cellular mechanisms between estradiol and DA (Numan et al., 2005). Administration of haloperidol in the mPOA (a D2-like and D1-like DA receptor antagonist) to lactating females during postpartum days 6-8 (PP 6-8), abolishes pup licking, nest building and pup retrieval (Giordano, Johnson, & Rosenblatt, 1990). Collectively, these results show that DA is involved in regulating the retrieval and nest building components of maternal behaviour but not in other components such as nursing and licking (Rita Silva, Bernardi, & Felicio, 2001).

The mPOA/vBST neurons that project to the mesolimbic DA system in the VTA are implicated in the responsiveness to pup-related stimuli and so unilateral damage to mPOA/vBST can greatly influence maternal behaviour (Numan et al., 2005). For example, a unilateral knife cut that severed the lateral fibers of the mPOA, paired with a contralateral electrical lesion of the neuronal connections within the VTA disrupted retrieving and nest building behaviours to a much greater extent than did a variety of control lesions in the same area (Numan & Smith, 1984). In the same manner, when postpartum maternal rats received an excitotoxic amino acid lesion in the mPOA paired with an excitotoxic amino acid lesion of the VP on the contralateral side of the brain, there was a greater disruption in retrieval behaviour during the post-operative period when compared with ipsilateral lesions (Numan et al., 2005). The aforementioned examples illustrate the manner through which neuronal outputs of the mPOA to the VTA and/or NAc interact with the mesolimbic DA system; where interferences (through

pharmacologic neuronal agents) between these connections negatively influence the proactive components of various types of maternal behaviour.

The regulation of maternal behaviour in rats can also be influenced by the lateral efferent output of the mPOA neurons (Stack et al., 2002). Activity of the efferent neurons of the mPOA is required for the mesolimbic dopamine system to release dopamine into NAc and for the c-Fos activation response that occurs in the ipsilateral NAc (Numan et al., 2005; Stack et al., 2002). As a marker of neuronal activation, c-Fos can be used to determine areas that are activated during physical interaction with pups (Matsushita, Muroi, Kinoshita, & Ishii, 2015). Researchers found that lactating dams engaging in maternal behaviour had elevated c-Fos mRNA expression in the mPOA and in forebrain sites connected to it (Hyder, Stancel, & Loose-Mitchell, 1991; Numan et al., 2010). Increased c-Fos activity was also observed in the medial NAc as a result of the activation of the mesolimbic dopaminergic systemic (Numan et al., 2005; Stack et al., 2002). It is no surprise then that a unilateral cut severing the lateral mPOA/vBST connections decreased Fos gene expression within the ipsilateral and contralateral mPOA and NAc (Stack et al., 2002). Consequently, the prevention of D1 activation in NAc, blocks both maternal behaviour and Fos gene expression (Stack et al., 2002).

The activation of the mesolimbic DA system is essential for the occurrence of maternal behaviour (Numan & Stolzenberg, 2009). Dopamine in the NAc contributes to the formation of mother–offspring bonds through the control of attentional mechanisms that allow the mother's behaviour to change, in response to contextual cues or the actions of the pup (Olazábal et al., 2013b). For example, associative learning processes, catalyzed by the release of dopamine in the NAc, is used to stimulate the behaviour in response to a new situation (Olazábal et al., 2013b). As a result, dopamine promotes behavioural selection by allowing the animal to be attentive to biologically significant stimuli (Olazábal et al.,

2013b). The transmission of dopamine in the NAc region permits a flexible response when an organism encounters a salient stimulus (Olazábal et al., 2013b). In the VTA, dopamine is essential for the consolidation of maternal memory (Numan & Stolzenberg, 2009; Stack et al., 2002). Another major neurotransmitter implicated in maternal behaviour is GABA.

1.2.2 GABA and maternal behaviour

GABA is typically known as an inhibitory neurotransmitter; it can be released by mPOA afferent neurons (Arrati, Carmona, Dominguez, Beyer, & Rosenblatt, 2006). GABA mediates the proactive aspects of maternal behaviour, such as pup-seeking and the retrieval response, via the projection of GABAergic neurons from the mPOA region to the Bed nucleus of the Stria Terminalis and the VTA (Arrati et al., 2006; Olazábal et al., 2013a). These neurons produce GAD_{67}, the enzyme that synthesizes GABA from glutamate - recognition of this enzyme with an antibody enables the identification of GABAergic neurons (Stone, Walsh, & Benes, 1999). The mPOA GABAergic afferent neurons, from the paraventricular nucleus of the hypothalamus, can also depress neural circuits which regulate rejection, withdrawal, and avoidance responses to infant-related stimuli which increases the likelihood of positive maternal responses to young pups (Numan & Stolzenberg, 2009). Changes in GABA circuitry associated with parturition can be implicated in some postpartum neuropsychiatric disorders. For example, changes at the level of the GABAergic neurons from the mPOA to the VTA and vBST could be involved in anxiety and mood alterations experienced by some women after giving birth (McHenry, Rubinow, & Stuber, 2015). It should be noted that the GABA receptors are not the only factors thought to affect maternal behaviour and neuroplasticity, but also hormones such as oxytocin and estrogens that will be discussed in the next sections.

1.2.3 Neuroplasticity of the maternal brain

Neuroplasticity is defined as the natural ability of the brain to modify its organization and/or processing in order to accommodate changing environmental demands (Pascual-Leone, Amedi, Fregni, & Merabet, 2005). There are several neurobiological and neurochemical changes that serve to sharpen a mother's memory as to aid in the care, protection, and nurturing of the vulnerable offspring (Kinsley et al., 1999). The neurochemical changes that occur during pregnancy include upregulation of estrogen (E_2), prolactin, and oxytocin, all of which are key modulators of hippocampal changes (Brunton & Russell, 2011). During pregnancy, there are also neurobiological changes that occur at the hypothalamic-pituitary-adrenal (HPA) axis (Galea et al., 2000). Studies in humans, rats, and mice indicate that overall basal activity is reduced, within the HPA axis, while the threshold for response to physiologic, psychologic, and environmental stressors is raised (Fleming, Steiner, & Corter, 1997). Both of these alterations enable the mother to be better able to handle the stressors that arise while caring for her young (Fleming et al., 1997). Furthermore, pregnancy significantly alters the olfactory system, one of the primary sensory systems of mammals involved in the regulation of maternal behaviour responses (Fleming et al., 1997). In rat dams, parity has been associated with hyper-responsiveness to olfactory cues, with mothers preferring cages with pup-soiled bedding over non-soiled bedding (Fleming et al., 1997). Finally, studies have also shown that cognitive functions, namely spatial, object recognition and prospective memory are improved with pregnancy (Bodensteiner, Cain, Ray, & Hamula, 2006; Macbeth, Scharfman, MacLusky, Gautreaux, & Luine, 2008; Pawluski, Vanderbyl, Ragan, & Galea, 2006).

Drastic changes in hormones and neurochemistry, after pregnancy and the initiation of lactation, lead to neuronal structural plasticity and neurogenesis (Bodensteiner et al., 2006; Galea et al., 2000; Kazuhito Tomizawa et al., 2003). Adult neurogenesis and neuroplasticity in the hippocampus are associated with the hormonal and experiential changes associated with the parturition and postpartum pregnancy

periods (Leuner & Sabihi, 2016). These hormone sensitivities are due to the various hormonal and neurochemical differences between primiparous and multiparous rats that allow for comparisons and observations to be made (Bridges, 2015). There have been observed variations in spatial abilities as well as improvements in various learning and memory functions resulting from changes in hippocampal neurogenesis during pregnancy and post parturition (Cameron & Glover, 2015; Tomizawa et al., 2003). For example, studies have found that primiparous female rats, during the late postpartum period, outperform nulliparous females on spatial tasks (Kinsley et al., 1999; Leuner & Gould, 2010; Pawluski & Galea, 2007). This could be due to increased hippocampal spine density in pregnant rats that continues to develop during the postpartum period (Pawluski & Galea, 2007). Similarly, oxytocin also plays a role in neurobiological mechanisms of neuroplasticity.

1.2.4 Oxytocin and neuroplasticity

The dramatic behavioural changes that occur during the postpartum period are mediated by a number of neuromodulators such as oxytocin, a neuropeptide synthesized in the supraoptic and paraventricular nuclei of the hypothalamus (Sabihi, Dong, Durosko, & Leuner, 2014). Prior to labour, increased estradiol levels result in the proliferation of oxytocin receptors in the uterus and mammaries, thereby rendering those areas of the female more sensitive to the effects of the oxytocin that is soon to be released (Kristal, 2009). In animal models, oxytocin has been found to enhance hippocampal synaptic plasticity and cognitive functions of mother rats when administered centrally during pregnancy (Tomizawa et al., 2003). Oxytocin stimulates neurogenesis and protects hippocampal plasticity by shielding against the suppressive effects of stress hormones (Leuner & Sabihi, 2016). In rat models, prolonged treatment with weekly peripheral 1mg/kg doses of oxytocin doses administered during gestation (levels equivalent to 50 µg/mL found in maternal blood samples collected 8 weeks postpartum) stimulated cell proliferation, seen as an increased number of bromodeoxyuridine (BrdU)-labeled cells in the ventral portion of the hippocampus (Leuner & Sabihi, 2016; Prevost et al., 2014). As an analogue of thymidine, Brdu is

incorporated into the DNA of cells during the synthesis stage of the cell cycle; evaluation of the survival time showed an increase in the time spent in the different stages of adult neurogenesis in the hippocampus (Leuner & Sabihi, 2016).

The actions of oxytocin, via activation of the oxytocin receptors in key regions of the brain, induces certain maternal behaviours (Cohen et al., 2010; Gimpl & Fahrenholz, 2001). In the peripartum period, oxytocin controls important reproductive functions such as labour induction and milk ejection (Neumann, 2008). In response to a variety of stimuli such as suckling, parturition, or certain kinds of stress, oxytocin is released from the posterior pituitary into the systemic circulation (Gimpl & Fahrenholz, 2001). Moreover, the central and peripheral activation of oxytocin receptors in the hippocampus has been linked to social behaviours and it has been shown to protect against the negative effects of stress by diminishing HPA axis responsiveness and decreasing levels of circulating glucocorticoids (Cohen et al., 2010; Windle et al., 2004). During the postpartum period, oxytocin receptors, which mediate the actions of oxytocin, are increased in the bed nucleus of the stria terminalis and mPOA (Insel, 1990). Oxytocin receptor regulation is influenced by estrogen (Choleris et al., 2003). Effects of oxytocin and estrogen can alter the hypothalamic–pituitary–adrenal (HPA) system during pregnancy and the postpartum period such that circulating corticosterone levels increase during late gestation and remain elevated during lactation (Leuner & Sabihi, 2016; Stern & Voogt, 1973). Activation of ERα can increase HPA axis activity and also modulate oxytocin transcription, whereas activation of ERβ can decrease HPA activity, as seen by reductions in adrenocorticotropic hormone and corticosterone levels, while also directly altering oxytocin mRNA levels in the same area (Lund, Munson, Haldy, & Handa, 2004). High expression of ERα in the amygdala, the brain area important for the convergence of socially relevant information, is necessary for the activation of the oxytocin receptor (Young, Wang, Donaldson, & Rissman, 1998). Evidently, oxytocin released during the postpartum period

is capable of protecting hippocampal neurogenesis from high glucocorticoid levels by reducing the binding capacity of glucocorticoid receptors in the hippocampus (Leuner, Mirescu, Noiman, & Gould, 2007).

The effect of oxytocin in the brain can be observed in other brain areas as well. In the central NAc, higher levels of oxytocin have been attributed to reduced anxiety associated during the postpartum period (Bosch, Meddle, Beiderbeck, Douglas, & Neumann, 2005). Among female adult rats, social recognition varied with the high and low phases of the estrous cycle, with improved social recognition when levels of circulating estrogens were high; ovariectomized female rats treated with estrogen were shown to have improved social recognition (Hlinák, 1993; Markham & Juraska, 2001). Lesion and inactivation studies performed in the pre-limbic region of the medial prefrontal cortex (mPFC), have shown that oxytocin can regulate social and anxiety behaviours (Ninan, 2011; Sabihi et al., 2014). In fact, blocking oxytocin receptors in the prelimbic region of the mPFC, using bilateral infusions of a highly specific oxytocin receptor antagonist, showed a decrease in the expression of maternal behaviour and an increase in anxiety-like behaviour, but only among postpartum females (Sabihi et al., 2014). Oxytocin receptor inhibition, in the postpartum prelimbic region of the mPFC, impaired pup retrieval behaviour by increasing the latency to retrieve the first pup and decreasing the number of pups received (Sabihi et al., 2014). The receptors also enhanced maternal aggression shown as a decrease in the latency to attack the intruder and an increase in the number of intruder attacks (Sabihi et al., 2014). For example, oxytocin receptor knockout female mice showed heightened aggression toward both familiar females and unfamiliar male and female intruders, whereas oxytocin administration reduced infanticide and agonistic aggression in these female mice (McCarthy, 1990; Ragnauth et al., 2005). To illustrate a reduction in anxiety, postpartum rats spent a greater percentage of time in the open arms of an elevated plus maze and made more entries into the open arms, which is indicative of less anxiety (Cruz,

Frei, & Graeff, 1994; Pellow, Chopin, File, & Briley, 1985). The ability of the lower, but not higher, dose of the oxytocin receptor antagonist to modify maternal behaviour shows a dose-dependent, but non-linear, effect (Figueira, Peabody, & Lonstein, 2008; Leuner, Caponiti, & Gould, 2012). Analogous to other physiological systems, the maternal immune system is carefully regulated to protect the mother and fetus from microbes and to tolerate the semi-allogenic fetus with various direct factors and environmental circumstances impacting the maternal immune response (Prins, Eskandar, Eggen, & Scherjon, 2018).

1.2.5 Neuro-immune changes in the maternal brain

Estrogen has both dose- and cell type-specific effects on immune cells to act in both a pro-inflammatory and an anti-inflammatory manner (Bruce-Keller et al., 2000). These neuro-immune adaptations are not only limited to neurons but also occur in other brain cells. High levels of estrogen suppress microglial proliferation and activation and decrease the secretion of pro-inflammatory cytokines, which in turn leads to a reduction in the number of microglial cells, immune cells of the central nervous system (Haim et al., 2017; Bruce-Keller et al., 2000).

Due to their transient contact with synapses, microglia might also play a crucial role in synaptic remodeling and neuroplasticity in the healthy maternal brain during the peripartum period (Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). Since microglial cells express receptors for hormones that fluctuate greatly during pregnancy, they may mediate the neuroimmune changes often observed with pregnancy (Tanaka et al., 1997). When microglial densitometry and microglial numbers are observed in dams across four brain areas- Basolateral Amygdala, medial Prefrontal Cortex (mPFC), NAc, dorsal Hippocampus at multiple time-points (GD20, PP1, PP8) the results showed a reduction in microglial density, with a decrease in only the number of thin and ramified microglia and not of other

microglial morphological sub-types (Haim et al., 2017; Posillico & Schwarz, 2016). Microglia with long, ramified processes are thought to carry out the role of immune surveillance and a reduction in this morphological sub-type indicates that peripartum hormonal shifts might have contributed to the observed neuroimmune changes (Haim et al., 2017; Wake et al., 2009) Thus, these neuroplastic modifications that occur in the peripartum brain are essential for enabling the various motivational, cognitive and emotional changes displayed by new mothers (Haim et al., 2017).

1.2.6 Estrogen and Estrogen Receptors

Estrogen, a gonadal steroid hormone, is the primary female sex hormone responsible for the development and regulation of the female reproductive system and secondary sex characteristics (Freeman, 1994). As briefly mentioned above, estrogens are involved in maternal behaviour, the processing of socially relevant information and also the detection and integration of socially relevant olfactory information to more complex social behaviours, including social preferences, aggression and dominance, and learning and memory for social stimuli (Ervin et al., 2015; Murakami, 2016). Estrogen mediates its effects by binding and activating estrogen receptors (ERs) (Pettersson & Gustafsson, 2001). Activated ERs, in the form of estrogen receptor complexes, are responsible for modulating the expression of many genes, the classic mechanism being the regulation of transcription via specific binding sites on promoter regions of DNA (hormone response element) of target genes (Pettersson, 2001).

Estrogens' effects on behaviour are indeed mediated by their action at estrogen receptors (ERs). Estrogen receptor α, also known as ESR1, NR3A1 (ERα) and Estrogen receptor β, also known as ESR2, NR3A2 (ERβ), are the most commonly studied receptors which have structural similarities but are encoded by genes on different chromosomes (Heldring et al., 2007). ERs contain three major functional

domains, an N-terminal domain that harbors a transcriptional activation function (AF-1), a DNA-binding domain, and a C-terminal ligand-binding domain hosting a ligand-dependent transcriptional activation function (AF-2) (Fawell, Lees, White, & Parker, 1990). The ligand binding domain is multifunctional, containing ligand recognition sites as well as regions for dimerization and ligand-dependent transactivation (Fawell et al., 1990). Hormones that bind to the ligand binding domain change the conformation of the receptor as to initiate the activation or repression of responsive genes (Tsai & O'Malley, 1994). Inter-domain crosstalk between the AF-1 and AF-2 domains allows the recruitment of a variety of transcriptional co-regulatory proteins that can act individually or synergistically to mediate the effect of ERs on gene expression (Kobayashi et al., 2000; Métivier, Penot, Flouriot, & Pakdel, 2001). The action of estrogens at these receptors can cause both long-term effects through their effect on gene transcription and rapid effects on behaviour caused by changes in intracellular signaling (Choleris, Clipperton, Phan, & Kavaliers, 2008; Frick, 2012).

Both types of ERs help regulate the oxytocinergic system; ERβs regulate the expression of the gene encoding the oxytocin neuropeptide and ERαs regulate expression of the oxytocin receptor gene (Patisaul, Scordalakes, Young, & Rissman, 2003; Vasudevan et al., 2001). Therefore, differences in the expression of both ERα and oxytocin receptors in the hypothalamus result in the natural variations in maternal care (Champagne & Curley, 2016). Estrogen receptors are involved in the regulation of the oxytocinergic and serotonergic systems, which affect the expression and function of oxytocin and serotonin receptors, respectively (Bethea, Lu, Gundlah, & Streicher, 2002). Oxytocin and serotonin receptors can be found in various locations in the brain (Ogawa et al 1998). These receptors have been shown to be indispensable for rat maternal behaviours, such that pup retrieval and grooming are severely impaired in ERα knockout mice and in gonadally intact female mice with decreased ERα expression (Ogawa et al., 1998). Overall, observed changes in maternal behaviour and differences in the

quality of maternal care have been shown to be influenced by changes in the neuronal and endocrine systems that are mediated by estrogenic effects on ERs (Krezel, Dupont, Krust, Chambon, & Chapman, 2001). Modifications to these aforementioned systems may be linked to important health conditions, in particular mental health in the peripartum period.

1.3 Postpartum Depression

Because gestation and lactation are physiologically demanding, there are often changes in disease susceptability after pregnancy (Galea, Leuner, & Slattery, 2014). For example, there is an increased risk for developing depression and anxiety along with other mental health conditions (Brummelte & Galea, 2016b; Galea et al., 2014). According to the International Statistical Classification of Diseases and Related Health Problems 10th Revision, Postpartum Depression (PPD) is depression that occurs after birth (World Health Organization, 1992). Symptoms of PPD include sad mood, restlessness and/or agitation, and impaired concentration (World Health Organization, 1992). It is associated with many negative outcomes for mothers and infants, including reduced breastfeeding, lack of maternal emotional and behavioural sensitivity to the infant, poor bonding, negative infant temperament, atypical neurodevelopment, and later emotional and behavioural problems for the offspring (Field, 2010; Glasheen, Richardson, & Fabio, 2010; Stein et al., 2014)

There are many fluctuations in hormones during gestation and the postpartum period. Women with a predisposition for depression could be more sensitive to these fluctuations with a potentially higher risk of developing PPD. The etiology of PPD is described as fluctuating levels of steroid (such as estradiol and progesterone) and peptide hormones during pregnancy and the postpartum period (World Health Organization, 1992; Bloch, Daly, & Rubinow, 2003). In rodent models, PPD manifestations differ slightly

from those reported or known in humans and include elevated progesterone during the first two weeks of gestation (that decline a few days prior to parturition) and elevated estradiol levels that remain at modest levels throughout gestation, until just a few days prior to parturition where a dramatic increase is observed (Rosenblatt, 1980). A similar pattern is observed in humans with progesterone levels that are approximately 20× higher during gestation and remain elevated throughout pregnancy, and estradiol levels are very high by week 20 of gestation and remain elevated throughout the rest of pregnancy (Schneider, Davies, & Honour, 1993). A study by Fleming, Steiner, & Corter, (1997) showed that a high estradiol to progesterone ratio in the collected saliva samples, during the late gestational period, was associated with disruptions in human mother–infant interactions and depression in postpartum women, whereas women with a low estradiol to progesterone ratio during gestation reported higher feelings of attachment in the early postpartum. Estradiol interacts with certain aspects of the serotonin system, as to increase serotonin synthesis and decrease inhibitory feedback to serotonergic neurons in the dorsal raphe nucleus (Mehta et al., 2014). Serotonin is a pivotal neurotransmitter in the regulation of mood and affective behaviour with previous studies showing changes in the level of estradiol being related to serotonin neural function and depressive symptoms (Halbreich, 1997; Halbreich, Piletz, & Halaris, 1992). Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in serotonin synthesis and the level or activity of this enzyme likely plays a role in the intracellular concentration of serotonin achieved in either cell bodies or axon terminals (Bethea, Mirkes, Shively, & Adams, 2000). In rats, studies have found that estradiol treatment for 28 days postpartum increased TPH protein mass four to six fold in the dorsal raphe nucleus of the brainstem and the neocortex, when compared to treatment with progesterone only (Bethea et al., 2000; Kuroda et al., 2011). In another study, estradiol treated and progesterone treated monkeys were shown to have a nine fold increase in TPH mRNA in the dorsal raphe among the estradiol treated monkeys when compared to the group with only progesterone treatment (Bethea et al., 2000). Taken together, these

data suggest that estradiol levels can contribute in the regulation of TPH gene expression, with lower levels of estradiol resulting in decreased TPH gene expression (observed as lower TPH mRNA) which consequently leads to lower levels of the TPH protein and lower level of serotonin synthesis (Bethea et al., 2000). Lower serotonin levels found to be a biomarker indicative of depressive symptoms (Brummelte & Galea, 2016; Mehta et al., 2014).

The processing of infant-related cues among human mothers can be affected by maternal depression and PPD. Significant changes in neuronal activity, observed in rat models, using functional magnetic resonance imaging to visualize the morphological changes occurring in the female rat hippocampus (Engert & Bonhoeffer, 1999). Using functional magnetic resonance imaging, women with PPD showed significantly weaker connectivity in the amygdala and the hippocampus, when compared with non-depressed postpartum women (Moses-Kolko et al., 2012). Depression in pregnant and postpartum women impacts a mothers' 'hedonic' response to stimuli, executive function, and cognition (Akbari, Wonch, & Fleming, 2015). When compared with non-depressed mothers, depressed mothers tend to be more intrusive with and irritated by their infant, often responding less sensitively and contingently to their infants who was eloquently illustrated by decreased vocal and visual communications, touching interactions, and smiling in an earlier study (Fleming, Ruble, Flett, & Shaul, 1988).

Exposure to stress, particularly during the peripartum period, has shown to cause neurogenesis and dendritic plasticity alterations in the rat hippocampus (Pawluski et al., 2012). Epidemiological studies have shown a link between gestational stress and PPD (Leuner, Fredericks, Nealer, & Albin-Brooks, 2014; Smith, Seckl, Evans, Costall, & Smythe, 2004). In postpartum rats, chronic gestational stress resulted in maternal care deficits, increased depressive-like behaviour, and impaired performance on an attentional

set shifting task that relies on the mPFC (O'Mahony et al., 2006; Smith, Seckl, Evans, Costall, & Smythe, 2004). In order to investigate the vulnerability to depression during the postpartum period, it is useful to examine the neural mechanisms that are affected by gestational stress. The mPFC is a brain region that has been linked to stress, cognition, maternal care, and mood disorders including PPD (Febo, Felix-Ortiz, & Johnson, 2010; Sorra & Harris, 2000). In the mPFC, neurons undergo experience-dependent structural reorganization including changes in the size, shape, and number of dendritic spines, sites of excitatory synapses (Sorra & Harris, 2000). These findings suggest that some of the behavioural features associated with PPD may involve stress-induced spine modifications in the mPFC (Sorra & Harris, 2000). Furthermore, exposure to gestational stress, during the postpartum period, causes structural changes that compromises structural plasticity (Haim et al., 2017). There is an increase in spine density in the NAc, during the postpartum period, which may be necessary to enhance the motivation required to engage in maternal care (Li & Fleming, 2003; Wansaw, Pereira, & Morrell, 2008). In contrast, gestational stress leads to robust (approximately 35% change) and persistent morphological modifications which are observed as reduced dendritic length, branching, and spine density of neurons found in the shell of the NAc (Haim, Sherer, & Leuner, 2015). In rat dams, mothers exposed to gestational stress have shorter dendrites and fewer branch points, which is observed as reduced structural plasticity during both the early/mid and late postpartum time points (Haim, Sherer, & Leuner, 2014). Since the NAc receives extensive dopaminergic input from the VTA, reductions in the spine density and neuronal complexity in the NAc of gestationally stressed mothers could be indicative of less dopamine input (Baik, 2013; Haim et al., 2015). In contrast, un-stressed mothers had the highest density of dendritic spines (Radley et al., 2008).

Exposure to gestational stress could cause alterations in the mesocortical dopamine system that could lead to postpartum depression (Haim et al., 2015). Dopamine positively regulates neuronal morphology

in the NAc (Meredith, Ypma, & Zahm, 1995). Studies conducted among women with PPD, found dopaminergic system dysregulation in the NAc which resulted in fluctuations of dopamine levels and thus an increased likelihood of developing PPD (Lavi-Avnon et al., 2008; Moses-Kolko et al., 2012). However, the altered morphology of the medial spinal neurons in the NAc could involve other mediators of structural plasticity (Meredith et al., 1995). Medial spinal neurons in the NAc are also susceptible to modifications caused by glutamatergic input from the mPFC, hippocampus, and basolateral amygdala (Haim et al., 2015; Russo & Nestler, 2013). Projections from the NAc to the mPFC encompass the mesocortical dopamine pathway; both areas receive dopaminergic projections from the VTA with afferent excitatory input regulating dendritic structure (Tzschentke & Schmidt, 2000). Structural alterations in these aforementioned areas have been linked to depressive behaviour in both rodent models and humans (Pittenger & Duman, 2008; Russo & Nestler, 2013; Tzschentke & Schmidt, 2000). Consequently, alterations in the structural plasticity in the mesocortical dopamine system following gestational stress could underlie postpartum depressive-like behaviour and also interfere with reward and motivational processes necessary for maternal behaviour (Haim et al., 2015).

Chapter 2. Environmental Toxicants

Pollution is a worldwide problem that can greatly affect the health of all living organisms (Liberda et al., 2014). Environmental toxicants are organic and inorganic substances that are harmful to human health and development, with exposure occurring at home, in the workplace, or in the community via contaminated soil, air, water, or food (Barrie et al., 1992). While some environmental exposures are ubiquitous and difficult to avoid others are more common to particular populations (Barrie et al., 1992). For example, the Indigenous population depends on traditional lifestyles, with their diet typically consisting of the consumption of a lot of fish; this poses a higher risk of exposure to certain contaminants found in aquatic systems (Batal, 2001) as reviewed by (Kuhnlein & Chan, 2000). The results of some studies suggest that maternal exposure to environmental toxicants is a threat to women's health as well as to fetal health and development (reviewed in Pawluski et al., 2016). Exposure that occurs during pregnancy and/or different stages of motherhood can disrupt the many typically occurring neurobiological changes or adaptations during these stages (Pawluski et al., 2016). Furthermore, the ubiquity and persistence of toxicants, whether within the environment or the body, causes unique challenges when trying to limit exposure among vulnerable populations (Liberda et al., 2014). Thus, this chapter explores the individual and combined effects of polychlorinated biphenyls (PCB), methyl mercury (MeHg) and organochlorine pesticides on neurodevelopment, maternal behaviour and the maternal brain in both animal and human models. An overview of the effects of each toxicant mixture component can be found in table 1.

Table 1. Summary of the neuromolecular and behavioural effects of perinatal exposure to three categories of environmental toxicants on offspring or mothers in animal and human studies.

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
Offspring							
MeHg	Human	Early prenatal	7 years postnatal	Reduced visual and spatial memory development			(Orenstein & Shamir, 2014)
MeHg	Human	Early prenatal	8 years postnatal	Neurocognitive deficits;	mesocorticolimbic dopamine/glutamate pathways		(Weston et al., 2014; Sagiv, Thurston, Bellinger, Amarasiriwardena, & Korrick, 2012)
MeHg	Human	Gestation	2-4 years postnatal	Developmental delays & psychomotor response to cues	Growth inhibition (due to cell death and inhibition of cell divison) of glial cells	Walking and speech development	(Cox et al., 1989; Prasad et al., 1979)

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
MeHg	Rats	During development	12 weeks postnatal	Reduced motor coordination	Decreased striatal dopamine release and inhibited glutamate reuptake		(Bourdineaud, Marumoto, Yasutake, & Fujimura, 2012; Coccini et al., 2011)
PCB	Human	Gestation	1 year postnatal	Language delays and altered short memory		delayed developmental milestones	(Patandin et al., 1999; Boucher et al., 2009; Caspersen et al., 2016).
PCB	Rats	Gestational day 7 to postnatal day 21	3-4 months postnatal	Decreased ability to perform spatial tasks &reduced Motor ability	Increased extracellular GABA in the cerebellum, Impaired cGMP pathways, reduced the number of NMDA		(Boix, Cauli, & Felipo, 2010; Cauli, Piedrafita, Llansola, & Felipo, 2013; Sager, 2004)

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
					receptors in cerebellum		
Organochlorine pesticides	Rats	Postpartum	7 weeks postnatal	abnormal stimulation of reproductive tract development	Neuronal cell death from retraction of GnRH neuronal processes in the hypothalamus		(Chapin, 1997; León-Olea et al., 2014)
Organochlorine pesticides	Rats	Postnatal days 1-4	Adult	Behavioural disturbances	changes in serotonin, arginine-vasopressin and oxytocin levels in the hypothalamus		(Aldridge, Meyer, Seidler, & Slotkin, 2005)
Toxicant Mixture	Rats	Perinatal exposure (gestation and	5 months postnatal	Changes in learning behaviours	Elevated levels of the pro-inflammatory cytokine, IL-1b and IL-10. moderately		(Hayley, Mangano, Crowe, Li, & Bowers, 2011)

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
		lactation)			increased hypothalamic levels of IL-12 and tumor necrosis factor α		
Mothers							
MeHg	Rats	2 weeks prior to breeding	After offspring weening	Changes in regions of the maternal brain (ie. Frontal cortex, olfactory bulb)	Reductions in DA, noradrenaline and DOPAC levels, reduced hypothalamic serotonin levels & dopamine turnover rate		(Weston, et al., 2014)

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
MeHg	Human	A few hours postpartum	48 hours Postpartum	Neuronal death, astrogliosis, microglial activation, and demyelination	Inhibition of neuronal Ca^{2+}ion channels, disruption of presynaptic transmitter release and postsynaptic receptor function, destruction to DNA structures and altered Na+/K+ ATPase and mitochondrial function		(Harada, 1995; Juárez et al., 2005; Peng, Hajela, & Atchison, 2002)
PCB	Rats	Gestational days 6-18	Post-weening of	Alters maternal grooming and		Levels of estrogen and	(Cummings, Nunez, & Clemens, 2005;

Toxicant	Pop'n	Age of exposure	Age assessed	Behaviour assessed/ Change observed	Molecular changes assessed	Other indicators assessed	Reference(s) supporting this effect
			their own offspring	licking of the pups Less high-crouch nursing among exposed group		dopamine that may be affecting the maternal-offspring bond	Cummings et al., 2008)
PCB	Rats	Gestational days 6-18	Postpartum day 14	More time on the nest and more time grooming (and licking) the pups		Changes in gonadal hormones in the male offspring	Cummings et al., 2008
Organochlorine pesticides	Human	Gestation up to 3 days postpartum	8 months postpartum	Depression, hostility and phobic behaviours	Weak estrogen and androgen receptor agonists altering expression	Changes in the dopaminergic and serotonergic systems	(Gore, 2008; Slotkin, Seidler, & Fumagalli, 2007; Yalçın, Örün, Yalçın, & Aykut, 2015)

2.1 Blood Brain Barrier

The developing brain is particularly sensitive to the effects of pollutants via maternal exposure (Landrigan & Goldman, 2011). For the embryo and fetus, protection from pollutants is provided by the placenta and the blood brain barrier (BBB). The placenta provides additional protection in the developing brain (Landrigan & Goldman, 2011). Three barrier layers contribute to the separation of the blood and neural tissues: (1) a highly specialized endothelial cell layer comprising the blood–brain barrier (BBB) that helps partition the blood and brain interstitial fluid and tightly regulates CNS homeostasis (Daneman & Prat, 2015), (2) The blood-CSF barrier with the choroid plexus epithelium which secretes the specialized cerebral spinal fluid into the cerebral ventricles, and (3) the arachnoid epithelium, separating the blood from the subarachnoid cerebrospinal fluid (Abbott, Rönnbäck, & Hansson, 2006). The barrier responds to regulatory signals from both blood and brain with tight junctions between adjacent cells, while also restricting diffusion of polar solutes through the intercellular cleft. The barrier is permeable to oxygen, carbon dioxide and other gaseous molecules and anesthetics (Grontoft, 1954). Water as well as lipid soluble substances can pass the barrier by diffusion. Carrier-mediated influx, which may be passive or secondarily active, provide transport into the Central Nervous System (CNS) of essential polar molecules (such as glucose, amino acids and nucleosides) that are unable to diffuse through the cell membrane (Saunders, 1992). The BBB plays a crucial role in the maintenance of a strict extracellular environment around synapses and axons (Saunders, 1992). Neuronal interfaces within the CNS depend on chemical and electrical signals and thus a steady neural function is dependent upon the barrier capacity (Serlin, Shelef, Knyazer, & Friedman, 2015). The barriers also regulate the recruitment and entry of leukocytes and innate immune elements and involve in both the reactive and surveillance functions of CNS immunity (Serlin et al., 2015).

2.2 Toxicant Mixture and its' components

2.2.1 Methylmercury

Mercury is a potent environmental contaminant that exerts toxic effects on a variety of vital organs in the human body. It is naturally found in three predominant forms: elemental, organic and inorganic mercury (Clarkson, 2002). One example of the organic form of mercury is methyl mercury, with exposure to high levels resulting in microcephaly, seizures, mental retardation and cerebral palsy (Davidson et al., 1998; Clarkson & Magos, 2006). It is a potent neuro-toxicant, to which humans are mainly exposed through consumption of contaminated fish and marine mammals, particularly large long-lived predators at the top of the food chain (Weston et al., 2014). Ingested MeHg is almost completely absorbed via the digestive tract (Hong, Kim, & Lee, 2012). CH_3Hg^+ is the ionized form of MeHg and the major source of organic mercury for all humans. Transport of CH_3Hg^+ across the blood–brain barrier is because of the *S*-Cys structure (CH_3Hg-*S*-Cys) of MeHg that is structurally similar to methionine (Landner, 1971). With increasing daily fish consumption as a part of a healthy diet, the chronic low-level dietary intake of MeHg has become more common and as such may pose a significant toxicological problem (World Health Organization, 1990). Furthermore, knowledge regarding the cellular and molecular processes underlying the neurotoxic effects are still limited (Davidson et al., 1998). Levels related to disasters exposure have been used to estimate the threshold level for MeHg-induced paresthesia to be 25 to 40 mg of total body burden, corresponding to blood levels of 250 to 400 µg/L and hair levels of about 50 ppm (Davidson et al., 1998).

2.2.2 Polychlorinated Biphenyls

Polychlorinated Biphenyls (PCB) are a class of chemical toxicants that can affect cerebral function (Cauli et al., 2013). These industrial contaminants persist in the environment because of their stable chemical

structure and long half-life which give way to varying mechanisms of toxicity (Cauli et al., 2013; Cummings, Clemens, & Nunez, 2008). PCBs include 209 chemical congeners that differ in the extent and pattern of chlorination of the biphenyl structure (Agency for Toxic Substances and Disease Registry, 2000). For example, exposure to PCB 52, 138 or 180, often occurring through environmental exposures (inhalation of particles, ingestion of contaminated foods and water) reduces motor activity (Boix,et al., 2011). The reduction is caused by altered modulation of extracellular dopamine by metabotropic glutamate receptors in the NAc (Boix,et al., 2011).

In nature, exposure to PCBs most often results from ingestion of contaminated foods such as fish, meat and milk (Cummings et al., 2008). In addition to *in utero* exposure through placental transport, PCBs can also reach the developing offspring by accumulating in maternal milk; thus increasing the likelihood of lactation transfer (Dewailly, Nantel, Weber, & Meyer, 1989). There are two type of PCBs. Dioxin-like PCBs, which are coplanar dioxins, bind to the aryl hydrocarbon receptor (Ah-receptor) and act as endocrine disrupters. Non-dioxin-like PCBs, which are not coplanar, do not bind to Ah-receptors and have been associated with developmental disturbances (Dallaire et al., 2014; Tan, Loganath, Chong, & Obbard, 2009), immuno- and neuro-toxicity (Maervoet et al., 2007; Park et al., 2010), reproductive function, endocrine disrupting effects (Abdelouahab et al., 2008; Maervoet et al., 2007), metabolic effects (Tanaka et al., 2011), and tumour promotion (Edwards, 2005). Furthermore, research findings have not been able to consistently correlate increasing non-dioxin-like PCB exposure with altered spermatogenesis and altered reproductive hormone levels in environmentally exposed men (Skakkebaek, Rajpert-De, Meyts, Main, 2001; Toppari, Larsen, Christiansen, Giwecman, Grandjean, Guilette et al, 1996). Dioxin-like PCBs are more toxic due to higher chlorination of congeners (six or more chlorines), they have been shown to require lower doses to cause cognitive deficits (Newman, et al., 2009). These typically make up the bulk of body burden of PCBs in humans (Newman, et al., 2009).

The mechanism of human neurotoxicity of PCBs is not well understood due to variations in the PCB congeners (usually more than one is present). Differences in the neuropsychological processes of study participants and limited analytic capability of attributing an observed effect to a particular PCB congener has also been noted as a drawback in allowing us to understand their underlying mechanisms of toxicity (Newman, Wagner, Cameto, & Knokey, 2009).

2.2.3 Organochlorine pesticides

Since the 1970s, widespread use of organochlorine pesticides has been drastically restricted because of concerns about their tendency to bio accumulate, their environmental persistence and ability to cause adverse effects on humans (Reid et al., 2013). Examples of organochlorine pesticides include Hexachlorobenzene, Hexachlorocyclohexanes, Hexachlorobenzene as well as the Dichlorodiphenyltrichloroethane group of chemicals (DDT, DDE and DDD) (Jaacks et al., 2016; Reid et al., 2013). Despite this fact, exposure to organochlorines persists. Their exposure has been found to have both acute and chronic effects on human and animal health, with acute health effects in both humans and animals including headaches, nausea, vomiting, dizziness, blood disorders and possible changes in the levels of sex hormones in the blood after breathing in or ingesting these pesticides (Registry, 2002; Reid et al., 2013). Prenatal exposure to organochlorines, in both animals and humans, is associated with a variety of harmful outcomes, including delayed neurodevelopment, poor attention in early infancy, reduced birth size, weight, and head circumference (Samra & Selim, 2009).

2.2.4 Toxicant Mixture

While there exist many toxicant mixtures, the one of interest to us is made up of methyl mercury chloride (1.997 mg/kg), organochlorine pesticides (1.9044 mg/kg) and polychlorinated biphenyls (PCBs) (1.0992 mg/kg); this mixture comprises components measured in the blood of women in a Canadian Arctic region (Bowers et al., 2004; Pelletier et al., 2009). MeHg, PCBs, and organochlorines are known to

affect various physiological parameters including oxidative stress, endocrine functions, levels of neurotransmitters and neuro-receptors, intracellular calcium homeostasis, and related signaling pathways (Fonnum, Mariussen, & Reistad, 2006; Newland & Paletz, 2000; Schantz, Widholm, & Rice, 2003). Changes in any of these parameters could potentially adversely affect brain development and function. Transcriptional perturbation of the genes involved in neuronal differentiation (via Tetrasapin5 and Purkinje Cell Protein 2) and in cell migration (myelin basic protein) may affect the functionality of the cerebellum later in life (Baumann & Pham-Dinh, 2001; Guan, Luo, & Denker, 2005).

2.3 Neurodevelopmental effects from exposure

Exposure to toxicants, during gestation or the early stages of development, can have various effects on the neurodevelopment of the offspring. Given that these toxicants often have additive effects with other co-occurring neurotoxicants, the mechanisms of action often differ. Therefore, this sub-section aims to summarize the neurodevelopmental effects from exposure to the major components of the toxicant mixture among children.

2.3.1 Methylmercury exposure

The appearance of the neurodevelopmental effects following prenatal exposure is often delayed, which makes it difficult to estimate the threshold dose or accurately determine the lowest dose of MeHg that impairs neurodevelopment (Mahaffey, 1998). In fact, the immature central nervous system is extremely sensitive to MeHg neurotoxicity; widespread nervous system damage can thus ensue from exposure (Cernichiari et al., 1995). The human foetus may be five times more sensitive to the effects of MeHg than the adult (Rice, 1995). According to World Health Organization (1990), there is a 5% risk of neonatal neurological damage associated with peak methylmercury exposures measured at 10–20 μg/ g in maternal hair. Some of the neurological damage from exposure includes damage to the

mesocorticolimbic dopamine/glutamate pathways that mediate complex cognitive functions as well as neurocognitive deficits; these have been shown to be related to increased diagnoses of attention deficit hyperactivity disorder and attention-related behaviours, impaired visual recognition memory and cognition and IQ reductions (Weston et al., 2014; Sagiv, Thurston, Bellinger, Amarasiriwardena, & Korrick, 2012). Even at lower doses, prenatal exposure has been associated with deficits in vision and hearing, delayed walking and speech development, and other more subtle developmental delays (Prasad et al., 1979). A direct correlation between the maternal peak level of exposure during pregnancy and psychomotor retardation in the offspring was found, where upon analysing 81 fetal contamination cases of MeHg, the estimated lowest effect level for delayed walking was 7.3 ppm (intake of about 11.2 µg/kg/ day or 1.2 mg/kg) with a 95% upper confidence limit of 14 ppm (intake of about 22.4 µg/kg/ day or 2.4 mg/kg), while that for neurological signs was 10 ppm (intake of about 17 µg/kg/ day or 1.8 mg/kg) with an upper 95% confidence limit of 287 ppm (intake of about 515 µg/kg/ day or 55.2 mg/kg) (Cox et al., 1989).

Prenatal MeHg exposure was also associated with lower scores for the MeHg associations were statistically significant for the Visual Memory Index ($p = 0.01$, compared with 0.14 and 0.08 for the verbal memory and learning indices, respectively) (Orenstein et al., 2014). A 1-µg/g increase in exposure level of MeHg was associated with significantly lower scores on the Finger Windows subtest of the Visual Memory Index, measuring the ability of a child to manually reproduce a demonstrated spatial sequence, and the Visual Learning subtest of the Learning Index, measuring recall of a fixed number of abstract images on a grid (Orenstein et al., 2014). Since the Visual Learning, and Visual Memory subtests primarily measure skills related to visual– spatial memory, the aforementioned findings suggest that low-level prenatal MeHg exposure affects memory development in this domain (Orenstein et al., 2014).

In animal models, MeHg exposure causes alterations in dopamine turnover rates in the hippocampus and corpus striatum, reductions in the number of cortical D1 and D2 dopamine receptors and decreased striatal dopamine release (Bourdineaud et al., 2012; Coccini et al., 2011). MeHg also impacts glutamatergic function by inhibiting the uptake of glutamate into astrocytes and over-activation of NMDA glutamatergic receptors in several brain regions including the basal ganglia, cerebellum, brainstem, and occipital cortex (Pedersen et al., 1982). Developmental exposure of rodents to 8 mg/ kg MeHg on GD 15 protracted HPA axis hyper-activation, as indicated by 4-fold increases in corticosterone at PND90 (Farina, Rocha, & Aschner, 2011). Moreover, developmental exposure to 0.5 mg/kg day MeHg reduced motor coordination in males and females at 7 months (Cauli et al., 2013). Note that MeHg exposure reduced vertical activity in female offspring at 3 months, observed as less exploration of the surrounding areas of the actimeter chamber and thus decreased interruption of infrared detectors (Cauli et al., 2013).

2.3.2 Polychlorinated Biphenyls exposure

PCB exposure during development has been linked to a number of physiological and behavioural deficits in experimentally-exposed animals, with effects linked to particular types of PCB congeners. For example, exposure to different types of PCB congeners can elicit differential motor alterations. For example, exposure to PCB 52 and PCB 153, from GD 7 to postnatal day 21, increases extracellular GABA in the cerebellum and also impairs motor coordination; GABAergic alterations were shown to be positively correlated with changes in cognitive function (Boix, Cauli, & Felipo, 2010; Cauli et al., 2013). Cognitive function was assessed by evaluating the ability of the exposed rats to learn a Y-maze conditional discrimination task, which is dependent on the function of the brain oxide–cGMP pathway. NMDA receptors in the brain play a crucial role in some types of learning

(Bernabeu et al., 1997; Sager, 2004). Activation of NMDA receptors leads to increased calcium in the post-synaptic neuron that activates neuronal nitric oxide synthase for the expression of both the NMDA receptors and nitric oxide (Sager, 2004). Studies have shown that inhibition of the enzyme nitric oxide synthase reduces the learning of some spatial tasks (Sager, 2004). Soluble cGMP is needed for passive avoidance learning and memory with time-dependent increases in cGMP associated with learning (Bernabeu et al., 1997). Exposure to PCB52, PCB138 or PCB180 has been shown to alter the function of the glutamate-nitric oxide (NO)-cGMP pathway as to result in PCB-induced cognitive impairment (Llansola, Montoliu, Boix, & Felipo, 2010). PCB180 and PCB138 impair the function of the glutamate-nitric-oxide-cGMP pathway at nanomolar concentrations, and PCB52 impairs the function of the glutamate- nitric-oxide -cGMP pathway at micromolar concentrations (Llansola et al., 2010). Perinatal exposure to all three PCB congeners, in rat models, increases nitric oxide; PCB52 and PCB138 increases basal cGMP, which then decreases the activation of soluble guanylate cyclase by nitric oxide (Llansola et al., 2010). In contrast, PCB180 decreases basal cGMP which then increases the activation of soluble guanylate cyclase by nitric-oxide (Llansola et al., 2010). Long-term exposure to PCB52, PCB180, or PCB138 reduces the activation of nitric-oxide synthase and the whole glutamate- nitric-oxide -cGMP pathway in response to activation of N-methyl-D-aspartate receptors (Llansola et al., 2010). Rats perinatally exposed to PCB52, PCB138 or PCB180 needed more trials (69±4, 96±4 and 92±4, respectively) than control rats that took 50±4 trials to learn a Y maze conditional discrimination task; which is indicative of impaired learning (Boix et al., 2010).

A study conducted among 44 mother-child pairs recorded dietary intake, from pregnancy to one year following gestation, by combining information on food consumption with a database of concentrations of dioxins and PCBs in Norwegian foods in order to assess exposure potential (Caspersen et al., 2016). The study found that perinatal exposure to PCBs was associated with sex-dependent

effects, cognition and language development at 3.5 years (Caspersen et al., 2016). High exposure to PCB-153, from ingestion of contaminated meat or milk, (median of 2.6 μg/kg body weight/day) was associated with higher odds of children speaking with incomplete grammar. There was an increased likelihood of observing language delays and a lower communication score following both very low (>0.7 μg/kg body weight/day) and high exposure to PCB-153, mainly from the consumption of contaminated fish, with the mechanism of developmental neurotoxicity remaining relatively unclear (Caspersen et al., 2016). Delayed language development, measured using a pre-established grammar rating, was more common in boys (5.8%) than girls (1.9%), with the boys having a cut-off for 'low score' that was at 2 standard deviations below the mean (Caspersen et al., 2016). In a 2 year longitudinal study, after controlling for potential confounding variables, prenatal PCB exposure, as measured from PCB levels in maternal plasma, was significantly associated with altered short-term memory as expressed by lower scores for both verbal and numeric components of the Kaufman Assessment test for Children at 42 months and on the full-scale of the Kaufman assessment test as well as the verbal IQ scores at 11 years of age (Patandin et al., 1999). There was a 4 point deficit between the highest exposure levels (where total maternal PCB exposure was greater than 3 μg/L in maternal blood plasma measured during pregnancy) and lowest exposed group (where total PCB maternal exposure was less than 1.5 μg/L in maternal blood plasma measured during pregnancy) (Patandin et al., 1999). Effects of prenatal PCB exposure were observed on visual recognition memory during the first year of life using the digit cancellation test which measures various aspects of prefrontal cortex functioning, including information processing speed, the ability to focus attention, and executive functioning by assessing the ability of a person to use visual selective attention and visual scanning for numbers shown at different speeds (Boucher et al., 2009). The results found omission errors indicative of delayed developmental milestones, deficits on formal developmental testing, and abnormalities on behavioural assessment in 12 (10%) of the exposed group compared with only 3 (3%) of the children in the control group (Rogan et al., 1988). Decreases in the Mental

Development Index values were found among mothers with maternal breast milk levels of PCBs at 2 weeks postpartum, which is indicative of changes in cognition seen as altered scores on the memory, problem solving, and early language sections of the test, when compared to mothers with lower levels of maternal breast milk levels (Boucher et al., 2009).

2.3.3 Organochlorine pesticide exposure

Organochlorines have been shown to exert specific and diverse effects on well-defined neuroendocrine cells in animal models (León-Olea et al., 2014). For example, human exposure to low levels of methoxychlor comes from inhaling dusts and aerosols in air surrounding areas where methoxychlor is used as well as the consumption of fish caught in water contaminated with methoxychlor (Agency for Toxic Substances and Disease Registry, 2002). For humans, the maximum acceptable daily intake is 0.1 mg/kg/day and the reference dose that bears no appreciable risk of deleterious lifetime effects is 0.05 mg/kg/day with similar values acceptable across the world (Agency for Toxic Substances and Disease Registry, 2002). Both methoxychlor and chlorpyrifos (organochlorine pesticide derivatives) affect reproductive maturation and fertility by altering gonadotropin releasing hormone (GnRH1) biosynthesis at the GnRH neuron, which is responsible for the regulation of GnHR and thus stimulation of the gonads (Gore, 2002). In the female reproductive system, chlorinated pesticide exposure can result in abnormal stimulation of reproductive tract development and function while exposure in males decreases reproductive tissue weights, delays puberty and inhibits testosterone production (Chapin, 1997). Furthermore, a qualitative analysis of cell morphology, using cells cultured from rats revealed that methoxychlor, administered at very low doses similar to the reference and accepted daily intake doses of 10 µM and 100µM, caused retraction of GnRH neuronal processes in the hypothalamus (a brain area part of the reproductive axis) that resulted in increased cell death after 24h exposure during gestational days 1-18 in both sexes (León-Olea et al., 2014). Early developmental exposure to chlorpyrifos on the

other hand, exerts neurotoxic actions by interfering with neuron replication and differentiation which appears to result in behavioural disturbances in exposed rodents; changes in serotonin, arginine-vasopressin and oxytocin levels in the hypothalamus appear to be the targets in this case (Aldridge et al., 2005). One hypothesized mechanism underlying these changes is from the interference of oxytocin synthesis in the hypothalamic paraventricular nucleus, resulting from chlorpyrifos exposure during critical developmental windows, which would in turn affect the arginine-vasopressin-system in other areas of the hypothalamus (Tait, 2006).

2.3.4 Toxicant Mixture exposure

Studies on animal models confirm that the individual components of the contaminant mixture affect brain development, behaviour and cognitive functions (Newland & Paletz, 2000; Schantz et al., 2003). In contrast, gestational exposure to the mixture was shown to produce long term elevations of several cytokines within the hypothalamus of female rat offspring (Hayley et al., 2011); cytokines play a role in the modulation of neurogenesis in the hippocampus with consequent effects on learning behaviours (Arisi, 2014). Administration of the toxicant mixture, at ecologically relevant doses mimicking concentration measured in humans, elevated levels of the pro-inflammatory cytokines IL-1b, and the IL-12, as well as the anti-inflammatory cytokine, IL-10 (Hayley et al., 2011). All of these effects were evident in adulthood, 120 days following the last day of the dosing period (Hayley et al., 2011). Alterations in the cytokine balance within the hypothalamus, during a critical time in neurodevelopment, could contribute to a range of pathological states hindering neuro-immune and hormonal communication with potential consequences on behaviour (Hayley et al., 2011).

2.4 Effects on maternal brain and behaviour

Mothers across all mammalian species undergo a host of changes in their neurobiology and behaviour as they embark on motherhood. Evidently, there are dramatic postnatal changes in maternal behaviour

and the maternal brain that occur during this period. Consequently, the present section synthesizes growing insights from both animal and human research to provide an overview of the plasticity of the mother's brain, following exposure to the components of the toxicant mixture and the toxicant mixture itself.

2.4.1 Methylmercury exposure

Methylmercury neurotoxicity has similarities between adults and children. MeHg neurotoxicity in the adult has a long latent period between exposure and the onset of symptoms, with paresthesia appearing as the first symptom after low exposure (Prasad, Nobles, & Ramanujam, 1979; Lapham et al., 1995). In one study using animal models, they recorded the 4 major components of maternal behaviour in rats following the administration of doses of 0, 0.5 or 2.5 ppm MeHg (equivalent to 0, 0.94 and 5.8 µg/ kg body weight/ day, respectively), given two to three weeks prior to breeding until offspring weaning (Weston et al., 2014). Changes in maternal behaviour, attributed to MeHg exposure alone, were extremely limited with post hoc tests failing to show any significant group differences (Weston et al., 2014). However, changes in multiple regions of the maternal brain were observed following the MeHg exposure. These consisted of - reductions in dopamine, noradrenaline and 3,4-dihydroxyphenylacetic acid (DOPAC) levels in the frontal cortex, as well as a reduction in the hypothalamic dopamine turnover rate (Weston et al., 2014). Furthermore, the maternal olfactory bulbs were also affected, showing reduced serotonin levels and significant reductions in 5 hydroxyindoleacetic acid levels following exposure to 2.5 ppm of MeHg or about 5 µg/kg/ day or 0.5 mg/kg (Weston et al., 2014).

In humans, MeHg exposure has been shown to be related to various changes in the maternal brain. Neuronal death, astrogliosis, microglial activation, and demyelination have been observed with high

exposure levels (Harada, 1995). MeHg has a wide range of cellular targets for their toxic effects such as the inhibition of neuronal Ca^{2+}ion channels, disruption of presynaptic transmitter release and postsynaptic receptor function, destruction of DNA structures and alteration of Na+/K+ ATPase pumps and mitochondrial function (Juárez et al., 2005; Peng et al., 2002). MeHg exposure adversely affects the cerebellum by generating oxidative stress, binding to sulfhydryl groups, depolymerising microtubules, and altering neurotransmission (Prasad et al., 1979). Exposure to polychlorinated biphenyls can also have adverse effects.

2.4.2 Polychlorinated Biphenyl exposure

Alterations in maternal care related to PCB exposure in rodent dams affects the reproductive development of the offspring as maternal care is important for offspring development (Cummings et al., 2008; Cummings, Nunez, & Clemens, 2005). Exposure to PCBs significantly alters maternal licking and grooming of the pups (Cummings et al., 2008). Dams exposed to PCBs spent more time on the nest and more time grooming (and licking) the pups, when compared to dams not exposed to PCBs (Simmons, Cummings, Clemens, & Nunez, 2005). Increased licking and grooming by mothers has been shown to reduce harmful effects of early stressors or to perinatal exposure to contaminants and therefore, these findings suggest that the dams used this maternal strategy to overcome the deleterious effects of the PCBs on their offspring (Meserve, Murray, & Landis, 1992). Furthermore, this altered behaviour decreases the number of motor neurons in the sexually dimorphic spinal nucleus of the bulbocavernosus, in male offspring, as well as reduces the total dendritic arbor in the same region (Cummings et al., 2008). Gonadal hormones are known to act in the establishment of sex differences in the number of motor neurons found in the spinal nucleus of the bulbocavernosus by regulating motor-neuron death during offspring development (Niel et al., 2009). Therefore, changes in the structure result in decreased responsivity to testosterone, because of greatly reduced androgen hormone binding relative to normal males that underwent regulated motor-neuronal death during development (Naess et

al., 1976; Niel et al., 2009). Additionally, exposure to PCBs has been shown to alter the important temporal dynamics of oxytocin synthesis or release that fosters the attachment between mother and offspring (Cromwell et al., 2007).

In animal models, the dioxin-like PCB congener 3,4,3',4'-tetrachlorobiphenyl (PCB 77) can have both estrogenic and anti-estrogenic actions and under some conditions can enhance central dopaminergic activity (Numan et al., 2005), with both estrogen and dopamine playing a role in the initiation and continuation of maternal behaviour in rats (Numan & Stolzenberg, 2009). One study found that dams given 4 mg/kg daily (s.c.) during the gestation and postpartum periods, spent significantly more time licking and grooming their pups than the control dams or those having received 2 mg/kg of this PCB on gestational days 6 through 18 (Simmons et al., 2005). Although the proportion of time nursing was unaffected by the PCB treatments, there was a statistically significant difference in the proportion of total time nursing in the high-crouch posture between the exposed and control groups (Simmons et al., 2005). Both PCB groups (2mg/kg and 4mg/kg PCB) showed significantly less high-crouch nursing than the control group, with no significant difference between the two PCB groups (Simmons et al., 2005). The high-crouch posture involves a distinct rigidity of the limbs and a salient arching of the back and is associated with optimal milk letdown (Lincoln et al., 1980). The reduction of high-crouch nursing posture may be responsible for the lack of normal weight gain shown by the PCB 77 litters (when compared to the weight-gain of the vehicle group), and perhaps decreased capability of providing sufficient stimulation to sustain the high-crouch position among the dams exposed to PCB 77 (Simmons et al., 2005).

2.4.3 Organochlorine pesticide exposure

In humans, both high and low heptachlor epoxide levels, an organochlorine derivative, were positively correlated with psychiatric illnesses as described by the Maternal Brief Symptom Inventory, a 53-item

self-report questionnaire designed to evaluate a range of psychiatric symptoms. The study evaluated 75 human breast milk samples, collected at the postpartum eighth month in cases from a previous Mother–Infant Care Cohort Study taking place in the suburban area of Ankara, Turkey (Yalçın et al., 2015). Self-administered questionnaire about characteristics of infant and mother was given to these lactating women within the first three days after delivery; psychological tests also administered at the postpartum second month (Postpartum Bonding Questionnaire and the Mother-to-Infant Bonding Scale) and Edinburgh Postnatal Depression Scale at the eighth month. The four indexes of the Maternal Brief Symptom Inventory that were reportedly affected following exposure to heptachlor include depression ($p = 0.040$), anxiety ($p = 0.032$), hostility ($p = 0.015$) and phobic behaviours ($p = 0.017$) (Yalçın et al., 2015). These findings suggest that mothers with high measured heptachlor epoxide levels (over 22.1 ng/g lipid weight in breast milk samples) might show signs of psychopathologies which may in turn eventually lead to perturbations in infant-mother bonding (Yalçın et al., 2015). Furthermore, some organochlorines are also known to act as weak estrogen and androgen receptor agonists (Gore, 2008). Thus, one mechanism of these compounds' direct effect on the neuroendocrine system may be via activation of nuclear or membrane bound estrogen receptors (Heldring et al., 2007). Since these neuro-hormones regulate social behaviour and reproduction, any altered expression, caused by organochlorine exposure, could be associated with the behavioural alterations mentioned. Organochlorines can also adversely affect the dopaminergic and serotonergic systems, important players in maternal behaviour (Slotkin et al., 2007).

2.5.4 Toxicant Mixture

Early life manipulations, often through the expression of maternal behaviours like pup licking, can alter gene expression in several brain regions, through modified DNA methylation patterns (Kosten & Nielsen, 2014). The resulting changes in the phenotype may lead to epigenetic modifications in gene expression

(Kosten & Nielsen, 2014). Another study conducted by Bowers examined the impact of DNA methylation with gestation and postnatal day 21 exposure to high or low doses of organochlorine, methylmercury chloride and polychlorinated biphenyls. These results show that mRNA abundance for DNA methyltransferase was significantly reduced in the high PCB and MeHg groups, whereas organochlorine pesticides had no significant effects on levels compared with control (Desaulniers et al., 2009). Other studies have shown causal evidence that maternal behaviour of licking or grooming affects DNA methylation levels of Nr3c1 or ERα promoter regions in offspring (Edelmann & Auger, 2011; Weaver et al., 2004). Taken together, these results demonstrate the link between maternal behaviour and altered DNA methylation levels and the epigenetic changes that can occur following gestational and lactational exposure to a mixture of PCBs, Organochlorines and MeHg.

Few studies have assessed outcomes from human co-exposure to some of the components of this toxicant mixture. These show that gestational exposure to combined PCBs and mercury can cause cognitive disturbances in infants and children (Stewart, Reihman, Lonky, Darvill, & Pagano, 2003). For example, 212 children exposed to both PCBs and MeHg from contaminated fish consumption during gestation, (with exposure levels measured using maternal blood and maternal hair MeHg samples) were assessed in the Oswego Newborn and Infant Development Project (Stewart et al., 2003). Cognitive functioning and motor development assessments were measured using the McCarthy Scales of Children's Abilities, at 38 months of age. Using this assessment, researchers found statistically significant predictors of small but measurable deficits in McCarthy performance (Stewart et al., 2003) from exposure greater than 0.52 ng/g/wt in maternal blood and 0.60 ng/mg in maternal MeHg hair samples. However, upon reassessment at 54 months of age, no relationship between toxicant exposure and McCarthy performance was found; note that inspection of the age-related trajectory of McCarthy

performance revealed that the more highly exposed children caught up with the least exposed children by 54 months of age (Stewart et al., 2003).

2.5 Objectives

The aim of our work is to gain a better understanding of peripartum maternal brain plasticity particularly upon exposure to an ecologically relevant toxicant mixture by:

- Characterizing the effects of the toxicant mixture on the number of estrogen receptors in the VTA, mPOA and NAc in the postpartum maternal brain.
- Assessing whether any effects in these brain areas relevant to maternal behaviour are due to exposure to the MeHg component in particular.

2.6 Hypothesis

We hypothesize that we will observe a reduction in the levels of estrogen receptors in the mPOA, and potentially in the other brain areas of interest, in rat dams having been exposed to the toxicant Mixture and MeHg perinatally, when compared to the vehicle control group. Support for this hypothesis, stems from the results of several studies that have shown that *in vitro* exposure to MeHg increases the levels of reactive oxygen species and induces dyshomeostasis in calcium and glutamate levels, all of which contribute to permanent neurochemical changes in the central nervous system (Brookes & Kristt, 1989; Reynolds & Hastings, 1995). Furthermore, *in utero* exposure to MeHg in rat pups has been shown to impair development, learning, memory and response to injury, with such effects being attributed to neuronal injury from the accumulation of glutamate in the synaptic cleft (Falluel-Morel et al., 2007; Ozawa, Kamiya, & Tsuzuki, 1998).

With respect to the histological changes observed after MeHg gestational exposure, a significant reduction in the number of neurons has been described (Carvalho, Nazari, Farina, & Muller, 2008; Falluel-Morel et al., 2007). When compared to the Mixture exposed group, we hypothesize that dams exposed to MeHg will show the greatest loss of ER protein expression. The addition of the other mixture components would either have no additional effect or may be additive to the effects shown by MeHg, as typically shown with exposure in rat pups (Bowers et al., 2004).

Chapter 3. Methodology

3.1 Animals

All treatment procedures and housing conditions complied with the Canadian Council on Animal Care guidelines and had been reviewed and approved by Health Canada's Institutional Animal Care Committee prior to the start of the study. The rats were kept and reared at the Health Canada laboratory facilities.

Seventy nulliparous female Sprague-Dawley rats, weighing 200-230 g at the time of arrival and 38 males, weighing 320-350 g were purchased for breeding from Charles River Laboratories (St Constant, QC). They were housed in polycarbonate hanging cages and kept on a reverse light cycle (12h: 12 hour, light: dark with lights on at 20:00h). The rats were mated by placing 2 females in the cage of a male; the females were checked twice a day for a vaginal plug. The day of detection of a vaginal plug was denoted as gestation day 0 (GD 0) at which times the females were returned to individual cages.

3.2 Treatment

In order to habituate them to the dosing methodology, all dams were given Teddy Graham cookies (Nabisco Ltd., Toronto, ON) dosed with 250μl of clean corn oil (Mazola brand, Bestfoods, Canada) for 5 days prior to breeding. This dosing technique was found as an effective, non-stressful and accurate method of delivering measured doses of the chemicals based on body weight (Bowers et al., 2004).

Pregnant rats were then randomly assigned to one of the treatment groups (see Table 2) and dosed daily via a Teddy Graham cookie from GD 1 to postnatal day 20 (PND20), with a body weight-adjusted

volume (1.0 ml/kg body weight). The pre-measured dose of the toxicant mixture or MeHg was dissolved in corn oil as the vehicle. The section below details the composition of the contaminant mixture. Body weight of the pregnant rat was taken daily and used to determine the volume of the contaminant dose to be administered. The levels of fat in the cookies and oil used as vehicle caused a slight increase in dam body weight, but this procedure was consistent between treatments (Bowers et al., 2004).

The high doses of 4.0 mg and 1.0 mg /kg body weight of the mixture and MeHg, respectively, were decided upon as the maximum dose, given that earlier trials with higher doses were associated with a high offspring mortality rate. The mixture concentrations used for this experiment were similar to those measured in human blood samples (Chu et al., 2008). Starting on GD 18, the dams were monitored three times daily for parturition at 07:00, 15:00, and 23:00h.

Table 2. Treatment groups and doses (1ml/kg body weight). Concentrations of dosing solutions are 4.0, 0.04 µg/ml for the mixture, 1.0 and 0.01 µg/ml for MeHg where the doses shown are also the concentration of the dosing solutions in mg/ml corn oil - (Bowers et al., 2004).

Treatment	Dose (mg/kg/day)	
	High	Low
Full mixture	4.0	0.04
Methyl mercury	1.0	0.01
Vehicle	0	

3.3 Contaminant Mixture

The contaminant mixture was created based on previously reported values for each component among Canadian Arctic Populations (Bowers et al., 2004; Chu et al., 2008). The contaminant mixture is made up of MeHg chloride (1.997 mg/kg), organochlorine pesticides (1.9044 mg/kg) and PCBs (1.0992 mg/kg). PCB congeners and organochlorine pesticides that make up the mixture are described in Table 3.

Table 3. The full chemical mixture with PCB, MeHg and organochloride pesticides. The values shown are for individual doses in mg/kg/day. The relative concentrations are based on measurements in fish consumers living in Great Lake/ St. Lawrence Basin (Bowers et al., 2004).

PCB Congener	**Dose (mg/kg)**	**Organochlorine pesticides**	**Dose (mg/kg)**
28	0.0072	Aldrin	0.0049
52	0.0154	-HCH	0.0746
99	0.0973	Cis –Nonachlor	0.0525
101	0.0145	p,p'-DDE	0.9187
105	0.0165	p,p'-DDT	0.0569
118	0.0727	Dieldrin	0.0223
128	0.0071	Hexachlorobenzene	0.2961
138	0.2146	Heptachlor epoxide	0.0232
153	0.3177	Mirex	0.0291
156	0.029	Oxychlordane	0.1359
170	0.0562	Toxaphene	0.0699
180	0.1522	trans-Nonachlor	0.2203
183	0.0193		
187	0.0795	Methyl mercury Cl	**1.0**
Sum PCB	1.0992	OC pesticides	1.9044
% PCB	27.5	% non-PCB	72.5
Total Dose			**4.0036**

3.4 Harvesting and preparation of brain tissue

Dams were sacrificed on postpartum day 21 (PPD21). Briefly, they were anesthetized with isofluorane, ex-sanguinated via cardiac perfusion with 0.09% saline and the brain fixed by perfusion with 4% paraformaldehyde. The brains were then removed and further fixed in 4% paraformaldehyde overnight followed by a minimum of 48h in 20% sucrose. Rat brains were sectioned coronally at 30µm at -21°C using a cryostat (Leica model 1580). Sections were sequentially placed in a 12-well dish filled with 0.01M PBS. The 12^{th} section was thaw-mounted onto a slide for future Nissl staining. Once the free-floating sections were further rinsed in PBS, they were transferred to labelled microtubules filled with cryoprotectant (0.01M PBS, polyvinyl pyrrolidone, saccharose, and ethylene glycol) and stored in a -20°C freezer until the time of immunohistochemistry.

3.5 Immunohistochemistry

Immunohistochemical labeling techniques use an antibody to bind specific antigens in tissues of interest, which in our case are brain sections. This primary antibody is then recognized by a secondary antibody that is biotinylated. To better visualize the signal, it is amplified and finally visualized with the 3'-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) chromagen. The exact protocol for immunohistochemistry on free floating sections using a rabbit anti-estrogen receptor α monoclonal antibody (Millipore: dilution of 1:5000) is presented in Appendix A; this protocol has previously and successfully been employed in our laboratory.

The Immunohistochemistry for ERα was completed on equivalent sets of sections for 35 animals. Briefly, sections were rinsed 3x5min in 0.01M phosphate buffer (PBS, pH: 7.2-7.3) and 3x5min rinses in PBS containing 1% Triton X-100 (PBS-T) in order to help permeabilize the cell membranes. Denaturing and

permeabilization of the cell nucleus was achieved by incubating the sections for 10 min in 5% dimethyl sulfoxide (DMSO). This step allows the reagents to penetrate inside the cells. There were 3x5min rinses in 1% PBS-T then 20 minute incubation in 0.6% H_2O_2 to block endogenous peroxidases. Since endogenous peroxidases can react with the substrate solution (hydrogen peroxide and the chromogen, DAB) and lead to false positives. Incubation in 0.6% H_2O_2 followed by incubation in PBS containing 0.4% Triton X-100and 10% normal goat serum (NGS) for 1h, (Vector Laboratories) allows the reduction of this non-specific background staining. Afterwards, sections were incubated with a primary antibody, rabbit anti-ERα monoclonal antibody (Millipore: dilution of 1:5000 in PBS containing 0.4% Triton X-100) (MC-20, sc-542, Santa Cruz, USA) for 1 hr at room temperature and 24 hours at 4°C to allow enough time for binding to the target antigen.

Following primary antibody incubation for 24 hours, the sections were rinsed 3x5min in PBS containing 0.4% Triton X-100, incubated with the secondary antibody, biotinylated anti-rabbit made in goat (Vector Laboratories: dilution of 1:1000 in PBS-T for 2 hours as to allow binding of the secondary to the primary antibody. Following three rinses in PBS-T (3×5min), the sections were incubated for one hour in an Avidin-biotin complex (Vector Laboratories Vectastain ABC kit) which amplified the target antigen signal and increased detection efficiency of ERα in the nucleus with the chromagen stain. After a final rinse of 3x5min in 0.01M PBS, the tissues were then stained with 3,3' –diaminobenzidine (DAB) (1.7mM, Sigma-Aldrich, USA) at room temperature in the fume hood for approximately 3 minutes. Distilled water was used to stop the chromagen reaction. ThreeX5min rinses in PBS were completed.

Following immunohistochemistry, brain sections were carefully mounted on slides. Once dried, the sections were dehydrated in increasing concentrations of ethanol followed by two rinses in xylene and finally cover slipped.

3.6 Microscopy and Cell Counting

Brain sections on the slides were visualized using a Zeiss Axio Imager microscope and black and white photographs were taken using a Zeiss AxioCam camera coupled to a computer.

Anatomical structures of interest namely the mPOA (see figure 1A), NAc (see figure 2A), and VTA (see figure 3A) were identified using the Paxinos and Watson (2007) rat brain atlas with the bregma values for each area described in table 4.

Table 4. Bregma values from the Paxinos and Watson (2007) rat brain atlas used to photograph brain areas of interest for quantification

Brain area	**Bregma**
mPOA	0.48 to -0.12
NAc	3.00 to 0.48
VTA	-4.68 to -5.04

Bilateral photographs were taken of all sections where the structure of interest appears, using the 40x objective. Missing data (missing section, torn section and debris) were noted. Counts were done for the mPOA (see figures 1B and 1C), the NAc (see figures 2B and 2C) and the VTA (see figures 3C and 3D) from each brain area provided that they had no obstructions in or near the brain region of interest. Examples of obstructions include rips, folds and air bubbles trapped during the coverslip adhesion process. Following the elimination of sections that contained any of the obstructions, additional Immunochemistry was completed as to ensure each region had at least 6 animals per treatment group to allow for robust statistical analysis values.

Immunopositive cell counts were assessed with the help of the Image J software (NIH). Bilateral representations of each section, for each brain region, were counted separately and the bilateral counts were averaged over the multiple sections. A rectangle of (4inch by 5.5inch) was drawn on the image and the immunopositive cells fully appearing within this rectangle were counted and recorded. Only cells that were fully grey in colour and fully appeared within the rectangle were counted. Cells that were excluded from the counts met one or more of the following criteria: More than 60% of the cell size was found outside the rectangle; cells where the staining was same colour as the background and finally cells with a diameter that was larger than 0.8cm as these were most likely debris or other material.

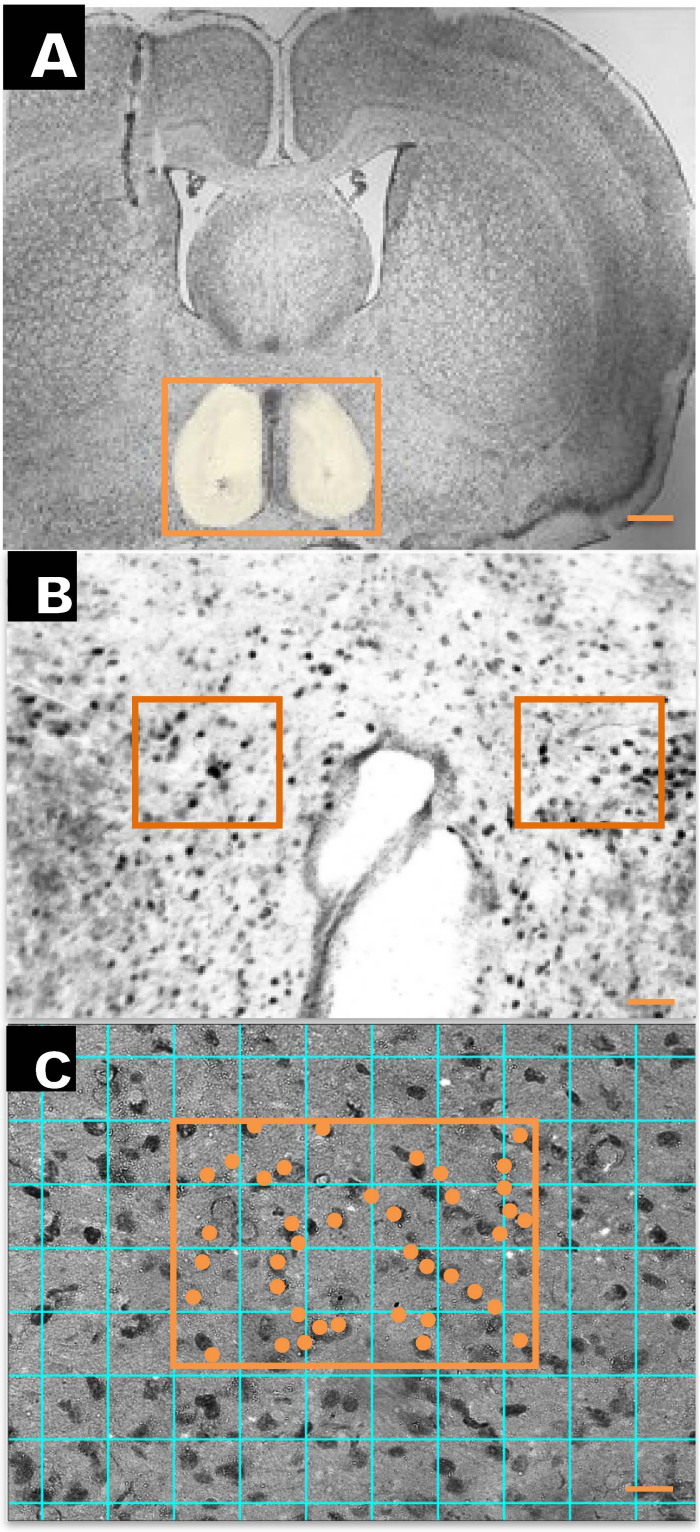


Figure 1. Photomicrograph of the mPOA region at A) 40x B) 200x and C) 400x. The orange square shows the area where cell counts were completed with the orange dots indicating positive ER cells. Scale bar, 50 µm

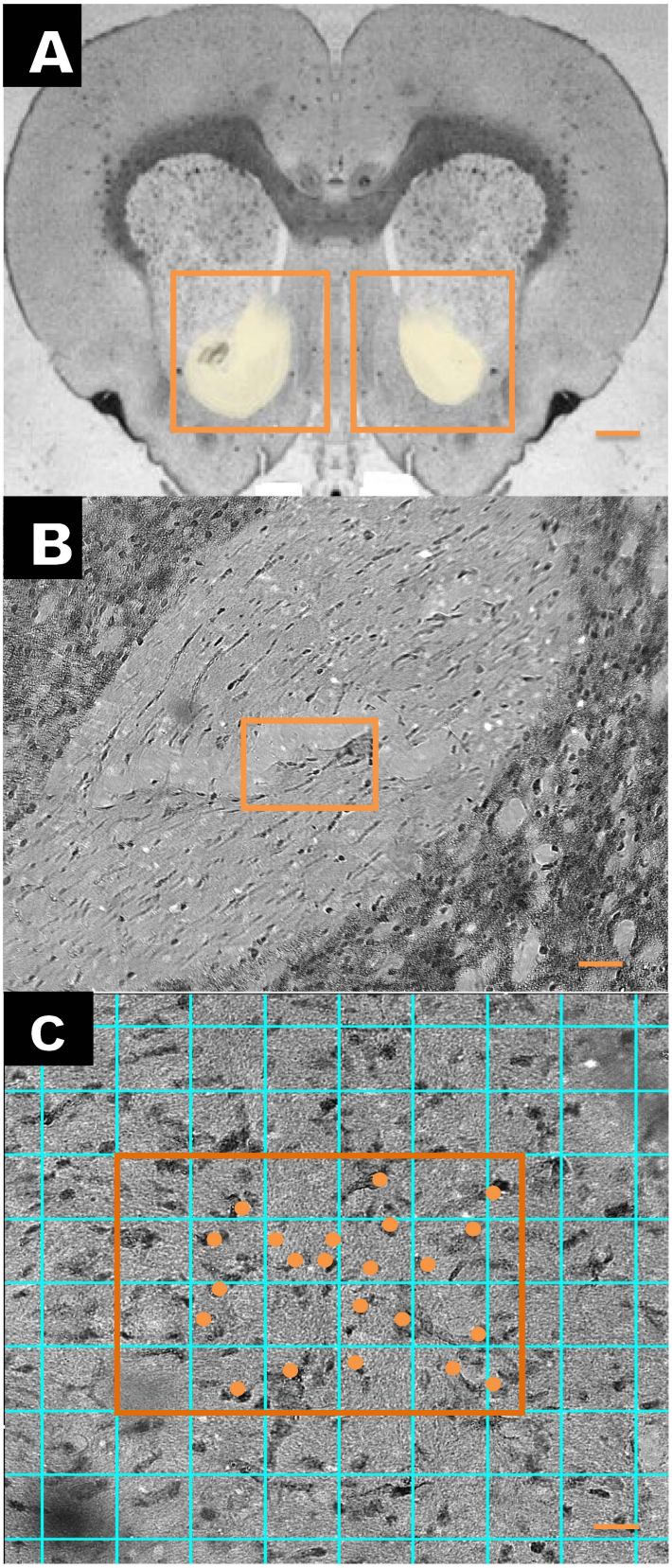


Figure 2. Photomicrograph of the NAc at A) 40x B) 200x and C) 400x. The orange square shows the area where cell counts were completed with the orange dots indicating positive ER cells. Scale bar, 50 µm

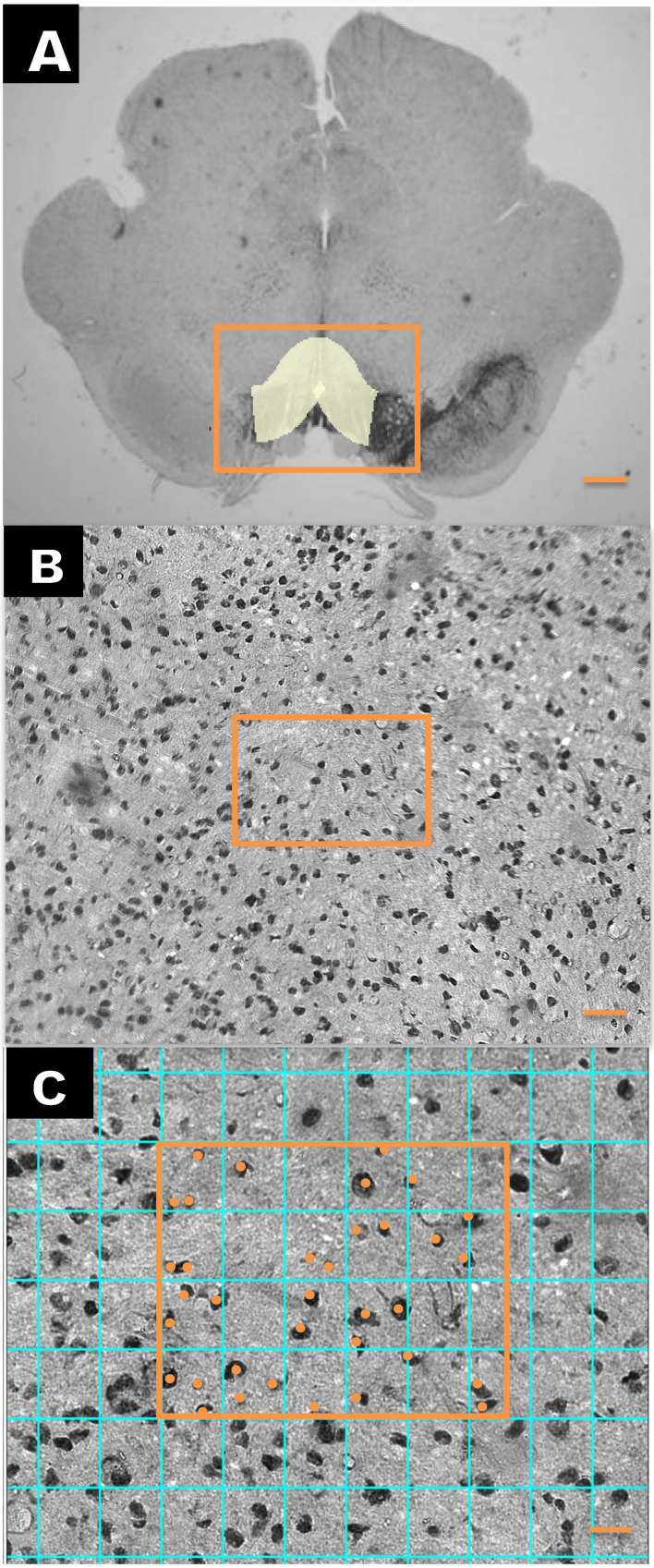


Figure 3. Photomicrograph of the VTA region at A) 40x B) 200x and C) 400x. The orange square shows the area where cell counts were completed with the orange dots indicating positive ER cells Scale bar, 50 µm.

3.7 Data analysis

Homogeneity of variance was verified using a Levene's test using SPSS software (IBM Corp, 2015). Any deviation from homogeneity (indicated by a p value less than .05) was dealt by transformation of data. A square root transformation was to be used; the data were checked again for normality and homogeneous variance.

A one way analysis of variance (ANOVA) was first conducted to see if there were any effects of treatment. This was done separately for each brain area. Following this, we conducted a factorial ANOVA with the two independent factors of Treatment (MeHg vs Mixture) and Dose (Low vs High) to assess whether we could detect any interaction of these on the cells counts. Further analysis consisted of verifying whether the Low dose was significantly different from the Vehicle, using a simple ANOVA. When these were deemed to not be different, we further pooled the values to compare the Control Dose versus the High Dose on the number of immunopositive cell counts.

Chapter 4. Results

This section will summarize our findings for the three brain regions, the mPOA, VTA and NAc. A synthesis of all statistical results appears in Table 5.

Table 5. Mean counts of ER staining in brain areas for all experimental animals of all groups ± SEM (n = 6 in each group), $p<0.05$ for the low and high dosage groups of both MeHg and the Toxicant Mixture. For abbreviations of brain areas, see first page; for statistical comparisons, see text.

	Low MeHg	Low Mix	High MeHg	High Mix	Vehicle
mPOA ERα Count	97.20 ± 22.62	93.65 ± 17.99	53.62 ± 7.85	54.77 ± 12.48	85.85 ± 15.67
NAc ERα Count	48.21 ± 6.74	46.87 ± 6.91	44.67 ± 5.21	42.69 ± 7.56	54.56 ± 3.54
VTA ERα Count	47.50 ± 10.69	41.12 ± 11.24	24.62 ± 5.03	26.10 ± 4.27	32.292 ± 19.60

4.1 medial Preoptic Area

ERα positive cells were counted in the mPOA (Figure 1C). Levene's test for homogeneity of variances indicated no heterogeneity between the groups ($p=0.18$). An overall ANOVA revealed no significant effect of treatment in the number of ERα+ cells (see Figure 4A). A two way ANOVA revealed no interaction between Treatment and Dose ($F_{1,20} = 0.02$; $p=0.89$) nor a main effect of Treatment ($F_{1,20} = 0.005$; $p=0.94$) on the number of immunopositive cells. However, we did see a significant difference between the High and Low Doses ($F_{1,20} = 6.45$; $p=0.02$), with the high dose reducing the number of ERα+ cells in the mPOA (see Figure 4B). A verification between the Low Dose and the Vehicle showed that there was no significant difference between these two ($p=0.68$). This allowed us to pool the vehicle

animals and those having received the Low Dose in order to compare this pooled Control Dose to the High Dose. Results of the analysis show that there is a significant difference in the number of ERα+ cells between Control and High doses, where the High Dose reduces the number of ER receptors in the mPOA ($F_{1,28} = 7.56$; $p=0.01$), see figure 4C to 4G.

4.2 Nucleus Accumbens

While group variances were homogenous (Levene's test $p=0.79$), statistical analysis with a simple ANOVA revealed no significant effect of treatment ($F_{4,25} = 0.34$; $p=0.84$). A further factorial ANOVA with Treatment and Dose revealed no interaction between these two factors, nor any main effect of either factor (Treatment: $F_{1,20} = 0.06$; $p=0.81$; Dose: $F_{1,20} = 0.34$; $p=0.57$), see Figure 5A and 5B. Figure 5C to 5G shows ERα positive cells in the NAc region for all five treatment groups.

4.3 Ventral Tegmental Area

ERα positive cells in the VTA are shown in Figure 3 of the different treatment groups. Analysis via a Levene's test indicates that group variances were homogeneous ($p=0.16$). A simple ANOVA revealed no significant effect of treatment ($F_{4,25} = 1.38$; $p=0.27$), see Figure 6A. A two way ANOVA looking at the interaction of Treatment and Dose revealed no significant interaction between these two factors ($F_{1,20} = 0.22$; $p=0.65$), and no significant effect or Treatment ($F_{1, 20} = 0.08$ $p=0.77$). However, we did detect a significant effect of Dose ($F_{1,20} = 5.05$; $p=0.036$), with the high Dose significantly reducing the number of ERα+ cells (see Figure 6B). Since a simple ANOVA revealed no difference between the Vehicle and Low Dose ($p=0.47$) we pooled these together as a Control Dose. Further analysis did not reveal any significant difference between the Control and High Dose in the number of ERα+ cells in the VTA, see figures 6C to 6G.

Verification between the low treatment groups and the vehicle showed that there was no difference between the two groups ($F_{1,16} = 0.99$; $p=0.33$). This allowed us to pool the Vehicle animals and Low Dose animals to compare to the High Dose. Results of this analysis indicate that there is no significant difference in the number of ERα+ counts between the Vehicle and High Dose ($F_{1,28} = 1.29$; $p=0.27$).

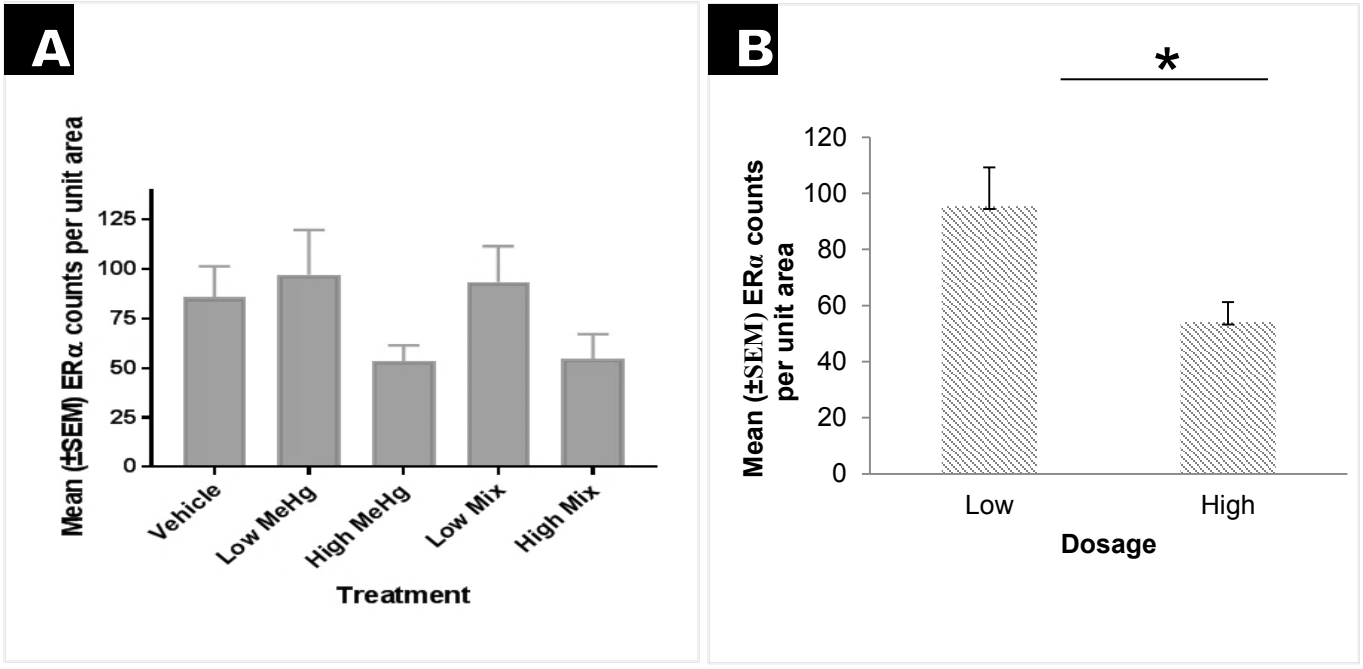


Figure 4. A) Mean (± SEM) ERα+ Cell counts shown for the mPOA region; n=6/group. No significant effects of treatment on average ERα numbers was detected ($p<0.05$). B) Mean (± SEM) ERα+ Cell counts shown for the mPOA region of high and low dosage groups ; n=6/group. A significant effect of Dose (High vs Low) on average ERα numbers was detected ($p<0.05$).

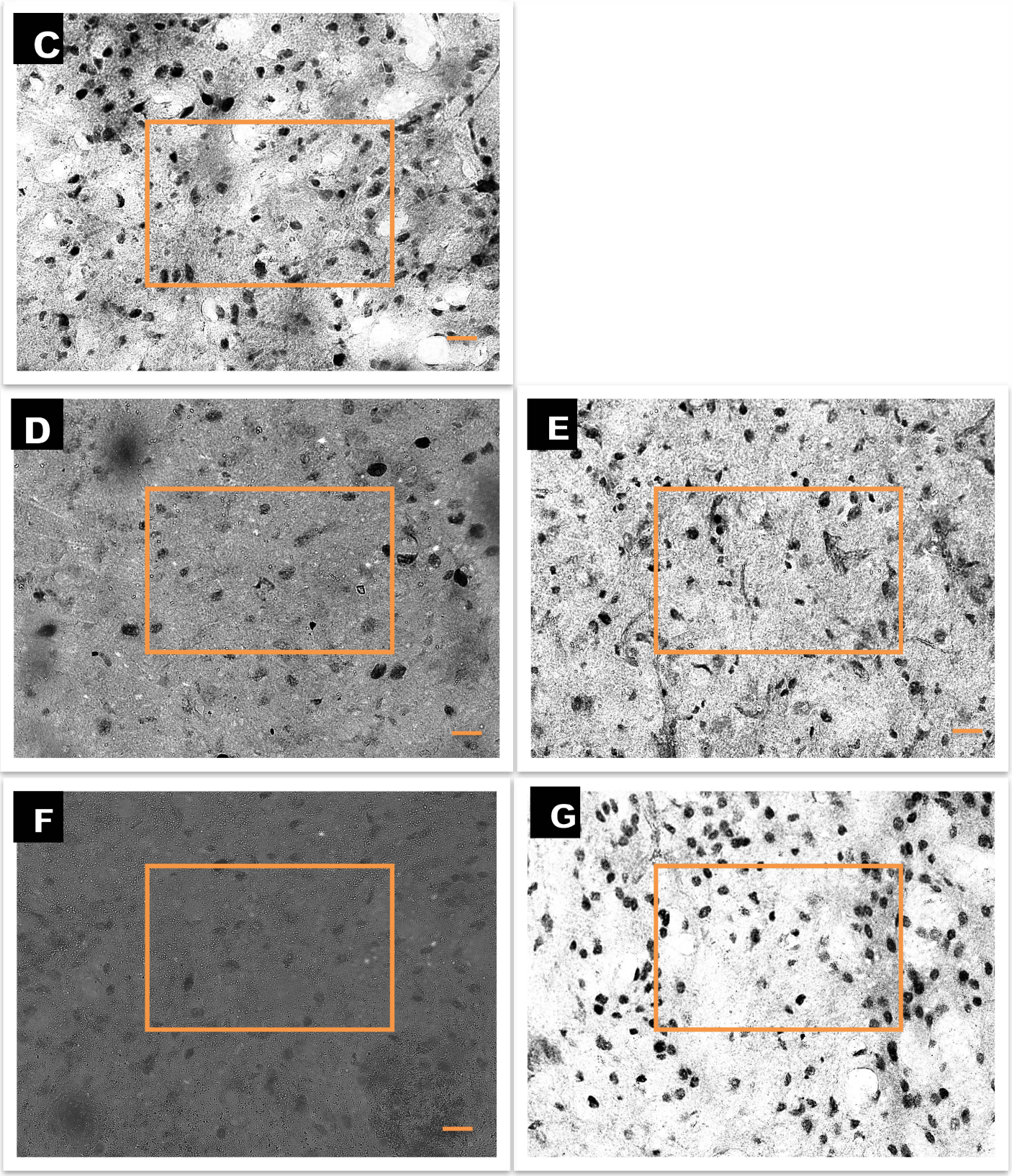


Figure 4. Representative photomicrographs of ERα+ cells in the mPOA at 400X for the C) vehicle, D) high toxicant mix and E) low toxicant mix, F) low MeHg and G) high MeHg. The orange square shows the area where the ERα cell counts were completed. Scale bar, 50 µm

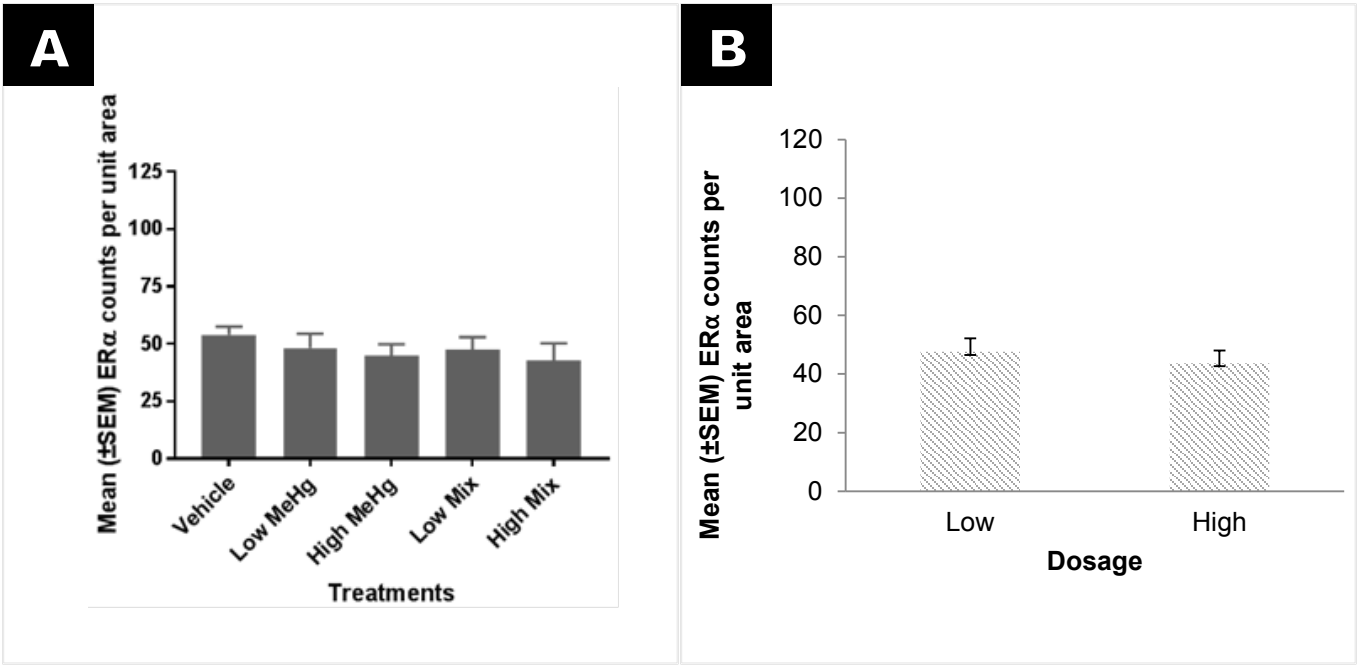


Figure 5. A) Mean (± SEM) ERα+ Cell counts shown for the NAc regions; n=6/group. No significant effects of treatment on average ERα numbers was detected ($p<0.05$). B) Mean (± SEM) ERα+ Cell counts shown for the NAc region of high and low dosage groups ; n=6/group. No significant effects of treatment on average ERα numbers was detected ($p>0.05$) between the low and high dosage groups.

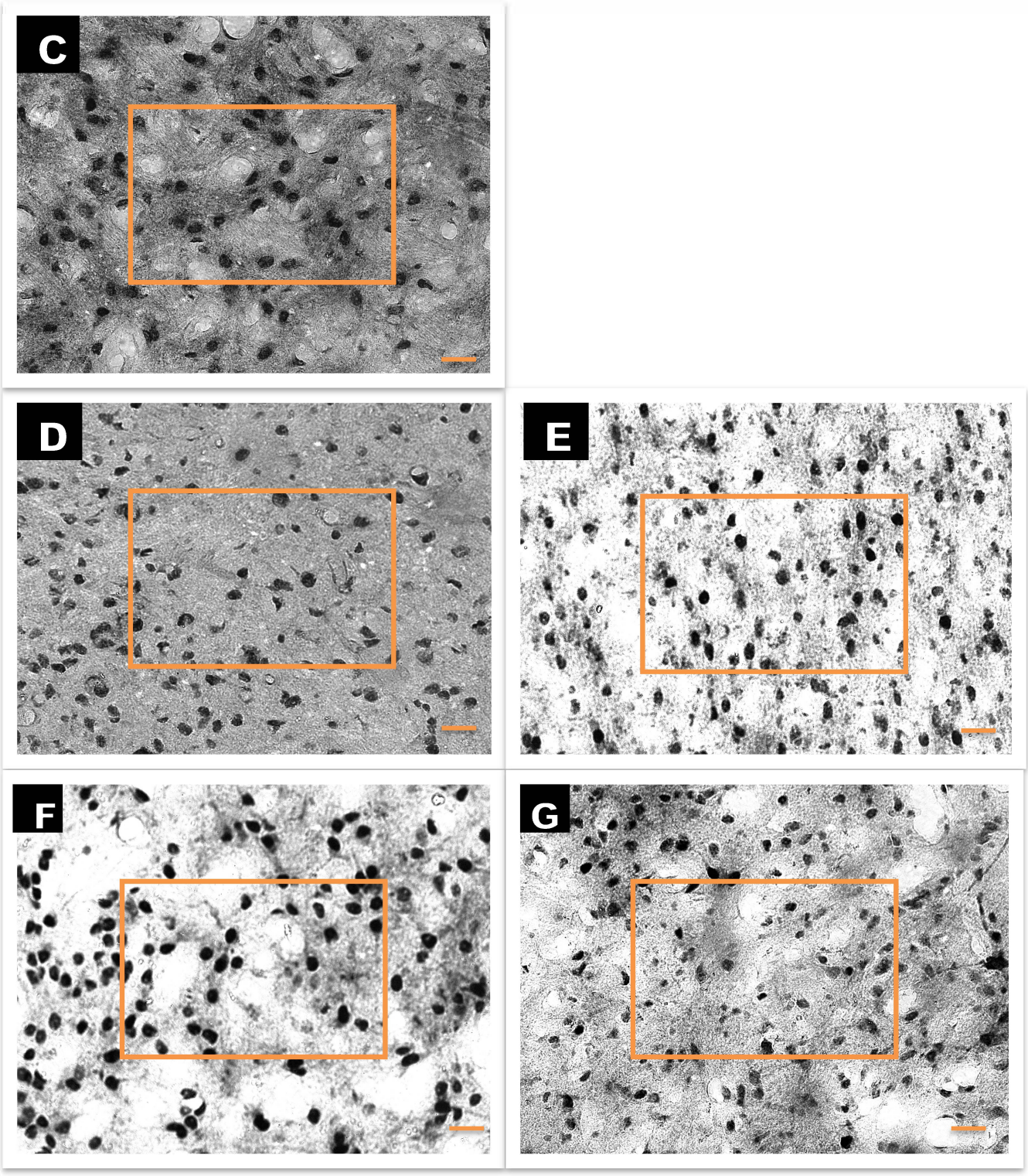
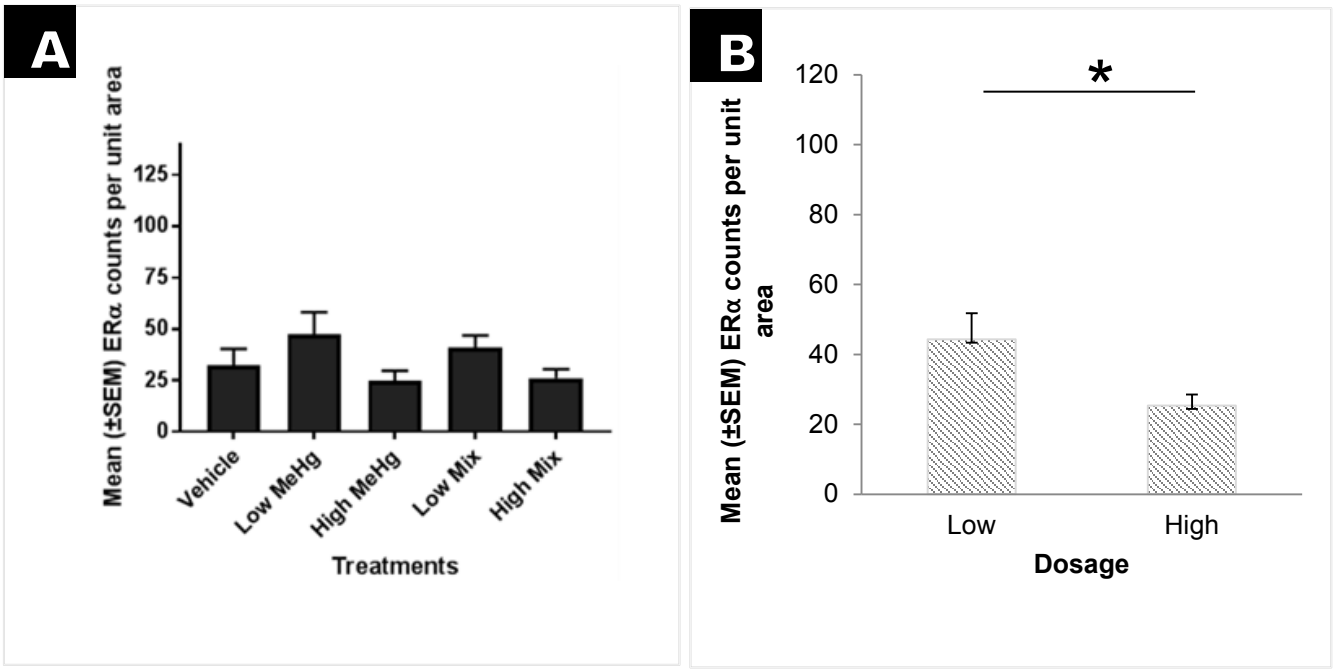


Figure 5. Representative photomicrographs of ERα+ cells in the NAc at 400X for the C) vehicle, D) high toxicant mix and E) low toxicant mix, F) low MeHg and G) high MeHg. The orange square shows the area where the ERα cell counts were completed. Scale bar, 50 µm.



F**igure 6.** A) Mean (± SEM) ERα+ Cell counts shown for the VTA regions; n=6/group. No significant effects of treatment on average ERα numbers was detected ($p<0.05$). B) Mean (± SEM) ERα+ Cell counts shown for the VTA region of high and low dosage groups ; n=6/group. Significant effects of treatment on average ERα numbers was detected ($p>0.05$) between the low and high dosage groups.

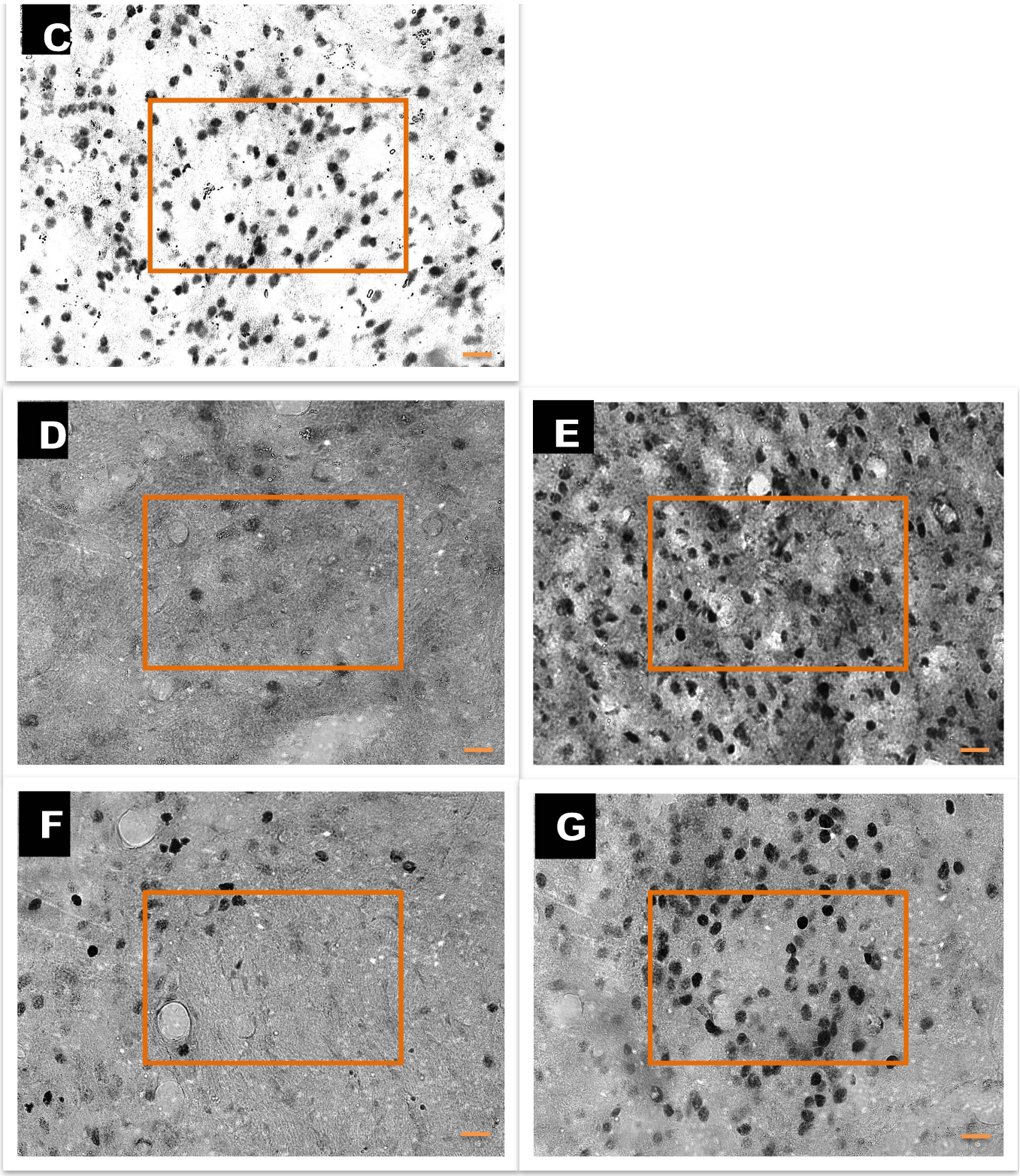


Figure 6. Representative photomicrographs of ERα+ cells in the VTA at 400X for the C) vehicle, D) high toxicant mix and E) low toxicant mix, F) low MeHg and G) high MeHg. The orange square shows the area where the ERα cell counts were completed. Scale bar, 50 µm

Chapter 5. Discussion

The characterized behaviours that constitute maternal behaviour all promote the survival of offspring and can be affected by physiological and environmental factors. Perinatal and/or gestational exposure to environmental toxicants can either directly or indirectly alter offspring neurodevelopment (Grandjean & Landrigan, 2014; Rice & Barone, 2000). Brain regions essential in the regulation of these maternal behaviours include the mPOA, VTA and the NAc. The presence of estrogen, that binds to the ERα receptors in these areas, facilitates maternal behaviour (Bridges, 1996; Numan et al., 2005). It would therefore be expected that neurotoxicants that change the number of ERα would have a great impact on maternal behaviour, thus influencing the care given to the offspring (Pawluski et al., 2016).

5.1 mPOA

Given that previous studies have shown permanent neurochemical changes and neuronal injury caused by *in vitro* and *in utero* exposure to MeHg respectively, we hypothesized the lowest expression of estrogen receptors, depicted as fewer immunohistochemically stained cells in brain areas of interest, would be found in the brains of the dams exposed to the high dose of MeHg (Bowers et al., 2004; Stavenes Andersen, Voie, Fonnum, & Mariussen, 2009; Weston et al., 2014). Analysis of our results on the effects of exposure to high and low doses did yield statistically significant differences in the number of ERα in the mPOA.

Although there were no studies that examined the effects of the toxicant mixture components on ER expression, one study examined the effects of methylmercury on maternal behaviour. ERα protein

expression levels were determined following maternal exposure to low, environmentally relevant doses (0.02, 0.2, and 2 μg/g mother bw/day) of the estrogenic pesticide Methoxychlor over gestational days 11 to 17 (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). Methoxyclor is a mixture composed of organochlorine compounds; with these organochlorine compounds making up 72.5% of the toxicant mixture used in this study. Both studies had similarities in the doses used and method of dosing administration. However, outcomes from the Palanza (2002) study focused on behaviour and not receptor expression. Generally, dams showed an increase in the time spent in self-maintenance behaviours (grooming, eating, and drinking) and decreased time spent performing nest-related behaviours, following exposure to methoxychlor (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). Over the eight observational days, the dams treated with 2000 μg/g had the lowest amount of time spent inside the nest and nursing and more time eating and resting outside the nest (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). Analysis of the time-related changes in maternal behaviour, using within-group post hoc analysis tests, showed females of the control group decreased the time spent in nest and nursing from PPD 11 and increased time eating and resting from PPD 15 (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). In comparison, gestational dosing of 2000 μg/g of MeHg resulted in dams exhibiting these behavioural changes much earlier; PPD 4 for nursing, PPD 5 for eating and PPD 7 for resting (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). Similarly, dams administered the increasing doses of 20 μg/g and 200 μg/g groups exhibited earlier behavioural pattern changes with similar time frames as those observed among the 2000 μg/g treatment group (Palanza, Morellini, Parmigiani, & Vom Saal, 2002). As our study found variations in ERα expression between the high and low treatment groups, the findings from Palanza, Morellini, Parmigiani, & Vom Saal (2002) study suggest that the observed variations in maternal behavior are associated with differences in ERα expression with lactating, high licking and grooming mothers showing increased ERα

expression in the mPOA, compared with lactating low licking and grooming dams (Champagne, Weaver, Diorio, Sharma, & Meaney, 2003).

5.2 NAc

The results of this study found no statistically significant effect on treatment on the number of ERα in the NAc using factorial comparison of drugs and dosage. To our knowledge, there is no literature that examined the effects of different components of the toxicant mixture on maternal behaviour or expression of ERα in this area. However, other literature has examined the impact of changes in dopamine expression on maternal behaviour, a key neuromodulator in the NAc. Recall, that dopamine release in this brain area is crucial for the expression of motivated behaviours, while estrogen acting at ER found in this area, regulates normal dopaminergic function and protects against damage of dopamine neurons after neurotoxin exposure (Morissette et al., 2008). As demonstrated with *in vivo* studies, rats engaged in pup-licking and nursing behaviours have increased dopaminergic activity in the NAc (Champagne & Curley, 2016). In the absence of maternal hormones, studies have found increased dopamine release among female rats, in NAc postpartum, following experiences with their own pups and recent experiences with donor pups (Afonso, Grella, Chatterjee, & Fleming, 2008). Considering the importance of dopamine in this brain area, a possible reason that no treatment effects were found in our study could be because we did not examine the effects of dopamine in the NAc region, which seems to show greater changes, following toxicant exposure, than ERα expression. As maternal care is disrupted with dopamine receptor antagonists, it is possible that altered dopamine levels interfered with the reward pathway (which associates infant response as a rewarding behaviour), which in turn may have altered the expression of ERα and thus the production of maternal behaviour (Champagne et al., 2004; Gammie et al., 2008). Observing the variations of dopaminergic activity in the

NAc could have been one mechanism by which the expression of ERα is affected. Therefore, further studies simultaneously reducing dopamine levels in this area would clarify whether sensitization of maternal behavior is dependent solely upon ERα activity in the NAc or whether elevated dopaminergic activity alone is sufficient to promote this behavior.

The NAc has been implicated in a variety of associative processes that are dependent on the integrity of the hippocampus as it is one of the few brain areas with continuous neurogenesis (Eriksson et al., 1998). Recall that neurochemical changes that occur during pregnancy; such as the up-regulation of E_2, prolactin, and oxytocin, are key modulators of hippocampal changes (Brunton & Russell, 2011). Given the link between the NAc and the hippocampus, it is possible that dosage or treatment effects could be more pronounced in the hippocampus, which could possibly explain the lack of treatment effect found in our study.

One mechanism that could have been used to assess MeHg neurotoxicity, with variying doses would be through cell apoptosis in the hippocampus. One study administered injections of Methylmercury chloride (0.1–10 μg/g in a 100 μL bolus) to the offspring on perinatal day 7 (Sokolowski, Falluel-Morel, Zhou, & DiCicco-Bloom, 2011). Examination of cortical slices, following immunohistochemistry revealed the greatest changes in protein levels of caspase-3 occurring in vivo after 5μg Methylmercury chloride exposure- where cleavage of caspase-3 indicates an advanced and irreversible apoptotic cascade (Riedl & Shi, 2004). In comparison, exposure to 0.6 μg/g Methylmercury chloride elicited a 5-fold increase in caspase-3 immunoreactive cells in the hippocampus 24 h after the injection, with a trend for exposure at 0.2 μg/g eliciting a smaller increase (Sokolowski et al., 2011). Furthermore, the study found a 3–4-fold increase in pyknotic nuclei at both 0.6 μg/g Methylmercury chloride and 5

μg/g Methylmercury chloride doses 24 h after exposure (Sokolowski et al., 2011). In addition, previous studies have also reported similar magnitudes of effect with 5 μg/g dosage of Methylmercury chloride on apoptotic markers with an 8-fold increase observed with exposures of 10 μg/g (Falluel-Morel et al., 2007). These findings show that parallel increases in caspase-3 immunoreactive cells and pyknotic nuclei in the hippocampus are associated with an increase in cell death induction, observed here as caspase-3 activation with increasing MeHg exposure. Given the deficits in spatial learning and memory often associated with MeHg exposure, these findings suggest that the hippocampus may be more susceptible because of ongoing neurogenesis and thus neuronal apoptosis in the hippocampus could be indicative of MeHg toxicity (Sokolowski et al., 2011).

5.3 VTA

The VTA is part of the mesolimbic dopamine circuit that plays an important role in the motivation, regulation and reinforcement of maternal behaviour. The system that extends from the mPOA to the VTA is important for the active motor components of maternal behaviour (retrieving and nest building) more so than for the passive motor components (nursing) (Terkel, Bridges, & Sawyer, 1979). Results from our work showed that there was a statistically significant difference in the number of ERα receptors in the VTA, across the high and low doses. Findings from our study coincide with those from previous studies that have found *in vitro* exposure to the PCB mixture Aroclor1254 (A1254) interferes with normal synaptic transmission and plasticity in hippocampus by altering the synaptic transmission between Schaffer collaterals and CA1 neurons of the rat hippocampus (Bang et al., 2011). Recall that the dopaminergic mesocorticolimbic pathway arises from the VTA (Steinfels, Heym, Strecker, & Jacobs, 1983) and dopamine is required for hippocampal-dependent memory formation (Rosen, Cheung, & Siegelbaum, 2015). Taken together, these findings suggest that changes to synaptic transmission in the hippocampus, from exposure to Aroclor 1254, would affect maternal brain plasticity with these changes

subsequently affecting the dopaminergic-mediated plasticity of the maternal brain.

While there is no real comparable literature, as no one has looked at ERα in the maternal brain following perinatal exposure to such toxicants, other studies have examined changes in maternal behaviour following gestational exposure to a PCB mixture containing similar congeners to the ones found in our study. When considering the maternal behaviours sub served by the VTA, others have found changes in these behaviours, on PND 1, 2, 4 and 6 (Cummings, Nunez, & Clemens, 2005). Among the low PCB exposed group, there was a significant difference in the amount of licking and grooming directed to the litter and number of times auto-grooming behaviours were performed; with enhanced pup grooming seen in a group with combined PCBs (Cummings et al., 2005). Given the important role of dopamine in the VTA, it is possible that elevated VTA dopamine neuron levels, exhibited in the study as licking and grooming, would have likely also contributed to the enhanced maternal behavior observed in the same study (Peña & Champagne, 2015). Thus, we speculate some similarities in their findings with those of our study, despite the differences in dosage and treatment administration. Since the neuroendocrine system and the mesolimbic dopamine system are anatomically connected and implicated together in maternal behavior, the findings of both of these studies suggest that these two systems may be linked at a molecular level by ERα expression, particularly during early postnatal development. Since elevated levels of maternal licking and grooming induce increased ERα and estrogen sensitivity in the mPOA (Champagne et al., 2003), we would suspect that low levels of licking and grooming to have lowered ERα and estrogen sensitivity within other hypothalamic regions (Peña, Neugut, Calarco, & Champagne, 2014), such as the VTA and NAc found in the lateral hypothalamus region. In addition, while our findings show that mPOA ERα expression decreases with increased doses of the toxicant mixture and its components, it has yet to be determined whether exclusively increasing the number of dopamine neurons in the VTA would have similar effects on ER expression or on any subsequent behavioural outcomes.

5.4 Link between the mother and offspring

As previously mentioned, maternal behaviour is the major source of sensory driven environmental stimulation with multi-sensory maternal cues that influence the development of the sensory systems in the newborn rats (Sarro, Wilson, & Sullivan, 2014). Evidently, the quality of this care can dramatically impact neurobehavioural development in the offspring (Landers & Sullivan, 2012). For example, within the nest environment, the mere presence of the mother and littermates in the nest, has an impact on offspring cortical neural activity, with maternal/littermate absence inducing de-synchronization in the organization of subsequent pup behaviours (Melo et al., 2006). A reduction in tactile, thermal, auditory, and other sensory stimuli, from maternal and littermate deprivation, can alter the development of the sensory systems used to process social cues; hence, the observed abnormalities in maternal behaviours and social learning for both the dam and offspring (Melo et al., 2006). Thus, any impact on maternal care, from perinatal exposure to environmental factors, may also impact the offspring.

Environmental factors, such as maternal gestational stress, have been found to induce changes in maternal behaviour (Langenhof & Komdeur, 2018). For example, exposure to maternal gestational stress (via restraint stress at 1 h/day, during days 10 to 20 of gestation) was shown to alter maternal behaviour, observed as reduced arched-back nursing and nesting/grouping pups, in dams over post-natal days 1–10 (Smith, Seckl, Evans, Costall, & Smythe, 2004). Furthermore, variations in maternal care can induce short-term or long-term neurobiological effects (Champagne & Curley, 2016). For example, maternal licking/grooming is an important source of tactile stimulation for the developing pup and can affect somatic growth and neural development (Morreale de Escobar, Obregon, & Escobar del Rey, 2004). The arched-back nursing posture of the mother can either increase or decrease tactile stimulation derived from nipple switching by pups, which in turn can affect hippocampal volume of the

offspring (Morreale de Escobar et al., 2004). For example, one study evaluated the effects of maternal behaviour on neuronal development of the male offspring of high-licking and grooming mothers (females whose frequency scores for high-licking and grooming were >1 significant digit above the mean) and low-licking and grooming mothers (females whose frequency scores for low- licking and grooming were greater than 1 significant digit below the mean) after brdU injection at P21 and P90 to identify neurogenesis (Bredy, Grant, Champagne, & Meaney, 2003). The offspring of the mothers exhibiting high licking and grooming were found to have higher levels of hippocampal bFGF protein expression at PD 21 than the offspring of the mothers exhibiting low licking and grooming. At both P21 and P90, there was a significantly higher number of the surviving BrdU-labelled new cells in the dentate gyrus of the offspring of high-licking and grooming mothers as well as increased astrocyte expression (Bredy et al., 2003) when compared to the low-licking and grooming mothers. Interestingly, this study also showed that environmental enrichment, imposed between P21 and P70, can partially reverse the effect of reduced maternal licking and grooming on spatial and non-spatial learning and memory by enhancing hippocampal-dependent learning in the offspring of low- licking and grooming mothers (Bredy et al., 2003). As a result, female offspring of low- compared to high- licking and grooming dams exhibit lower neuronal activation stimulated by ERα within the mPOA and thus lower levels of ERα (Champagne & & Curley, 2016).

Maternal behaviour also affects spatial learning and memory. In another study, on the second and third days of testing, the offspring of high licking/grooming and arched-back nursing mothers exhibited significantly shorter latencies and swim paths taken to locate the target platform when compared to the offspring of low licking/grooming and arched-back nursing mothers (Liu, Diorio, Day, Francis, & Meaney, 2000). Furthermore, when removing the platform in the Morris water maze test, the offspring of high licking/grooming and arched-back nursing mothers spent more time searching in the target quadrant

compared with the offspring of high licking/grooming and arched-back nursing mothers (Liu et al., 2000). The differences in spatial learning and memory continued into later phases, which was evidenced by the time searching differences at 24 months of age between the offspring of the high- and low-licking/grooming and arched-back nursing mothers (Liu et al., 2000). Consequently, these findings show that maternal behaviour, especially when provided at sensitive periods of offspring neuronal development, can in fact influence the developing brain. Maternal care itself, as an adult, can be influenced by the maternal care received during infancy (D'Cunha, King, Fleming, & Lévy, 2011).

In rodents, disruptions to the quality of mother-infant interactions can have long-term effects on the hypothalamic-pituitary-adrenal response to stress, cognition and social/reproductive behaviour (F. Champagne & Curley, 2016). The neural circuits that regulate maternal behaviour can be conserved across mammalian species; thus, the findings of this study would be relevant to the neural control of maternal behaviour in other mammals, including humans (Champagne & Curley, 2016).

5.5 Limitations

In the present study we examined the role of a toxicant mixture and one of its components, MeHg, on maternal brain plasticity. It should be noted that there are limitations with our study. First, most studies examining maternal brain plasticity usually report on the neuronal changes occurring early in the days of the peripartum period, thus overlooking other changes that could be occurring later in the postpartum period. As the regulation of maternal behaviour, in humans, is facilitated by circulating hormones that remain in the body up to 24 – 36 h postpartum (Bridges, 1984), the majority of the neuronal changes that occur during pregnancy return to pre-conception levels by postpartum day 28 (Oatridge et al., 2002). Even though the rat dam brains, used in this study were harvested much later in the postpartum period, high levels of treatment to the toxicant mixture or MeHg, reduced number of estrogen receptors

in the mPOA and VTA. From what has been shown in the literature, these findings are novel and suggest that exposure to doses that mimic those found in the human population can in fact be toxic. The risk of human exposure to such contaminants continues to be evident. Thus, our findings highlight the importance of studying the effects of such toxicants on the maternal brain and to include later postpartum times as well, in order to better understand the all-around toxic impacts on the maternal brain. Furthermore, our study supports the idea of evaluating the effects of ecologically relevant mixtures of toxicants.

Secondly, the effects of all the individual components of the mixture or pair-wise combinations were not explored. Accomplishing this task would have entailed a large number of groups (seven groups to represent all possible combinations) and subsequently even greater sample sizes that can be able to parse out any statistically significant true effects. Therefore, we may have missed important combinations, or individual effects that were masked by the presence of one of the components in the mixture that contained all three chemicals.

A third limitation to this study is that we only evaluated a sub-set of the neural modifications found in the maternal brain, amidst all that may occur throughout gestation and parturition. As ERα and ERβ are expressed in distinct yet overlapping regions of the female rat brain, their overlapping circuity found in many brain regions means that effects could have been observed in other regions (such as other hypothalamic nuclei and the amygdala) that were not explored (Shughrue, Lane, & Merchenthaler, 1999). And fourth, the lack of a behavioural component to assess any functional effects of the exposure to this mixture is an important limitation. Since the mother's response to the young is contextually driven, her behaviours towards her offspring rely not only on motor responses (regulated by alterations in her motivational and reward states)

but also the interactions between biological determinants, experiential events and the stimuli of her immediate environment. Evidently, a number of relevant neurological processes may be affected by exposure to toxic agents, i.e., neurogenesis, migration, neurotransmitter and receptor activity, with the functional consequences expressed differently in the mother and the offspring (MacLusky, Lieberburg, & McEwen, 1979). The detection of any effects following exposure may not necessarily be apparent until a critical age when the neurotoxic defect may be unmasked or precipitated by a subsequent injury or exposure (Gore & Dickerson, 2012). Thus, the incorporation of behavioural analyses, in addition to the molecular assessment, while screening the effects of potential neurotoxic substances would provide concomitant evidence.

Chapter 6. Significance

Direct causal links between exposures to the toxicant mixture and disease states in humans are difficult to draw. Results from basic research, conducted on human and rat models, as well as epidemiological studies following exposure at different time-points, makes it clear that there is a need to link basic research to basic human practices. The results from these types of studies can be used to understand the health implications caused from exposure in populations living in the Canadian Arctic.

6.1 Indigenous Health

The Indigenous people of Canada are Indians, Inuit, and Métis that reside in Yellowknife, Whitehorse and southern Canadian cities (Constitution Act of Canada, 1982). Six geo-political areas have been identified based on the Inuit groups that reside there. The Inuit regions include: Labrador, Nunavik (Northern Québec) and the Northwest Territories, the western Arctic and Nunavut (Van Oostdam et al., 1999). These groups are very heterogeneous with varying cultures, geographical locations and languages (Willows, Veugelers, Raine, & Kuhle, 2008). Approximately 79% of Canada's Indigenous people live in the Arctic region (Yukon, Northwest territories, northern Quebec and Labrador (Statistics Canada, 2015). The Indigenous population has a young population demographic with 39% of their population under age 15 (Statistics Canada, 2015). Because of their younger population demographic, problems with the safety of traditional foods poses a considerable challenge in both interpreting and communicating the well-known benefits of limiting consumption of food items contaminated with neurotoxicants.

6.1.1 Traditional foods

The Indigenous interpretation of health is focused on wellness which is defined as the combined state of well-being for both the individual and the entire community (Van Oostdam et al., 1999). For the Inuit,

traditional foods are directly associated with physical health and well-being or their ability to provide nutrients that imported foods cannot. For example, Caribou meat is the most frequently consumed traditional food by the Inuit, followed by fish (beluga and seal) and other marine mammals. Other commonly consumed foods include waterfowl (duck, goose, swan) (Batal, 2001; Van Oostdam et al., 1999). Traditional foods maintain the cultural, spiritual and physical integrity of the Indigenous people and in most cases, serve as the primary source of many important nutrients such as lipids, vitamins and minerals, and protein (Donaldson et al., 2010). Indigenous culture can influence health outcomes. For example, recent reports from several indigenous Arctic populations in Greenland and Alaska indicate that their mortality risk from ischemic heart disease is substantially below that of the non-Native population in the same region, due to the consumption of high amounts of lipids derived from marine fish and mammals (Devries & Free, 2011). Over the last 30 years, changes in the economic, cultural and environmental conditions have led to the gradual replacement of traditional food by processed foods, which has been linked to the declining health in Arctic Indigenous populations (Kuhnlein, Receveur, Soueida, & Egeland, 2004).

6.1.2 Cultural benefits of traditional foods

It is no surprise that food sustenance for the Indigenous people is not individualistic and so includes the participation of family/community members in the harvesting, processing, receiving and consuming of traditional food (Van Oostdam et al., 1999). Historically, Indigenous societies integrated food harvesting, processing, consuming, and sharing to the social, political, economic, domestic, spiritual, and cultural organization of their communities (Kuhnlein & Chan, 2000). Traditional food embodies the traditional way of living. Over many generations, extensive experience and observation of the land has led to a better understanding of the various species and diverse ecological interactions between them (Parlee et al., 2012).

6.2 Metal contamination of traditional food

While the risk of heavy metal contamination associated to wild food consumption has been extensively studied in the Arctic, data are scarce for specific effects on the Canadian Indigenous people (Bordeleau, Asselin, Mazerolle, & Imbeau, 2016). Sources of the contaminants include: atmospheric and water transportation of the pollutants from industrial activities, geological sources and anthropogenic sources that enter the North via atmospheric and oceanic long-range transport mechanisms (Bordeleau et al., 2016). Contamination of the Arctic marine ecosystem affects the species consumed by Indigenous people (Barrie et al., 1992; Muir et al., 1992). The ingestion of the neurotoxic compounds, through the consumption of contaminated traditional food, can result in the accumulation of hazardous chemicals causing detrimental effects to the maternal and fetal nervous system (Ceccatelli, Bose, Edoff, Onishchenko, & Spulber, 2013)(Ceccatelli et al., 2013). Specifically, mercury in its methylated form, remains a food contaminant of major concern (Ceccatelli et al., 2013). Mercury deposits in lakes, rivers and oceans are converted to methylmercury (MeHg) by the methylation activity of sulphate-reducing bacteria, thus allowing MeHg to enter the aquatic food chain and bio-accumulate in predatory fish (Ceccatelli et al., 2013). Bio-magnification of MeHg is common, with the highest levels found in predatory fish and shellfish (Ceccatelli et al., 2013). Once ingested, MeHg is almost completely absorbed in the gastrointestinal tract as it enters the blood stream and crosses the blood–brain barrier to distribute to all organs including the brain (Clarkson, 2002). MeHg undergoes a slow demethylation in the brain, which results in the accumulation of inorganic mercury (Clarkson, 2002). Due to the fact that the Northern population consumes an elevated proportion of fish and seafood, there is growing concern that MeHg levels could quickly exceed safe levels (Ceccatelli et al., 2013). Health Canada's Methylmercury Monitoring Program found that 57% of Inuit population sampled had blood methylmercury levels that exceeded the 20 μg/L guideline used to distinguish the increasing risk of health effects related with methylmercury exposure (Wheatley & Territories, 1994). The elevated levels

were linked to seal consumption, with those consuming more than six meals/week having a mean blood mercury value of 62.5 µg/L, while those eating one meal of seal or less/week having a mean mercury value of only 22.2 µg/L (Hansen & Wulf, 1983). Another study found that the geometric mean maternal total mercury concentrations ranged from 0.87 µg/L (SD=1.95) in the Caucasian group of participants (n=134) compared to 3.51 µg/L (SD=8.30) in the Inuit group (n=146); with the mean of the Inuit group 2.6-fold higher than that of other groups (1.35 µg/L, SD=1.60, n=92 ;*P*<0.0001) (Butler Walker et al., 2006).

6.3 Possible solutions

Across the Arctic, previously attempted culture-focused health intervention techniques originally intended to incorporate the Indigenous people in larger national political economies (Gone & Calf Looking, 2011; Moore et al., 2014). They often utilized techniques such as community relocation and settling of this nomadic group, which resulted in problems such as broken family ties or loss of language (Arias, Xu, & Jim, 2014; Mitrou et al., 2014). As a result, the consequences yielded poor health outcomes like substance abuse, Type 2 diabetes, depression and other mental health outcomes as well as suicide (Allen et al., 2014; Petrasek MacDonald et al., 2015; Reeve, Church, Haas, Bradford, & Viney, 2014; Wexler, Gubrium, Griffin, & DiFulvio, 2013). Currently, emerging research suggests that interventions should focus on cultural-based protective factors with emphasis on the potential for cultural participation to mitigate negative health outcomes (MacDonald, Ford, Willox, & Ross, 2013). A proposed solution could be integrating the First Nations in the development of evaluations and management strategies of assessing toxicant levels in traditional foods.

Another possible solution could include economic interventions to allow the purchase of traditional food. An approach that subsidizes the purchase of traditional food through freight, in-store pricing and/ or direct commodity subvention systems (Lee, Leonard, Moloney, & Minniecon, 2009). The economic interventions should incorporate models of sustainable development that are inclusive of the lifestyles in remote communities (Lee et al., 2009). According to the Government of Canada's Action Plan for Food Security (1998), priorities for action require improving access to traditional foods in remote northern communities by commercially producing and distributing some of the traditional foods through coordination between the federal, provincial and municipal governments and with non-governmental organizations focusing on Indigenous care (Lee et al., 2009). Additionally, it would be imperative to secure resources toward funding research initiatives to better understand the risks and impacts of toxicants on Indigenous people's Health and the development of methods that can accurately assess the level of contaminants in traditional foods.

Chapter 7. Conclusion

Analyses of behavioural, neuroendocrine, cellular, and molecular mechanisms in both experimental and epidemiological studies have advanced our understanding of the mechanisms through which maternal care influences the developing brain. In particular, this study aimed to advance our understanding of maternal brain plasticity following exposure to a toxicant mixture found in Canadian Northern populations. The data from this study follow the hypothesis that exposure to a toxicant mixture or one of its components, MeHg, can affect the expression of ERα in the maternal brain, with ERα necessary for normal maternal behaviour expression. In turn, modifications to typical maternal behaviour would negatively impact the offspring. Various windows of susceptibility of the brain exist, though the best documented is during fetal development or postnatal exposure. We believe that changes in hormonal and neurotransmitter systems during pregnancy and postpartum also constitute periods of brain vulnerability to such external factors. The results of a factorial ANOVA of Dose and Treatment did show lower ER counts with the treatments in the mPOA and VTA exclusively. This suggests that these areas of the maternal brain may be more susceptible to the effects of these toxicants when exposed during pregnancy an postpartum

In line with this, we believe that future studies should include the following: *(1) Study of sex differences.* The impact of early life experiences related to quality of maternal behaviour on long-term developmental outcomes differs between male and female offspring (D'Andrea, Gracci, Alleva, &

Branchi, 2010; Mueller & Bale, 2008). Despite this knowledge, studies that compare the impact of maternal behaviours on males *vs.* females are not systematically employed in many pre-clinical studies. *(2) Study of neuromodulators (GABA, dopamine, oxytocin) following toxicant exposure*. Oxytocin-dopamine interactions are necessary for the establishment and maintenance of social bonds, whereas GABA in key brain regions is needed for the postpartum initiation of maternal behaviours (Caldji, Diorio, & Meaney, 2003; Numan & Young, 2016). Studies that examine the effects of toxicant mixtures on the abundance of these neuromodulators could provide insight into the neurochemical changes that incite changes in maternal behaviour. The boundaries between the maternal and offspring environments is still not well understood so better understanding how environmental perturbations may impact the mother is a piece of the puzzle to better understanding how perturbations in the environment may impact the offspring.

Appendix A

ERα IHC procedure in rat brain

Date: _____

Name: _____ **Experiment: _____**

DAY 1

___ 3 X5 min rinses in **0.01M PBS** (to remove cryoprotectant)

___ 3 X 5 min rinses in **0.01M PBS-T** (to permeabilize cell membranes)

___ 10 min in **5% DMSO** in PBS (to help maximize penetration of reagents inside the nucleus)

___ 3 X 5 min in **0.01M PBS-T**

___ Incubate for 20 min in **0.6% H_2O_2** in PBS (see below - to block endogenous peroxidases)

___ 3 X 5 min rinses in **0.01 M PBS-T**

___ Incubate for 30 min in **10% normal goat serum** (Vector Laboratories) in PBS-T (to limit background staining)

(~ 1 ml per beaker cup)

___ Incubate in primary antibody (e.g. anti-ERα made in rabbit - Millipore **at a concentration of 1:5000 in 0.01 M PBS-T**) for 1 h at room temperature then 24-48 h at 4 C on shaker.

- do one without antibody to assess non-specific binding

(~1 ml per beaker cup)

Calculations:

H_2O_2

$C_1V_1 = C_2v_2$

To make 10 ml of 0.6% H_2O_2 from 30% stock:

C_1 = 30%

V_1 =?

C_2 = 0.6%

V_2 = 10 ml

(30)V_1 = (0.6) (10)

V_1 = 0.2 ml

So: add 200 µl (0.2ml) of stock 30% H_2O_2 to 9.8 ml of PBS

10% goat serum

10% is 10 ml into a total volume of 100 ml – thus, 10 ml + 90 ml = 100 ml

So: to make 1 ml total

100 µl of serum to 900 µl PBS-T

Eg Anti-ERα want a 1:5000 preparation (for one sample or 1 cup, make a total of 2 ml)

1:5000

X ← 2 ml

X = 0.04 µl (0.0004 ml)

0.04 µl of anti-ERα in 1999.6 µl PBS-T

DAY 2

___ 3 X 5 min rinses in **0.01 M PBS-T**

___ Incubate with **secondary antibody (in the case of ERα, the primary is made in rabbit, so I will use as a secondary anti-rabbit made in goat)** in PBS-T for 1 hour @ a concentration of 1:1000

(~ 1 ml per beaker cup)

a. make up ABC before rinses

(2 drops of A in 5 ml of PBS-T, mix then add 2 drops of B and mix again)

___ 3 X 5 min rinses in **0.01 M PBS-T**

___ Incubate with **ABC** for 1 hour in PBS-T (~1 ml per beaker cup)

- Half way through, take out a large enough quantity of DAB so that it is thawed in time for the last step. Keep covered with foil.

___ 3 X 5 min rinses in **PBS-T**

___ Develop in DAB in fume hood.

Directions: to make 5 ml total	IN FUME HOOD
100 µl of 2% DAB	
(prepare in disposable	4900 µl PBS
container)	Filter
	Add 1.67 µl of 30% H_2O_2 (only when ready for use)

___ Stop reaction by placing in **0.01 M PBS**

___ 3 X 5 min rinses in **0.01 M PBS**

___ Mount tissue on slides or store for short period in a well covered container (make sure there is sufficient PBS so section won't dry out, that there is parafilm to protect against evaporation and that the dish is labeled).

Eg. Secondary (goat anti-rabbit)

1:1000

X ← 5 ml

X = 0.005 ml (=5 µl)

So: 5 µl of horse anti-mouse in 4995 µl PBS-T

0.04% DAB

(already prepared at 2%)

$C_1V_1 = C_2v_2$

To make 5 ml of 0.04% H_2O_2 from 2% stock:

C_1 = 2%

V_1 =?

C_2 = 0.04%

V_2 = 5 ml (=5000 µl)

(2)V_1 = (0.04) (5000)

V_1 = 100 µl

So: add 100 µl of stock 2% DAB to 4.9 ml of PBS

Then add 1.67 µl of 30% H_2O_2 to activate right before using on sections

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