Thyroid hormone disrupting chemicals and their influence on the developing rat brain

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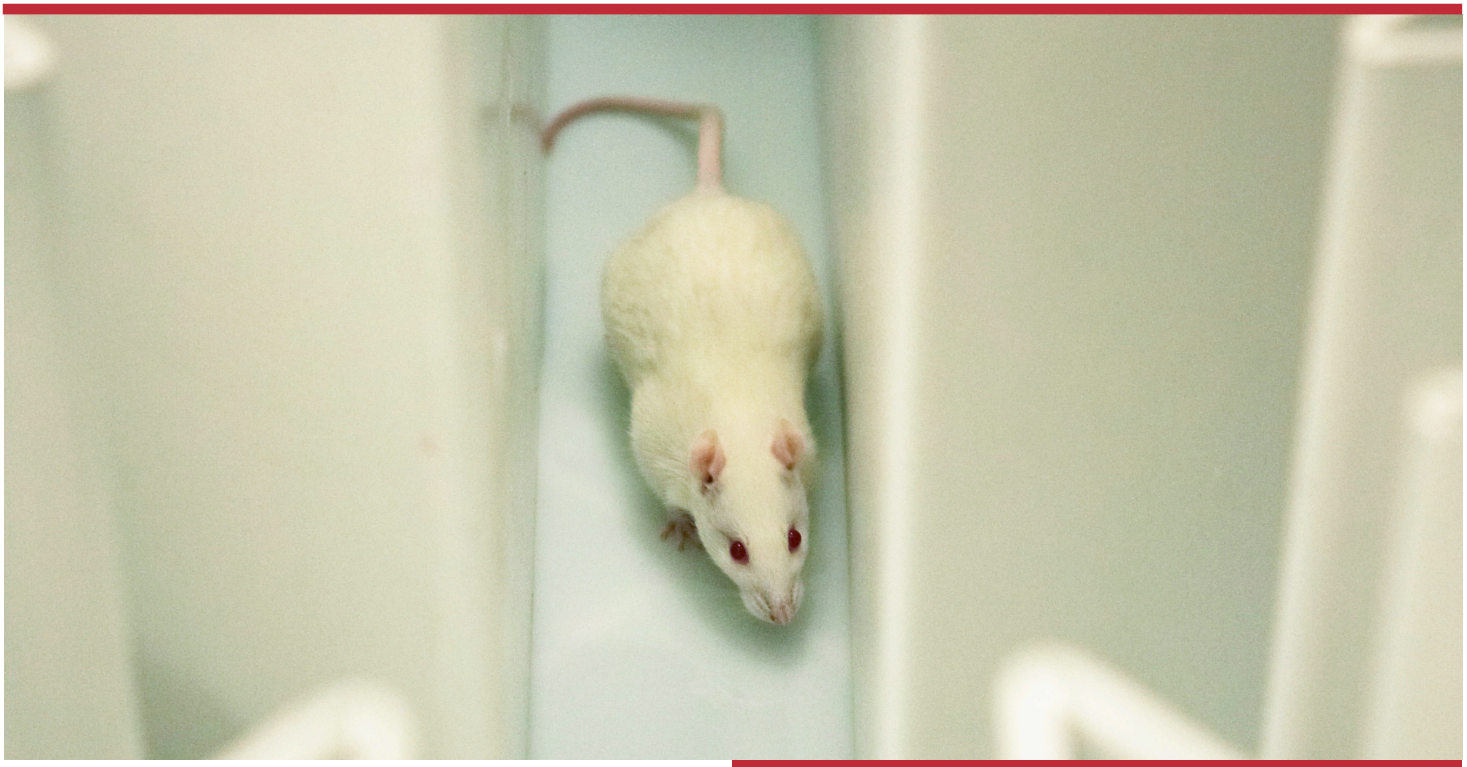

Marta Axelstad Petersen
PhD Thesis
2011

DTU Food
National Food Institute

Thyroid hormone disrupting chemicals and their influence on the developing rat brain

Ph.D. Thesis

Marta Axelstad Petersen

Division of Toxicology and Risk Assessment
National Food Institute
Technical University of Denmark

2011

Data sheet

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Abbreviations

AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
BP2	Benzophenone 2
BPA	Bisphenol A
BW	Body weight
CAR	Constitutive active/androstane receptor
CH	Congenital hypothyroidism
DIT	Di-iodotyrosine
DNT	Developmental neurotoxicity
ETU	Ethylene thiourea
fT_4	free T_4
GD	Gestation day
HPT	Hypothalamic-pituitary-thyroid
MIT	Monoiodotyrosine
MMI	Methimazol
NIS	Sodium-iodide symporter
NR	Nipple retention
OECD	Organisation for Economic Co-operation and Development
OMC	Octyl methoxycinnamate
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyls
PFOS	Perfluorooctane sulfonate
PND	Postnatal day
PTU	Propylthiouracil
PXR	Pregnane X receptor
SULT	Sulfotransferase
T_3	Triiodothyronine
T_4	Thyroxine
TBBPA	Tetrabromobisphenol A
TBG	Thyroxine binding globulin
TDC	Thyroid disrupting chemical
TG	Test guideline
TH	Thyroid hormone
ThX	Thyroidectomised
TPO	Thyroid peroxidase
TR	Thyroid receptor
TRH	Thyrotropin releasing hormone
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
UDPGT	Uridinediphosphate glucuronosyltransferase

Summary

The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) are produced in the thyroid gland, and besides their role in the body's metabolic rate, they also play a determining role during foetal and neonatal brain development. Because thyroid hormones (TH) are needed for proper nerve cell differentiation and proliferation, normal status of these hormones during early development is crucial, and in humans even moderate and transient reductions in maternal T_4 levels during pregnancy, can adversely affect the child's neurological development. In order to maintain correct levels of THs, the body is dependent on sufficient iodine intake but several substances in the environment may also affect thyroid status. These are called thyroid disrupting chemicals (TDCs), and they are xenobiotics that can change the levels of circulating THs. The TDCs are made up of a wide range of chemical structures and include industrial chemicals, pesticides and ingredients used in personal care products. A way of getting more insight into the causal relationship between exposure to endocrine disrupters, their effects on TH levels and subsequent adverse effects on brain development, is by investigating it in animal studies. Therefore, the overall purpose of this Ph.D. project was to use an animal model to examine how neurological development is affected by pre- and postnatal exposure to TDCs, and to find out whether behavioural changes in the offspring could be predicted simply by measuring TH levels in the blood from exposed dams. This knowledge could enable regulatory authorities to easier identify the risks of developmental neurotoxicity after exposure to TDCs.

The project included three developmental neurotoxicity studies in rats. In the first study, the known thyrotoxic drug propylthiouracil (PTU) was tested. This was done, in order to have a reference with regard to T_4 reductions and behavioural outcomes in our laboratory. Hereafter two environmentally relevant chemicals were investigated. The first was the ultra violet (UV)-filter octyl methoxycinnamate (OMC), the other was the fungicide mancozeb. Both compounds are very widely used, and for both, studies have shown thyroid disruption to be a very sensitive endpoint but no developmental neurotoxicity studies have previously been published. The overall experimental design was as follows: time mated rat dams (n=15-20) were dosed with the test substance from gestation day 7 until the end of lactation at postnatal day (PND) 16, thereby exposing the offspring indirectly to the test substances via placental and milk transfer. One week after dosing had begun, blood samples from the dams were collected and analysed for serum T_4 levels. On the last day of dosing some of the offspring were sacrificed, blood samples were collected and several organs were excised. From each litter a few offspring were weaned for further neurobehavioural assessment, which included tests of spatial learning and memory, motor activity levels and startle response. Hearing function was also assessed, along with additional reproductive toxicity endpoints.

The results from the first study showed that developmental exposure to the known thyrotoxic compound PTU, caused hypothyroidism in both dams and offspring, and adversely affected the offspring's brain development. Both dams and offspring had significantly decreased T_4 levels during the dosing period, and their thyroid glands were severely affected. The expected neurobehavoiral and auditory effects were seen, as learning and memory was impaired in the adult male offspring, while both males and female offspring showed hyperactivity and impaired auditory function. The observed changes in behaviour and hearing ability were significantly correlated to reductions in maternal T_4 levels during gestation, indicating that these could be an indicator of the severity of behavioural and auditory defects later in life. In the OMC study, all tested doses markedly decreased T_4 levels in dams, while the thyroid effects in the offspring were not very severe. OMC exposed male offspring showed reduced reproductive organ weights and decreased sperm counts in adulthood, effects that were probably mediated by OMC's estrogenic properties. The offspring's behaviour was also affected, however, differently than expected. In female offspring, motor activity levels were decreased in the highest dose group, while low and high dose males showed improved spatial learning abilities compared to controls. These behavioural changes were not correlated to maternal T_4 levels, and were probably not mediated by early T_4 deficiencies, as they differed substantially from effects seen in other studies of developmental hypothyroidism. In the mancozeb study, exposure of pregnant dams to the fungicide caused significant decreases in dam T_4 levels during gestation. However, on PND 16 the thyroid system in the offspring was unaffected. Furthermore, no effects on reproductive endpoints and no behavioural changes were observed.

The results from the three studies indicate that while PTU was readily transferred to the offspring, toxicokinetic factors may have affected placental and milk transfer of OMC and mancozeb, and thereby limited the offspring exposure. In the two latter studies, it was demonstrated that maternal T_4 deficits during gestation were not correlated to behavioural outcome in the rat offspring, and unlike what is seen in humans, were therefore not good predictors for adverse neurobehavioural development. Based on these results, it was concluded in the thesis that in rats, measurements of prenatal T_4 decreases alone cannot be used for regulatory purposes to predict developmental neurotoxicity, after exposure to chemicals that affect the TH system. A possible explanation for the observed differences between rodents and humans could be that while most of the brain maturation in humans happens prenatally, much happens postnatally in the rat. Prenatal T_4 deficits may therefore be more critical for neurobehavioural development in humans than in rats. In conclusion, at present we know too little about the timing of TH deficiency, and of the complex array of feedback mechanisms and compensatory processes in the thyroid hormone system, to use maternal T_4 measurements as predictors for adverse behavioural effects in offspring.

Dansk Resumé

Skjoldbruskkirtelen, også kaldet glandula thyreoidea, producerer skjoldbruskkirtelhormonerne triiodothyronin (T_3) og thyroxin (T_4). Udover deres rolle i kroppens stofskifteprocesser spiller disse hormoner også en afgørende rolle for bl.a. nervecelledifferentieringen under hjernens udvikling, og hos mennesker kan selv moderate og forbigående fald i moderens T_4-niveauer under graviditeten have negativ indflydelse på barnets neurologiske udvikling. For at opretholde korrekte hormonniveauer, er skjoldbruskkirtelen afhængig af et tilstrækkeligt jodindtag, men den kan også påvirkes af en række stoffer i vores miljø. Disse kaldes skjoldbruskkirtelforstyrrende stoffer. Det er kemikalier som kan ændre indholdet af T_3 og T_4 i blodet og de omfatter bl.a. en række industrikemikalier, pesticider og ingredienser der anvendes i produkter til personlig pleje. En måde at belyse årsagssammenhængen mellem udsættelse for hormonforstyrrende stoffer, effekten på skjoldbruskkirtelhormonerne og evt. negative påvirkninger af hjernens udvikling, er ved at undersøge det i dyreforsøg. Formålet med dette ph.d.-projekt var derfor, ved hjælp af en dyremodel at undersøge hvordan den neurologiske udvikling hos rotter påvirkes af præ-og postnatale skjoldbruskkirtelforstyrrelser, samt at finde ud af, om det er muligt at forudsige adfærdsændringer i afkommet ved hjælp af hormonmålinger i de eksponerede mødres blod. En sådan viden vil kunne bruges af myndighederne, til at identificere risikoen for skadelige virkninger på hjernen udvikling efter udsættelse for skjoldbruskkirtelforstyrrende stoffer.

Projektet omfattede tre store dyreforsøg i hvilke effekterne på rotters hjerneudvikling blev undersøgt efter præ- og postnatal eksponering for stoffer som påvirker skjoldbruskkirtlen. I det første forsøg blev det kendte thyreoideatoksiske stof propylthiouracil (PTU) undersøgt. Dette blev gjort for at have en reference fra vores eget laboratorium i forhold til T_4-nedsættelser samt adfærds-ændringer. Herefter fulgte undersøgelser af to miljømæssigt relevante kemikalier. Det første stof var UV-filteret octyl methoxycinnamat (OMC), mens det andet var svampemidlet mancozeb. Begge stoffer er meget udbredte og begge giver thyreoidea forstyrrelser, men der er ikke tidligere publiceret nogen adfærdsundersøgelser med stofferne. Det eksperimentelle design for de tre forsøg var som følger: drægtige rotter blev doseret med teststoffet fra dag 7 i drægtigheden, indtil slutningen af laktationsperioden, dvs. postnatal dag (PND) 16. En uge efter dosering var påbegyndt, blev blodprøver fra mødrene taget og analyseret for T_4-niveauer. På PND 16 blev noget af afkommet aflivet, der blev taget blodprøver og flere organer blev udtaget. Fra hvert kuld blev et par af ungerne fravænnet til senere adfærdsundersøgelser. Disse indebar bl.a. vurdering af afkommets indlæringsevne og motoriske aktivitet, samt deres ”forskrækkelses-respons”. Dyrenes hørelse blev også vurderet, og det samme gjorde en række reproduktionstoksikologiske endpoints.

Resultaterne fra den første undersøgelse viste, at eksponeringen for PTU forårsagede hypothyreoidisme i både mødre og afkom, og påvirkede afkommets hjerneudvikling. Både mødrenes og afkommets T_4-niveauer faldt som resultat af doseringen, og skjoldbruskkirtlerne var også kraftigt påvirket. Som forventet sås både adfærdsmæssige og auditive effekter, idet dyrenes indlæringsevne var nedsat i det voksne hanafkom, mens både han- og hunafkom viste hyperaktivitet og nedsat hørelse. De observerede ændringer var desuden signifikant korreleret med reduktioner i mødrenes og ungernes T_4-niveauer, hvilket indikerede at graden af T_4-nedsættelse under udviklingen kunne være en indikator for graden af adfærdsmæssige og auditive forstyrrelser. I OMC studiet medførte alle de testede doser massive T_4-nedsættelser hos mødrene, mens effekterne på ungernes skjoldbruskkirtelsystem var forholdsvis begrænsede. OMC eksponerede hanafkom viste reducerede organvægte af reproduktionsorganerne og reduceret sædcelleantal. Disse effekter var sandsynligvis medieret af OMC's østrogene egenskaber. Afkommets adfærd var også ændret dog anderledes end forventet. I hunafkommet var den motoriske aktivitet nedsat i den højeste dosisgruppe, mens hanner doseret med lav og høj dosis udviste forbedret rumlige indlæringsevne. Disse adfærdsændringer var ikke korreleret til T_4-niveauerne under udviklingen, og var sandsynligvis ikke forårsaget af tidlig T_4-mangel, da de adskilte sig væsentligt fra de effekter som er set i andre undersøgelser af hypothyreoidisme. I mancozeb undersøgelsen førte dosering af drægtige rotter med svampemidlet til betydelige fald i de mødrenes T_4-niveauer under drægtigheden. Men på dag 16 var ungernes skjoldbruskkirtelsystem helt upåvirket. Desuden blev der ikke observeret nogen forandringer i afkommets adfærd.

Resultaterne fra de tre undersøgelser tyder på, at mens PTU blev overført til afkommet via placenta og modermælken, så kan toksikokinetiske faktorer have påvirket overførslen af OMC og mancozeb og dermed have begrænset afkommets eksponering. I de to sidste undersøgelser blev det vist, at mødres T_4-nedsættelser under drægtigheden ikke var korreleret til negative effekter på hjerneudviklingen hos afkommet, og i modsætning til hvordan det forholder sig hos mennesker. Baseret på disse resultater blev det i afhandlingen konkluderet at, hos rotter kan målinger af prænatale T_4-reduktioner ikke alene anvendes til at forudsige effekter på hjernens udvikling efter udsættelse for skjoldbruskkirtelforstyrrende stoffer. En mulig forklaring på de observerede forskelle mellem rotter og mennesker kunne være, at mens hjernens modningsprocesser sker prænatalt hos mennesker, så foregår en del af disse efter fødslen hos rotter. Prænatale T_4-nedsættelser kan derfor være mere kritiske for hjerneudviklingen hos mennesker end hos rotter. Da vi i øjeblikket stadig ved meget lidt om hvilke tidspunkter der hos rotter er mest afgørende for hjernes udvikling i forhold til hormonforstyrrelser, samt om de komplekse feedback-mekanismer og kompenserende processer i skjoldbruskkirtelhormonsystemet, kan vi ikke p.t. bruge mødrenes T_4-målinger som parameter til at forudsige adfærdsforandringer hos rotteafkommet.

List of included papers

The Ph.D. thesis is based on the following three papers. They are referred to in the text by their roman numerals.

I. **Axelstad, M**., Hansen, PR., Boberg, J., Bonnichsen, M., Nellemann, C., Lund, S.P., Hougaard, K.S., Hass, U. (2008). Developmental neurotoxicity of Propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes. *Toxicol Appl Pharmacol* **232**, 1-13.

II. **Axelstad, M**., Boberg, J., Hougaard, K.S., Christiansen, S., Jacobsen, P.R., Mandrup, K.R., Nellemann, C., Lund, S.P., Hass, U. (2011). Effects of pre- and postnatal exposure to the UV-filter Octyl Methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring. *Toxicol Appl Pharmacol* **250**, 278-90.

III. **Axelstad, M**., Boberg, J., Nellemann, C., Kiersgaard, M., Jacobsen, P.R., Christiansen, S., Hougaard K.S., Hass, U. (2011). Exposure to the widely used fungicide Mancozeb causes thyroid hormone disruption in rat dams but no developmental neurotoxicity in the offspring. *Toxicol Sci* **120,** 439-446.

The following publications are not included in the thesis, but are related to the research areas covered herein. They include research on the thyrotoxic effects of nitrate and on the reproductive toxicity of widely used azole fungicides.

Taxvig, C., Hass, U., **Axelstad, M.**, Dalgaard, M., Borch, J., Andersen, H.R., Vinggaard, A.M. (2007). Endocrine disrupting activities *in vivo* of the fungicides tebuconazole and epoxiconazole. *Toxicol Sci* **100**, 464-73.

Taxvig, C., Vinggaard, A.M., Hass, U., **Axelstad, M.**, Metzdorff, S., Nellemann, C. (2008) Endocrine disrupting properties in vivo of widely used azole fungicides. *Int J Androl* **31**, 170-7.

Hansen PR, Taxvig C, Christiansen S, **Axelstad M**, Boberg J, Kiersgaard MK, Nellemann C, Hass U. (2009) Evaluation of endocrine disrupting effects of nitrate after in utero exposure in rats and of nitrate and nitrite in the H295R and T-screen assay. *Toxicol Sci* **108**, 437-44.

1. Introduction

When endocrine disruption is discussed, focus is often primarily on the adverse reproductive effects seen in both humans and experimental animals, after perinatal exposure to environmental xenobiotics. There are, however, a number of xenobiotics which affect the levels of circulating thyroid hormones (THs) rather than the reproductive hormones, and because THs are very important for nerve cell differentiation in the developing brain, normal TH status during foetal and neonatal life is crucial. It has been known for a long time that children with untreated congenital hypothyroidism or cretinism are at risk of developing growth retardation, hearing loss and severe mental retardation. During recent years, it has become evident that even mild changes in human thyroidal function in prenatal life can have negative consequences for a child's development, and be associated with impaired motor- and neurological function in childhood (Pop *et al.,* 1999; Kooistra *et al.,* 2006; Li *et al.,* 2010). During the first part of pregnancy the foetus does not produce THs itself and is therefore entirely dependent on maternal TH transfer. Xenobiotic exposure may therefore affect foetal brain development, either indirectly, by changing maternal thyroid function, or directly by impairing foetal thyroid system via placental or lactation transfer (Brucker-Davis 1998). Focus on thyroid disruption in relation to human neurological development has therefore increased substantially during recent years (Porterfield *et al.,* 2000; Howdeshell, 2002; Zoeller and Crofton, 2000; Schantz and Widholm, 2001).

In order to maintain correct TH levels, the body is dependent on sufficient iodine intake but several substances in the environment may also affect thyroid status. Such substances are called thyroid disrupting chemicals (TDCs), and are broadly defined as xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated with TH homeostasis, or change circulating or tissue concentrations of THs (Crofton, 2008). TDCs include a wide range of chemical structures that act through a variety of mechanisms. Some TDCs are industrial chemicals like polychlorinated biphenyls (PCBs), dioxins and flame retardants, while others are pesticides or ingredients used in personal care products. Several human studies report links between exposure to high background levels of xenobiotics like PCB and dioxin, and suboptimal neurological function in children, but it is still uncertain whether these effects are caused by thyroid disruption during development or by direct neurotoxicity (Brucker-Davis, 1998). In order to elucidate the causal relationship between exposure to endocrine disruptors and subsequent brain development, animal studies are needed. There are a number of uncertainties in the interpretation of such studies, as known species differences in the thyroid hormone systems exist. The advantages of animal studies are, however, very great as they make it possible to predetermine the doses of TDCs, measure TH levels continuously and subsequently assess the neurological development in the offspring through behavioural investigations.

2. Aims of the Ph.D. study

From the research done in animal studies this far, the relationship between chemical exposure, thyroidal dysfunction and neurobehavioral effects is still difficult to evaluate. Therefore, the overall purpose of this Ph.D. project was to gather further knowledge on how neurological development is affected by pre- and postnatal thyroid disruption, in order to conclude more generally on the risks of exposure to environmental TDCs during critical periods of nervous system differentiation.

The specific aims of the project were to:

1. investigate how neurological development and TH status in rats are affected after developmental exposure to xenobiotics that disrupt the thyroid hormone system, and whether observed neurobehavioural effects are correlated to developmental TH levels
2. assess whether reduced TH levels are good predictors for developmental neurotoxicity, and whether maternal TH reductions are determining for altered behaviour in rat offspring.
3. gather experience that could enable scientists and regulatory authorities to address whether risk assessment procedures should be modified to better take account for thyroidal dysfunction and its potential impact on the developing brain.

If a clear and consistent relationship could be established between TH disruption and adverse neurobehavioral effects, then chemicals known to disrupt the thyroid hormone axis could in the future be evaluated and classified for developmental neurotoxicity (DNT) based solely on their effect on THs. This would decrease the need for costly DNT studies and also reduce the use of experimental animals.

The Ph.D. project included three DNT-studies in rats. In the first one, the known thyrotoxic drug propylthiouracil (PTU) was tested, in order to have a reference with regard to T_4 reductions and behavioural outcomes in our laboratory. Hereafter studies on two environmentally relevant TDCs were performed. The first was with the UV-filter Octyl Methoxycinnamate (OMC), and the other was with the fungicide Mancozeb. For both compounds studies have shown thyroid disruption to be a very sensitive endpoint, but no DNT-studies have previously been published. In order to assess the neurobehavioural functions of the animals after perinatal exposure to the three xenobiotics, a study design based on the Developmental Neurotoxicity Study (OECD 2007) was used.

3. Background

3.1 Developmental neurotoxicity

Developmental neurotoxicity studies are a specialized type of animal studies, investigating potential adverse effects of pre- and post-natal exposure on the development and function of the nervous system. Because the nervous system has a long period of development with multiple 'critical windows', it is especially susceptible to toxic insults during development, and functional changes can be induced at a lower exposure level than those resulting in toxicity in adults (Hass 2006).

In humans developmental neurotoxicity has been seen after prenatal exposure to a number of chemical substances including ethanol, methyl mercury, PCBs and inorganic lead (Rice and Barone, 2000). However, establishing a causal connection between a suspected developmental exposure, and the diagnosis of a behavioural deficit much later in life, is very difficult due to the huge number of events that may occur during the time span. Therefore, developmental neurotoxicity studies in experimental animals are used. Here the appropriate controls are easier to establish, the exposure is well-defined, and the life span of the organism is much shorter than in humans. Recently a regulatory test-guideline for assessing developmental neurotoxicity (DNT) has been accepted in the OECD (OECD 2007). In this DNT Test Guideline (TG no. 426), the test substance is administered to animals (typically rats) during gestation and lactation. Cohorts of offspring are randomly selected from control and treated litters for evaluations of gross neurological and behavioural abnormalities during postnatal development and adulthood. These include assessments of physical development, behavioural ontogeny, motor activity, motor and sensory function, learning and memory and post-mortem evaluation of brain weights and neuropathology. The behavioural endpoints are assessed at different age periods during postnatal development and adulthood (OECD 2007). From such DNT studies several chemicals with widespread occupational or consumer exposure have been identified as possible developmental neurotoxicants. These involve a number of organic solvents, heavy metals and pesticides (Hass 2006). Furthermore, because hormonally mediated events play a central role in the central nervous system development and function, there is speculation that some of the cognitive deficits that arise from developmental exposure to environmental chemicals may be the result of endocrine disruption (Schantz and Widholm, 2001). Because the developing nervous system is sensitive to thyroid hormone deficits, a large group of chemicals that affects the TH system may also possibly act as developmental neurotoxicants.

3.2 The thyroid hormone (TH) system

The mammalian thyroid gland is located on each side of the trachea. It consists of two elongated oval lobes, joined by a thin isthmus crossing the trachea. It is composed of two distinct endocrine cell populations. The C-cells (or parafollicular cells) are concerned with the production of calcitonin that regulates calcium metabolism, while the follicular cells produce and secrete the thyroid hormones. Microscopically the gland is made up of thyroid follicles, surrounded by connective tissue. The follicles are composed of a single layer of cuboidal cells surrounding a lumen filled with colloid (NCM, 2002; Zoeller *et al.*, 2007). These follicles synthesize the two major thyroid hormones; 3,5,3'triiodothyronine (T_3) and thyroxine (T_4), which can be seen in Figure 1.

In adults, the THs stimulate metabolic rate in the liver, kidney, heart, nervous system and skeletal muscles (Randall 1997). T_3 is the more active of the two hormones, and the majority of biological actions of THs are believed to be mediated through receptors for T_3. In humans, the thyroid gland produces 100% of the T_4 found in the body, and 20% of the T_3. The other 80% are produced by peripheral conversion of T_4 to T_3 by deiodinase enzymes (Zoeller *et al.*, 2007). Three types of deiodinases exist. Type I and II remove an iodine atom from the phenolic ring of T_4 to create T_3, while type III deiodinase removes an iodine atom from the amino ring, thus converting T_4 to the biologically inactive reverse T_3 (rT_3). The deiodinase enzymes are mainly present in the liver, kidney and thyroid (type I), in the pituitary and brain (type II) and in the skin and placenta (type III) (Hood and Klaassen, 2000), and probably play key roles in the control of tissue/cellular levels of T_3 (Zoeller *et al.*, 2007).

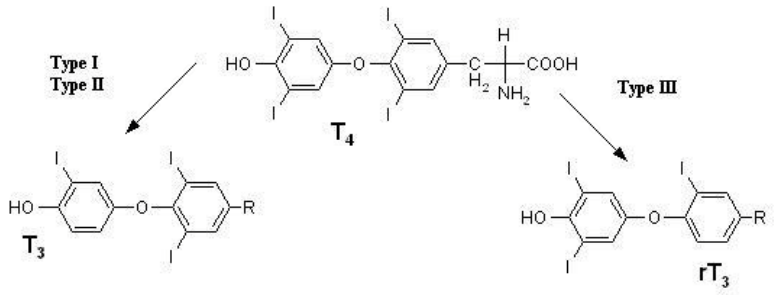

Figure 1. Structural drawings of T_4, T_3 and rT_3. Modified from: http://commons.wikimedia.org/wiki/File: Dejodasenfunktion.png

Thyroid hormones are lipid soluble and either bind to specific receptors on the inner mitochondrial membrane to activate energy metabolism, or to nuclear receptors to increase the transcription of specific genes, and ultimately alter the production of the proteins encoded by them.

In humans the thyroid gland contains large amounts of T_3 and T_4 incorporated in thyroglobulin, the protein within which the two hormones are linked when synthesised and stored in the colloid. Because of these stores, T_3 and T_4 can be secreted rapidly without the need for new hormone synthesis (NCM, 2002).

The production and release of the thyroid hormones is presented in Figure 2:

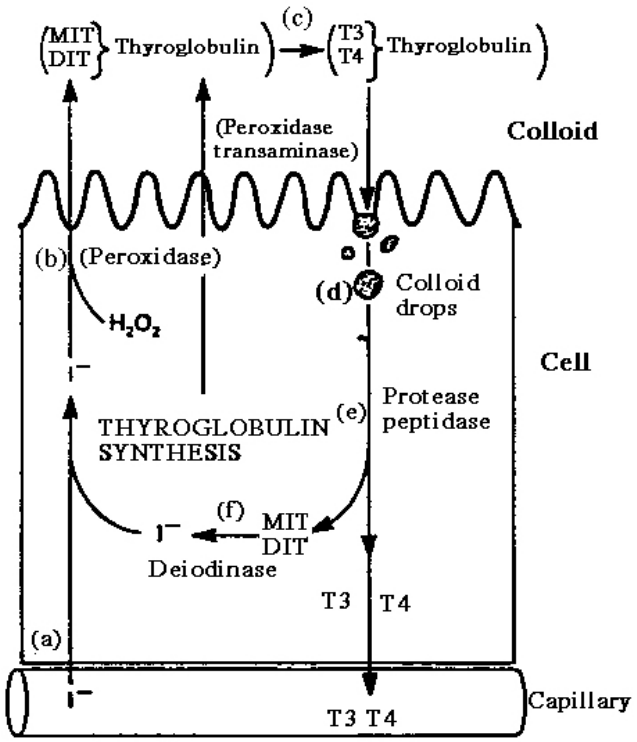

Figure 2. Production and release of thyroid hormones.
Modified from: http://www.druginformation.bc.ca/thyroid.htm

In the next paragraph, bold letters in parentheses refer to the letters in Figure 2, showing the different steps in the TH formation and release process. Iodide from the diet is actively transported across the cell membrane by the sodium-iodide symporter (NIS) (***a***). Under normal conditions the thyroid may concentrate iodine up to about 30-50-fold of the concentration in blood. The trapped iodide is oxidised to reactive iodine (by thyroid peroxidase, TPO) and transferred across the cell and into the colloid in the lumen (***b***). Here it is linked to tyrosine to form monoiodotyrosine (MIT) and di-iodotyrosine (DIT). These are contained within the large thyroglobulin proteins, which have been synthesized by the cells and secreted into the colloid. While still linked to the thyroglobulin, MIT and DIT are converted to T_3 and T_4 with the aid of the enzymes thyroid peroxidase (TPO) and transaminase (***c***). T_4 and T_3, while linked to thyroglobulin, are reabsorbed into the cell in the form of colloid droplets (***d***), are separated from thyroglobulin (***e***), and secreted into the circulation. Any uncoupled MIT and DIT is deiodinated to release tyrosine and iodine, which is then recycled (***f***) (Randall *et al.,* 1997; MCN, 2002; Zoeller *et al.,* 2007).

The thyroid hormones are cleared from the blood in the liver, following sulfonation by sulfotransferases (SULT) or glucuronidation by uridinediphosphate glucuronosyltransferase (UDPGT). The modified THs are then eliminated through the bile (Zoeller *et al.,* 2007).

Thyroid hormones in the circulation are bound to various serum proteins: thyroxine binding globulin (TBG), transthyretin (TTR) and albumin. The overall effect of the binding proteins is to maintain the free serum T_3 and T_4 concentrations within narrow limits, and to ensure that the hormones are continuously and immediately available to the organs. The proteins have both storage and buffer function, and it is the free T_3 and T_4 concentrations that determine the biological activity of the hormones (NCM, 2002). In humans, about 75% of T_4 is bound to TBG, 15 % to TTR and the remainder is bound to albumin. Albumin has a much lesser affinity for the THs than TBG and TTR but binds about 10% of the T_4 anyway because it is present in much greater proportions (Zoeller *et al.*, 2007). T_3 in human blood is mostly carried by TBG. In rodents, TBG levels are high during foetal and neonatal life, but are almost undetectable in adults (Zoeller *et al.*, 2007). Consequently TTR is the major TH transport protein in rodents (Crofton, 2008).

The levels of thyroid hormones in the blood are regulated by a negative feedback mechanism involving the hypothalamic-pituitary-thyroid (HPT) axis, presented schematically in figure 3. The hypothalamus releases thyrotropin releasing hormone (TRH), which stimulates the anterior pituitary to produce thyroid-stimulating hormone (TSH). TSH then prompts the thyroid to produce thyroid hormones. Cells in both the hypothalamus and pituitary respond to levels of circulating thyroid hormones. When these are high, the output of both TRH and TSH declines. When levels of thyroid hormones are low, the outputs of TRH and TSH are raised, prompting the thyroid to increase the output of T_4 and T_3. The negative feedback loop helps the body to respond to varying demands for thyroid hormone and to maintain hormone homeostasis. Interactions from several types of growth factors are also involved in stimulation of the follicular cells (Hurley *et al.*, 1998; MCN, 2002; Zoeller *et al.*, 2007).

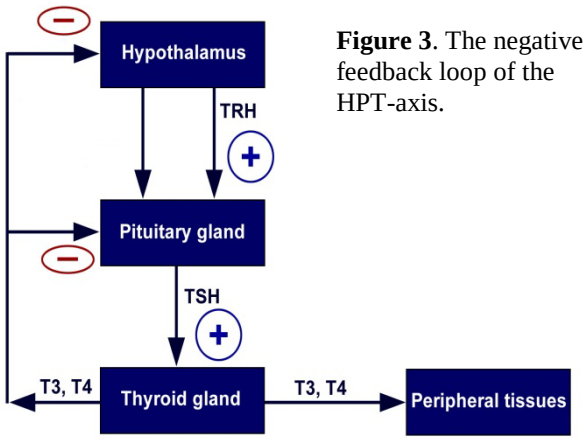

Figure 3. The negative feedback loop of the HPT-axis.

The thyroid gland is capable of meeting physiologic demands for T_4 and T_3 up to a point. However, beyond that point, continuous stimulation may result in changes that could eventually lead to disease. Persistent elevation of TSH levels stimulates the thyroid gland to deplete its existing stores of THs. When the thyroid is not able to keep up with the demand, the follicular cells hypertrophy and divide, leading to hyperplasia and nodular hyperplasia. Generally, the effects are reversible upon removal of the stimulus, at least early in the process (Hurley *et al.*, 1998).

3.3 Environmental thyroid disrupting chemicals (TDCs) and their modes of action

A large number of xenobiotics are known to affect the TH system. These include chemical classes like PCBs and dioxins, brominated flame retardants, several pesticides and ingredients in personal care products like UV-filters, hair dyes and antibacterial compounds (Brucker-Davis, 1998; Hurley *et al.*, 1998; Crofton, 2008; NCM, 2002; Boas *et al.*, 2009). However, many more yet unidentified TDCs may exist. The TDCs can perturb thyroid hormone homeostasis in a number of different ways. Some affect the thyroid gland directly, either by inhibiting the active transport of iodide into the follicular cell or by inhibiting the enzyme thyroid peroxidise, thereby affecting production of THs. Outside the thyroid, chemicals can cause transport disruption by altering binding to serum proteins. Others inhibit the conversion of T_4 to T_3 by affecting peripheral deiodinases, or enhance metabolism and biliary excretion of THs from the liver, through the action of the isoenzymes UDPGT or SULT (Hurley *et al.*, 1998; Crofton, 2008). Finally thyroid receptor agonists and antagonists can possibly also alter TH dependent gene transcription. In figure 4 the possible sites of disruption are illustrated, along with some examples of TDCs that affect them.

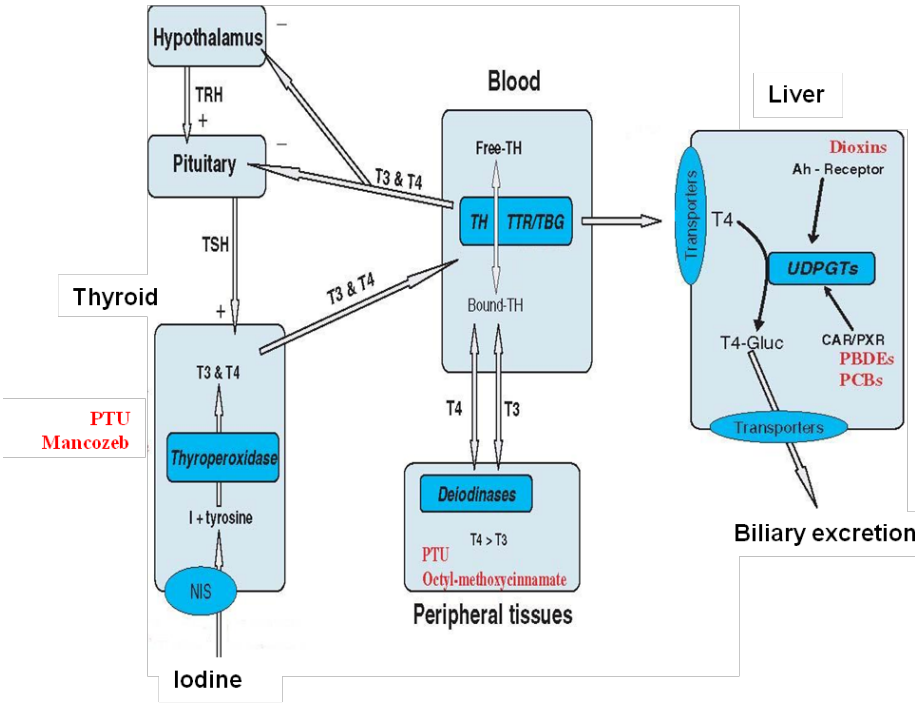

Figure 4. Thyroid hormone control pathways and possible sites of disruption. Inside the thyroid these include the NIS transporter and thyroid peroxidase enzymes. Outside the thyroid, the transport proteins in the blood (transthyretin and thyroxine binding globulin), and deiodinase enzymes in peripheral tissues can be affected. Furthermore metabolism by the UDPGT pathway in the liver can be affected, either through the Aryl hydrocarbon (Ah) receptor or the CAR/PXR receptor. Examples of chemicals affecting the different sites are presented in red, including the three chemicals tested in the present project (PTU, mancozeb and OMC), and three other types of well known TDCs. Modified from Crofton, 2008.

Based on a number of reviews dealing with xenobiotics that affect the thyroid (Brouwer *et al.*, 1998, Brucker-Davis, 1998; Howdeshell, 2002; Zoeller, 2007; Miller *et al.*, 2009; Crofton, 2008; NCM, 2002; Boas *et al.*, 2009, Kashiwagi *et al.*, 2009), a list of TDCs, grouped by their known or suspected mechanisms of action, is presented in Table 1.

Mechanism of action	**Effects on THs**	**Chemicals**
Blocking of iodine transport (NIS)	Decreased thyroidal synthesis of T_3 and T_4	Perchlorate, nitrate
Inhibition of thyroid peroxidase (TPO)	Decreased thyroidal synthesis of T_3 and T_4	MMI, **PTU**, ETU, resorcinol, **mancozeb**, soy isoflavones, BP2
Altered binding to serum proteins, mainly TTR	Disrupted T_4 transport	PCBs, PBDE, TBBPA
Inhibition of deiodinase acivity	Decreased peripheral synthesis of T_3	**PTU,** PCBs, dioxins, **(OMC)**
Enhanced hepatic catabolism by UDPGT	Increased biliary elimination of T_3 and T_4	PCBs, PFOS, PBDE, Azole fungicides, dioxins
Inhibition/activation of SULTs	Altered sulfation of THs	PCBs, triclosan
TR agonists and antagonists	Altered TR-dependent gene transcription	PCBs, bisphenol A, TBBPA

Table 1. Selected thyroid disrupting chemicals grouped by their mechanisms of action.
The xenobiotics presented in red are the three chemicals investigated in the present PhD thesis.
Abbreviated chemical names are explained in the text on the following pages.

Blocking of the NIS transporter and consequent inhibition of iodine uptake into the thyroid has been shown for perchlorate (Wolff, 1998), as well as for nitrate (Fozzatti *et al.*, 2007). Perchlorate salts are used in the pyrotechnics-, space- and military industry, and potential sources of human exposures are contaminated waste sites, polluted water supplies and polluted food sources (US Public Health 2008). Animal studies have shown perchlorate to decrease circulating T_4 levels in rats (Gilbert and Sui, 2008) and the mode of action is blocking of the NIS (Wolff, 1998). Nitrate exposure can also come from polluted water sites or from several food items, e.g. lettuce and spinach. In animal studies, adverse effects on the thyroid system have been reported after long-term nitrate exposure (Esciocak *et al.*, 2005; Zaki *et al.*, 2004). The effects are seen when nitrate is metabolised to nitric oxide, which inhibits the NIS (Fozzatti *et al.*, 2007; Costamanga *et al.*, 1998). In a recent study from our group (Hansen *et al.*, 2009), no effects on thyroid function were seen in dams or foetuses after nitrate exposure in the gestation period, but this may have been due to the relatively short dosing period compared to the 5-7 months of dosing investigated in the previous studies (Esciocak *et al.*, 2005; Zaki *et al.*, 2004).

Inhibition of thyroid peroxidase (TPO), the enzyme responsible for TH formation in the thyroid, is the mechanism of action by which the known thyrotoxic drugs PTU and methimazole (MMI), and the thyrotoxic chemical ethylene thiourea (ETU) act (Taurog, 1976; WHO,1988; Marinovich *et al.,* 1997). When TPO is inhibited, iodine is not transferred to thyroglobulin and consequently synthesis of new THs is reduced. In rats this quickly leads to decreased levels of circulating THs (Zoeller and Crofton, 2005), and because of this well characterised mode of action, both PTU and MMI have been used as model compounds to study the adverse effects of developmental hypothyroidism in experimental animals for many years (Davenport and Dorcey, 1972; Shalock *et al.,* 1979; Akaike *et al.,* 1991; Goldey *et al.,* 1995b; Kobayashi *et al.,* 2005; Noda *et al.,* 2005).
A group of commonly used fungicides from the ethylene bisdithiocarbamate family, including mancozeb, maneb and propineb also inhibit TPO. Maneb and mancozeb are metabolised to ETU while propineb is metabolised to PTU (Houeto *et al.,* 1995; Marinovich *et al.,* 1997; WHO, 1988). Mancozeb, which was one of the chemicals investigated in this Ph.D. study, is a very commonly used fungicide in large parts of the world. Due to its metabolism to ETU, mancozeb affects thyroid function and decrease T_4 levels in experimental animals. This has been shown in a large number of unpublished industry studies (JMPR, 1993), and in a few studies in the published literature (Trivedi *et al.,* 1993; Kackar *et al.,* 1997). Here, however, the dose levels needed to obtain significant thyroid effects were much higher than in the industry studies. Other TDCs which have been shown to adversely affect the TH system by inhibiting TPO include the commonly used UV-filter benzophenone 2 (BP2) (Schmutzler *et al.,* 2007), the hair dying chemical resorcinol (Arnott and Doniah, 1952) and naturally occurring substances like soy isoflavones (Doerge and Sheehan, 2002).

Some xenobiotics may impact the binding of TH to serum proteins, and consequently affect the TH transport. Polychlorinated biphenyls (PCBs) are persistent industrial chemicals, used earlier as fire retardant and cooling fluids in e.g. transformers. Even though their production stopped in the late 1970'ies, they are still leaching out into the environment where they bio-accumulate (Brucker-Davis, 1998). Numerous animal studies have shown thyroid effects after exposure to several PCB congeners (Barter and Klaassen, 1994; Gray *et al.,* 1993; Morse *et al.,* 1996; Ness *et al.,* 1993; Goldey *et al.,* 1995a) and the main mode of action for the coplanar PCBs is most likely induction of UDPGT metabolism, thereby increasing metabolism and excretion of T_4 (see page 10). There are however, *in vitro* studies showing that hydroxylated PCBs can also act by displacing T_4 from TTR (Lans *et al.,* 1993; Chauhan *et al.,* 2000). A group of chemicals that also seem to affect TTR are the brominated flame retardants. These chemicals are widely used in computers, television and other electronic devices (Kashiwagi *et al.,* 2009) and the group includes both polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA). Both compounds have been shown to reduce T_4 levels in animal studies (van der Ven *et al.,* 2008; Hallgren *et al.,* 2001; Zhou *et al.,* 2002),

and mechanistic studies indicate that PBDEs may act by down-regulating the transport protein TTR (Richardson *et al.,* 2008), while TBBPA has been documented to interfere with binding to TTR (Meerts *et al.,* 2000).

Disturbances of the metabolic pathways are also very common ways of thyroid disruption. TH metabolism involves mainly deiodination, glucoroconjugation and sulfation. Deiodination is the major route of metabolism for T_4 (82%) and contributes to 50% of T_3 metabolism (Brucker-Davis, 1998). A number of xenobiotics may alter thyroid hormone homeostasis via interference with deiodinases, which leads to decreased peripheral synthesis of the active thyroid hormone T_3. One example is PTU, which besides its direct inhibition of TPO, also inhibits deiodinase activity in peripheral tissues (Oppenheimer *et al.,* 1972; Leonard and Rosenberg, 1978; Visser *et al.,* 1979). Other chemicals which have been shown to affect deiodinase enzymes in mechanistic studies are the PCBs and dioxins (Hood and Klaassen 2000; Schuur *et al.,* 1998). The UV-filter OMC, which is a very frequently used UV-filter in sunscreens worldwide has also been shown to cause decreased hepatic type I deiodinase activity in rats (Schmutzler *et al.,* 2004; Klammer *et al.,* 2007). This effect is, however, probably rather a consequence of reduced TH levels in the blood than the explanation for the reduced T_4 levels seen in both the study performed by Klammer *et al.* (2007) and in the OMC study performed in the present Ph.D. project. Klammer and co-workers have investigated other possible modes of action of this chemical, and found that OMC did not affect the NIS or the TPO enzymes, but caused reduction in circulating levels of T_4, T_3 and TSH, and therefore hypothesised that OMC may reduce TH levels by a direct action on the thyrotropes in the anterior pituitary, resulting in reduced TSH release (Klammer *et al.,* 2007).

Another well-characterized effect of TDCs is their ability to increase biliary elimination of THs via induction of UDPGTs (Crofton 2008). Dioxins are highly toxic compounds, which are formed as by-products during combustion of clorine-containing waste. They induce hepatic UDPGT activity by binding to the aryl hydrocarbon receptor (AhR), while co-planar PCBs act through the nuclear receptors constitutive active/androstane receptor (CAR) and pregnane X receptor (PXR) to induce UDPGT activity (Crofton, 2008). This leads to biliary excretion and elimination of T_4 (Barter and Klaassen, 1994; Liu *et al.,* 1995), which in turn reduces circulating and tissue T_4 levels (Seo *et al.,* 1995; van Birgelen *et al.,* 1995; Schuur *et al.,* 1997; Morse *et al.,* 1996). Perfluorinated chemicals, and among these perfluorooctane sulfonate (PFOS), are very persistent chemicals. PFOS is no longer produced but was previously very widely used in industrial and consumer products due to its surface protection properties. Several animal studies have shown decreased levels of T_4 after PFOS exposure (Lau *et al.,* 2003; Thibodeaux, 2003; Yu *et al.,* 2009), and *in vitro* studies of exposed rat tissues have shown up-regulation of hepatic glucuronidation enzymes (Yu *et al.,* 2009). The flame retardant PBDE might also act by induction of hepatic UDPGT (Hallgren *et al.,* 2001),

and some azole fungicides have also been shown to affect thyroid function through increased UDPGT activity (Wolf *et al.,* 2006). In a recent study from our group, investigating effects of developmental triazol exposure, the azole fungicide teboconazol significantly reduced T_3 levels in dams (Taxvig *et al.,* 2007). Even though the mode of action was not investigated in this study, it was likely the same as seen with the other azole fungicides.

Finally some xenobiotics appear to affect metabolic pathways by affecting hepatic SULT enzymes. The antibacterial compound triclosan, which is present in many consumer products like detergents and toothpastes, has recently been shown to decrease T_4 levels at relatively low doses in animal studies (Zorilla *et al.,* 2009; Paul *et al.,* 2010a, b). Triclosan has been shown to inhibit SULTs (Paul *et al.,* 2010a), while PCBs up-regulate SULTs (Schuur *et al.,* 1998), so the impact of SULT alterations on circulating levels of THs is currently unknown (Crofton, 2008).

Whether xenobiotics bind directly to thyroid receptors (TR) is currently a controversial issue, and there are data to both support and refute the hypothesis (Crofton, 2008). In mechanistic studies the very widely used plastic additive bisphenol A (BPA) has been shown to prevent the binding of T_3 to the TR, which resulted in suppression of TR-mediated gene expression (Moriyama*et al.,* 2002; Sun *et al.,* 2009). In amphibian models TBBPA has been shown to bind directly to the TR and impair TH-dependent metamorphosis (Jagnytsch *et al.,* 2006; Kitamura *et al.,* 2005; Fini *et al.,* 2007). Because of their structural similarity to THs, PCBs have also for a long time been regarded as possible TH agonists or antagonists (Porterfield, 1994, McKinney and Waller, 1994). Although it now appears unlikely that PCB congeners, or their metabolites competitively bind to the TR, it may well be that they produce allosteric effects on TRs that alter their ability to mediate thyroid hormone action (Yamauchi and Ishihara, 2006; Crofton, 2008)

As can be seen from this literature review, there are a great number of chemicals that can affect the thyroid hormone system, acting through many different modes of action, but with the end result in almost all cases being reduced T_4 levels and sometimes also reduced T_3 levels. In adults, the thyroid is a relatively robust organ, normally able to compensate for mild or moderate disruption through compensatory thyroid enlargement caused by hyperplasia of the follicular cells (Brucker-Davis, 1998). However, the impact of thyroid disruption *in utero* should be distinguished from the impact on a fully grown individual. Because thyroid hormones have a pivotal role in the developing foetus, prenatal exposure to thyroid disrupters may potentially have very damaging and irreversible effects.

3.4 The role of thyroid hormones in the developing human nervous system

During the foetal and neonatal periods, thyroid hormones control neuronal and glial proliferation in the brain and regulate neuronal migration and differentiation. Because neurologic development occurs in discrete developmental windows, the role of thyroid hormones in orchestrating the timing of specific developmental events is crucial, and even transient disorders in TH availability can have profound and irreversible consequences on brain development (Porterfield, 2000). Many important aspects of brain development occur before the time of foetal TH synthesis. This is possible because maternal THs are transferred through the placenta and exert their effects long before the foetus's own thyroid hormone system is active (Porterfield and Hendrich, 1993). In humans, transfer of maternal THs to the foetus starts early in pregnancy and continues until birth. The foetal thyroid does not become functional until the 12^{th} week of gestation, so the foetus depends entirely on TH of maternal origin during the first trimester, but its own TH system assumes an increasingly greater role in producing THs as gestation progresses (Zoeller and Rovet, 2004).

It is well established that low levels of THs during development can cause irreversible effects on brain development, and the risk of suboptimal neurologic development is recognised in the following groups (Zoeller *et al.*, 2007):
- prematurely born babies, severed from the maternal TH supply during the third trimester
- children born with congenital hypothyroidism (CH), and therefore unable to produce THs themselves
- children suffering from endemic cretinism, caused by low maternal iodine intake during pregnancy
- children of women who suffered from untreated hypothyroidism during pregnancy

The longer the period of TH deficiency, the more adverse effects are seen. In children suffering from endemic cretinism TH levels are decreased both before and after the time of foetal thyroid functioning because of the low iodine intake. This can result in mental retardation, as well as problems with gross and fine motor control and with the auditory system. If postnatal hypothyroidism is present, there is also risk of growth retardation and delayed or absent sexual maturation (Porterfield, 2000). In most cases of congenital hypothyroidism (CH), symptoms are somewhat milder and include reduced intelligence, decreased learning ability and hyperactivity (Porterfield, 1994). If the disease is detected soon after birth, the child may be treated with thyroid hormones, thus reducing the risk and magnitude of long-term damage to the nervous system. CH can be detected if children are screened for thyroid function at birth, and systematic neonatal screening for CH has therefore been progressively implemented in industrialized countries during the last 15 to 25 years (Zoeller *et al.*, 2002; van Vliet 1999).

Among healthy women who do not have iodine deficiency in their diet, and whose children do not have CH, there may still be a good reason to be aware of TH levels during pregnancy. A number of interesting discoveries made over the past decade have shown a correlation between healthy pregnant mothers' thyroid status and their children's neurological development. In 1999 Pop *et al.* found that children of mothers, who in their 12^{th} week of pregnancy were in the lowest 10^{th} percentile with regard to concentration of free T_4 (fT_4), had significantly lower psychomotor development at the age of 10 months than was seen in the reference group. A clear correlation was seen between maternal fT_4 concentrations in the 12^{th} week and their children's developmental scores, and in addition infants of women whose fT_4 levels were low at both 12 and 32 weeks of gestation exhibited a poorer outcome than infants of women whose free T_4 levels were low only at 12 weeks. To further investigate the adverse effects, a new group of children from hypothyroxinemic mothers was investigated at 1 and 2 years of age, and compared to paired controls (Pop *et al.,* 2003). The results were similar to the ones from the 1999 study, namely that low maternal fT_4 concentrations (hypothyroxinemia) during pregnancy was associated with altered brain development, as the children performed worse in tests of motor and mental skills. The term "hypothyroxinemia" in pregnant women is defined as "a level of circulating T_4 that is lower than the norm in pregnant women in the same trimester, regardless of whether clinical hypothyroidism is present" (Morreale de Escobar *et al.,* 2000).

Other studies have demonstrated that children of healthy women who had very high TSH concentrations in the 17^{th} week of pregnancy (above 98 percentile) also had significantly lower IQ levels at the age of 7-9 year (Haddow *et al.,* 1999) and that the IQ in these children was inversely correlated with maternal TSH concentration (Klein *et al.,* 2001). Several more recent studies have further corroborated the above mentioned findings, as is has been shown that maternal hypothyroxinemia during the first trimester can result in adverse effects on a child's intelligence and motor development (Kooistra *et al.,* 2006, Li *et al.,* 2010), and be a risk factor for both verbal and nonverbal cognitive delay in early childhood (Henrichs *et al.,* 2010).

Whether the maternal hypothyroxinemia is in some way associated with endocrine disruption by environmental xenobiotics is presently unknown. There are however studies of human populations with high background exposures to PCBs and dioxins, showing that exposed children showed mild reductions in TH levels shortly after birth (Schantz, 1996). Furthermore, data from several independent cohorts of newborns have shown sub-optimal cognitive function in infants and children exposed prenatally to the highest degree of PCBs and/or dioxins, though still at "background" levels (Schantz and Widholm 2001). Whether the neurological effects are caused by TH disruption or direct neurotoxicity is, however, still unknown but here animal studies may provide important information.

3.5 Developmental hypothyroidism in animal studies

In rats, dams also transfer thyroid hormones to their foetuses before these start producing hormones themselves. This was shown, in a study where T_3 and T_4 levels were measured during gestation in foetuses from rat dams that had been thyroidectomised (ThX) before gestation. In these foetuses, circulating THs were only found after GD 17.5 that is when the foetuses began to produce the hormones themselves, while in control foetuses THs were present much earlier. Even after GD 18, TH levels in offspring from ThX females remained much lower in than in control offspring and the offspring were generally underdeveloped (Morreale de Escobar *et al.,* 1987). This indicated that rats could be useful models for studying the effects of developmental hypothyroidism or hypothyroxinemia in man.

From the animal studies performed this far, we know that reduced circulating levels of THs, lead to reduced TH levels in the brain (Oppenheimer,1983). This again increases the risk of permanent structural abnormalities in the brain, including defects in nerve cell migration and other structural abnormalities. A detailed review of the studies investigating how developmental hypothyroidism affects brain structure has been performed by Howdeshell (2002). These morphological and neuochemical changes may be associated with impairments in neurobehavioural function (Schantz and Widholm 2001). In most of the early animal studies investigating effects of developmental hypothyroidism on behaviour, severe TH suppression was obtained by thyroidectomy or by exposure to high doses of antithyroid agents like PTU or MMI. Regardless of the method used, effects on brain development were largely the same, as severe TH insufficiency during development was associated with impaired learning and memory abilities and increased spontaneous activity (Davenport and Dorcey, 1972; Davenport *et al.*, 1976a, b; Comer and Norton, 1982; Schalock *et al.,* 1979). In newer studies, in which more moderate developmental hypothyroidism has been induced, similar effects have been found. In almost all of the studies dealing with this issue, dams have been made hypothyroid during either part of or the entire gestation period, as well as the lactation period, and in most cases PTU has been used (Brosvic *et al.,* 2002; Noda *et al.,* 2005; Goldey *et al.,* 1995b; Kobayashi *et al.,* 2005). The consequences in the adult offspring have again been cognitive and motor deficits, seen as impaired spatial learning abilities and hyperactivity. One study investigating the effects of neonatal PTU exposure alone also showed impaired spatial learning and memory abilities in the offspring (Akaike *et al.,* 1991).

Impaired neural development and lowered TH levels has also been investigated in a few studies after developmental exposure to environmentally relevant TDCs, including PCBs and PBDE. Offspring from rats exposed to PCBs throughout gestation and lactation showed significant reductions in serum T_4 levels, as well as impaired learning ability (Provost *et al.,* 1999). Developmental exposure to the brominated flame retardant PBDE, which have been

shown to decrease circulating T_4 levels (Hallgren *et al.*, 2001; Zhou *et al.*, 2002) has also had negative effect on brain development, as it caused hyperactivity and impaired learning and memory in mice (Viberg *et al.*, 2003; Branchi *et al.*, 2003).

Hearing loss has also been demonstrated in several studies after early postnatal hypothyroidism, and hearing is impaired because of permanent structural damage and loss of function in the cochlea (Crofton, 2004). In most of these studies, hypothyroidism has been induced by developmental exposure to either PTU or different PCB congeners (Goldey *et al.*, 1995a; Goldey *et al.*, 1995b; Henley and Rybak, 1995; Crofton *et al.*, 2000a; Crofton *et al.*, 2000b; Crofton and Rice, 1999; Powers *et al.*, 2006), but developmental exposure to TBBPA has also resulted in cochlear effects in the exposed offspring (Lilienthal *et al.*, 2008). Furthermore, Crofton (2004) have shown that impaired hearing could be correlated to decrease in early postnatal T_4 levels, after PCB exposure. This inspired us to investigate if the same correlations could be found for behavioural effects and T_4 levels in the three performed studies.

3.6 Timing of TH insufficiency

From the great number of studies and case reports on developmental hypothyroidism and/ or hypothyroxinemia in humans, it now seems clear that not only severity but also the timing of TH deficiency can affect the child's neurological outcome. Generally, a prenatal TH loss contributes to difficulties in visual processing and visuomotor abilities, whereas an early neonatal TH insufficiency is associated with impaired visuospatial abilities. A TH insufficiency somewhat later in postnatal development is associated with sensorimotor and language deficits whereas hypothyroidism that extends even further in infancy is associated with poorer language, fine motor, auditory processing, attention and memory skills (Zoeller and Rovet, 2004).

On the other hand, few animal studies have focused on identifying the developmental windows of TH action in the rat brain, as the animals have usually been made hypothyroid throughout both pregnancy and postnatal development (Zoeller and Rovet, 2004). There are, however, a few studies in which hypothyroidism in rat dams has been induced during gestation only. Auso *et al.,* (2004) have reported that three days of MMI treatment to pregnant rats, caused a transient 30% decline in maternal T_4, and that this was enough to cause significant migration defects in the cerebral cortex of the offspring. Other neuroanatomic alterations (Gilbert and Sui, 2006; Gilbert and Sui, 2008; Lavado-Autric *et al.,* 2003; Sharlin *et al.,* 2008; Cuevas *et al.,* 2005), as well as changes in gene expression patterns in the brain (Dowling *et al.,* 2000; Dowling and Zoeller, 2000) have also been reported in rat offspring after moderate and transient maternal hypothyroxinemia. Furthermore, there are a few studies in the published literature where the behaviour of rat offspring, from mothers that were only rendered hypothyroid during gestation, was affected. In three of the studies hypothyroidism was induced by thyroidectomy (Hendrich *et al.,* 1984; Liu *et al.,* 2010; Friedhoff *et al.,* 2000), whereas prenatal MMI treatment was used in the fourth (Opazo *et al.,* 2008). In all studies, the effects in the adult offspring were hyperactivity and/or impaired maze learning. It has, however, been shown that maternal thyroidectomy during gestation does not only reduce maternal TH levels, but also significantly reduces the TH levels in the offspring during the neonatal period (Porterfield and Hendrich 1981). So the studies where maternal hypothyroidism was induced by thyroidectomy may not have shown the isolated effects of maternal hypothyroxinemia, but rather the combined effects of pre- and postnatal hypothyroidism in the offspring. In the study where MMI was used to obtain hypothyroidism some methodological shortcomings regarding behavioural testing were present. With this knowledge in mind, and based on the findings from the present project the hypothesises that moderate maternal hypothyroxinemia is not sufficient to induce behavioural effects in rat offspring, was presented. This point is discussed further in papers II and III as well as in the discussion section of this thesis.

3.7 Species differences

Although both brain development and the hypothalamic-pituitary-thyroid axis generally function similarly among the mammalian species, there are differences which are important to consider when extrapolating from rodents to humans.

Both rodents and humans need THs for differentiation and maturation of the central nervous system, but there are large species differences in the timing of these events. While most of the brain maturation happens prenatally in humans, a large part of it happens postnatally in rats (Howdeshell, 2002).This is also true for development of the hearing organ (Crofton *et al.,* 2000), and consequently these species difference could affect the importance of the timing of correct TH levels in the different species.

There are also species differences in the thyroid hormone system. For example, the effect of long-term hypothyroidism in adult rodents is thyroid tumour formation. These tumours are the result of long-term overstimulation of the thyroid gland, via up-regulation of TSH, and it is now widely recognized that this rodent mode-of-action is not relevant to humans (Capen, 1997; Crofton, 2008). Other important species differences between humans and rodents are found in the amounts of stored THs in the thyroid gland, as well as in transport proteins present in the serum. The adult rat stores only a few days worth of thyroid hormone, whereas the normal adult human thyroid stores perhaps several months' worth (Zoeller *et al.,* 2007). Furthermore, adult rodents lack thyroid hormone binding globulin (TBG), the specific high-affinity serum carrier protein that exists in humans (Dohler *et al.,* 1979). The absence of this carrier protein results in a greater proportion of free TH in the rat serum, which may be more readily available to metabolism and excretion. It is therefore often claimed that because of a shorter half-life of THs, rodents are more sensitive than humans to chemically induced thyroid disruption (Hurley *et al.,* 1998; NCM 2002) and may therefore not be suitable models for the purpose of reflecting thyroid disruption in humans (NCM 2002).

Others have, however, argued that the current animal models have not been characterized in a manner that allows us to determine whether rodents are more or less sensitive than humans to TH insufficiency (Zoeller and Crofton, 2005). There is no evidence that the lack of TBG in rodents necessarily makes them more sensitive to thyroid perturbation than humans, as this concept requires that we fully understand the physiological role of serum binding proteins for thyroid hormone and this may not be the case (Zoeller *et al.,* 2007). According to Zoeller *et al.* (2007), total T_4 levels in rodents are therefore valid and relevant measures of thyroid function in relation to effects in humans, as long as serum binding proteins are not being affected by the treatment under study.

Given the same dose of an antithyroid compound e.g. PTU (normalized to body weight), it is quite clear from the experimental data that rats would exhibit a significant reduction in circulating levels of THs sooner than humans (Zoeller and Crofton, 2005). Although this kind of study has not been performed directly, differences in thyroid economy and in the amount of stored TH leads to this prediction. Therefore, a decrease in TH synthesis would be manifested in reduced serum hormone levels in rats prior to humans. However, rats and humans may be similarly sensitive to the ability of PTU or other TDCs to reduce thyroid hormone synthesis. Likewise, considering that a human neonate has a serum half-life of T_4 of around 3 days (Vulsma *et al.,* 1989) and intra-thyroidal stores of T_4 are estimated to be less than 1 day worth (van den Hove *et al.,* 1999), it would be predicted that human neonates would exhibit a decrease in serum TH levels prior to that observed in adults. Thus, humans and rats may be similarly sensitive to the effect of TH synthesis, with rats exhibiting effects on hormone levels after shorter exposures than in humans, though the ultimate effect may be the same (Zoeller and Crofton, 2005).

To summarise, concordance between rodent and human modes-of-action therefore depends on the life stage in which exposure takes place, and there seems to be a good degree of concordance for developmental neurotoxicity using altered TH concentrations as a key event (Crofton and Zoeller, 2005; Crofton 2008). Even though a large number of animal studies investigating the effect of developmental hypothyroidism/hypothyroxinemia have been performed, we are still missing a lot of knowledge on the relationship between xenobiotic exposure during development, the effects on TH levels and the consequences for the developing nervous system - which is why this Ph.D. project was initiated. In the following section an overview of the animal studies performed during this project is presented.

4. Overview of the animal experiments

Table 2 gives an overview of the three studies performed in the present Ph.D. project, and of the endpoints investigated in each of the studies. In the first study, the thyrotoxic drug PTU was tested. Hereafter studies on the two environmentally relevant TDCs, OMC and mancozeb, were performed. Dose levels of the three tested compounds were selected to induce transient developmental hypothyroxinemia, without causing general maternal toxicity effects. For PTU and mancozeb range finding studies were performed before the main dose-response studies, while the dose levels for OMC were selected based on the published literature.

The experimental procedure for the three studies is described in more detail in papers I, II and III, but the overall experimental design was as follows: time mated rat dams were dosed with the thyroid hormone disrupting compound from implantation of the embryos on gestation day (GD) 7, to the end of lactation on postnatal day (PND) 16. On GD 15 dams were anesthetised and a blood sample from the tail vein was drawn. In all three studies blood samples were also collected from the offspring on the last day of dosing (PND 16), and from dams and offspring on the day of weaning (PND 24/27/28), to investigate if the effects on the TH system were reversible. In the OMC study blood samples from dams were additionally collected on PND 15. All blood samples were analysed for total T_4 levels, as these are more sensitive to thyroid disruption than T_3 levels. The neurological development of the offspring was investigated in a battery of behavioural tests. These included assessment of motor activity and habituation in the young offspring (PND 14, 17 and 23) and again at the age of 2-4 months. Spatial learning and memory was assessed in the Radial Arm Maze and the Morris Water maze at 3-7 moths of age, while acoustic startle testing and hearing function, including auditory thresholds, was assessed in collaboration with the National Research Centre for the Working Environment at 6-8 months of age. Furthermore the experimental set-up investigated additional developmental and reproductive toxicity endpoints. These included effects on pup survival and growth, sex hormone levels, semen quality and weights and pathology of several organs (thyroid gland, testis, ovary, prostate, epididymis, liver and adrenal). However, not all of these endpoints were investigated in all three studies. Statistical analysis of data was always adjusted, using litter as a nested factor, when more than one pup from each litter was examined.

In table 2 the doses of the test compounds, the number of animals and the investigated endpoint for each of the three studies are described.

Table 2. Overview of the animal experiments performed in the Ph.D. project.

Paper	**Compound**	**Investigated toxicological endpoints**				
	Test substance and doses (mg/kg bw/day)	general -	hormonal-	thyroidal-	reproductive-	behavioural-
I	PTU - Propylthiouracil (0, 0.8, 1.6, 2,4) N=16-17	Maternal weight gain - pup growth	Dams: T_4 on GD 15 and PND 27 Offspring: T_4 on PND 16, 27 and in adulthood	PND 16 and 27: thyroid weight and histology. Adulthood: thyroid weight and histology	AGD – NR	PND 14, 17, 21: motor activity Adulthood: motor activity - MWM - RAM - hearing
II	OMC - Octyl methoxycinnamate (0, 500, 750, 1000) N=14-18	Maternal weight gain - pup growth PND 16: liver and adrenal weights and histology.	Dams: T_4 on GD 15, PND 15 and 28 Offspring: testosterone, estradiol, progesterone and T_4 on PND 16 and 28 Testosterone and T_4 in adulthood	PND 16: thyroid weight and histology Adulthood: thyroid weight and histology	AGD - NR PND 16: gene expr., weight and histopath. of prostate, testes, epididymis and ovaries. Timing of sexual maturation. Adulthood: sperm counts, reproductive organ weights	Adulthood; motor activity - RAM - startle-hearing
III	Mancozeb (0, 50, 100, 150/100) N=9-21	maternal weight gain, pup growth, PND 16: liver and adrenal weights	Dams: T_4 on GD 15, PND 24 Offspring: testosterone, and T_4 on PND 16. T_4 on PND 24	PND 16; thyroid weight and histology Adulthood; thyroid weights	AGD - NR PND 16; weight and histopath. of prostate, testes, epididymis and ovaries Adulthood: ovary weights	PND 14, 17, 21: motor activity. Adulthood: motor activity - RAM - startle

Wistar rat dams (Han:Tac) were dosed by gavage, from gestation day (GD) 7 – postnatal day (PND) 16, vehicle was corn oil. In all offspring anogenital distance (AGD) was measured at birth and nipple retention (NR) was measured at PND 13. Some offspring were sacrificed on PND 16 while others were weaned on PND 24/27/28. Timing of sexual maturation was measured in study II. Motor activity levels were measured in young animals and in adulthood, and the animals were furthermore tested in the Morris Water Maze (MWM), the Radial arm maze (RAM), for acoustic startle response and hearing function. Some animals were also sacrificed after ended behavioural testing and reproductive organ weights, hormone levels and semen quality were measured.

5. Summary of the main findings from the three present studies

All of the results from the three studies are presented in papers I, II and III respectively. Below is a short summary of the main findings and points of discussion from each of the three papers. The findings from the studies are further presented in tables 3 and 4 on the following pages of the thesis.

Paper I (PTU).

The aim of this study was examine how neurological development in rats would be affected by pre- and postnatal thyroid disruption by PTU, and to find out whether behavioural changes in the offspring could be predicted by measuring TH levels in the blood from exposed dams. The known thyrotoxic compound PTU was tested in order to have a reference with regards to T_4 reductions and behavioural outcomes in our laboratory. The results showed that dams from the two highest PTU dose groups, and pups from all three dose groups (0.8, 1.6 and 2.4 mg/kg bw/day) had significantly decreased total T_4 levels when measured on GD 15 and PND 16 (see Table 3), and that the weights and histology of the thyroid glands on PND 16 and 27 were severely affected. In the highest dose group, pup body weights were significantly decreased on PND 23 and 27 but were no longer affected in adulthood. As expected from other developmental hypothyroidism studies, motor activity levels in the young exposed offspring were decreased on PND 14 and increased on PND 23. In the adult male offspring, maze learning was impaired in the two highest dose groups, while both males and females from the highest dose group additionally showed hyperactivity and impaired auditory function (Table 4). The observed changes in both behaviour and hearing loss were significantly correlated to reductions in T_4 levels during development, indicating that the degree of developmental T_4 levels could be an indicator of the severity of behavioural and auditory defects later in life.

Paper II (OMC).

In order to examine if hypothyroxinemia in the pre- and postnatal period, induced by an environmentally relevant chemical with more possible modes of action, was also related to subsequent neurobehavioural toxicity in rats, it was examined how developmental exposure to the UV-filter OMC would affect the offspring. All doses of OMC (500, 750 and 1000 mg/kg bw/day) caused total T_4 levels to decrease markedly in dams, when measured on GD 15 and PND 15 (Table 3). The thyroid effects in the offspring were much less marked than seen in the PTU study, as only a moderate decrease in male T_4 levels was seen on PND 16, while no significant effects were seen in the female offspring. Furthermore, no effects on thyroid histology were seen. In OMC exposed female offspring, motor activity levels were decreased in the highest dose group, while low and high dose males showed improved spatial learning abilities (table 4). The observed behavioural changes were not correlated to developmental T_4

levels, and were probably not mediated by early T_4 deficiencies, as they differed substantially from effects seen in other studies of developmental hypothyroxinemia - including the PTU study in the present thesis. Offspring body weights were reduced throughout the study in males from all dose groups, whereas female body weights were reduced in the highest dose group until PND 50. On PND 16 high dose male offspring showed reduced relative prostate and testis weights, histopathological effects in both organs, and a dose-dependent decrease in testosterone levels which was significant in all dose groups. At eight months of age, sperm counts were reduced in all three OMC-dosed groups, and prostate weights were reduced in the highest dose group. The reproductive effects were probably mediated by OMC's estrogenic properties. The reproductive and behavioural effects seen in the present study indicate that pre- and postnatal OMC exposure may be a cause of concern for human neurological and reproductive development, as humans are systematically exposed to OMC through usage of sunscreen products and other cosmetics. Since motor activity and learning behaviour in these rats was affected oppositely of what would be expected after developmental hypothyroxinemia, maternal T_4 deficit was not a good predictor for effects on the developing nervous system in the offspring after exposure to this xenobiotic.

Paper III (Mancozeb).

In this study exposure to another environmentally relevant xenobiotic compound, the fungicide mancozeb was tested. General toxicity effects of Mancozeb exposure, including weight loss and transient hind limb paralysis, were evident in the dams at much lower exposures than expected from the published literature. Therefore, mancozeb doses had to be lowered in mid study, which may have resulted in the absence of effects in the offspring. A significant and dose-dependent decrease in total T_4 levels (between 20-40%) was seen in dams from all three dose groups on GD 15 (Table 3). However, offspring total T_4 levels, thyroid weights and histology were unaffected when examined on PND 16. Furthermore no effects on reproductive organ weights were seen, and no behavioural changes were observed (Table 4). Possibly, toxicokinetic factors may have affected maternal transfer of Mancozeb and thereby limited exposure to the pups. Alternatively mancozeb may not have triggered the same toxicodynamic effects in the offspring as in exposed dams.

Based on the results from the mancozeb study, as well as on the OMC-study, it was hypothesized in this paper that in rats, prenatal T_4 decreases in dams may not in themselves be determining for adverse development of learning and motor skills in the offspring. It was however stressed that because of conflicting results found in the literature, more research in this area is needed. As mancozeb did cause significant T_4 reductions in the pregnant rat dams, exposure to this pesticide may still be a potential contributor to thyroid disruption in humans, and a risk factor for pregnant women, as developmental hypothyroxinemia in humans is known to adversely affect the developing human brain.

6. Discussion

6.1 Developmental T_4 decreases and their effects on rat behaviour

The first aim of this Ph.D. project was to assess behaviour and TH status in rats exposed to three different xenobiotics during development. We found that:
i) exposure to PTU, OMC and mancozeb caused varying effects on T_4 levels,
ii) dams and offspring were affected differently depending on which xenobiotic was used, and
iii) exposure to the three compounds resulted in very different behavioural outcomes.

In tables 3 and 4 the results from the three studies are presented schematically. Table 3 describes the reductions in T_4 levels as a percentage of concurrent control values, while table 4 shows how motor activity, maze learning, hearing ability and startle response were affected in the adult offspring from the three studies. The actual T_4 levels measured in the three studies, including the number of samples for each measurement and the standard errors are presented in each of the three papers (Table 2 in papers I, II and III), while the significant behavioural effects are presented in figures 3-7 in paper I (PTU) and in figures 3-4 in Paper II (OMC). As no behavioural effects were seen in the mancozeb study, no data on behaviour were presented in paper III.

Table 3. T_4 levels in dams and offspring from the three studies - % of control. Numbers in bold, represent T_4 levels which were significantly lower than in concurrent controls. In the young offspring the presented group means are based on litter means. In the papers these data are shown for male and female offspring separately, when relevant. Blanks represent measurements that were not performed.

	Dams			**Offspring**			
Dose mg/kg/day	GD15[#]	PND 15[#]	PND 27/28/24	PND 16 [#]	PND 27 / 24	Adult female	Adult male
PTU							
0	100%		100%	100%	100%	100%	100%
0.8	94%		82%	**50%**	115%	96%	85%
1.6	**55%**		103%	**23%**	108%	114%	85%
2.4	**41%**		136%	**16%**	89%	**81%**	92%
OMC							
0	100%	100%	100%	100%		100%	100%
500	**27%**	**30%**	97%	91%		95%	100%
750	**2%**	**3%**	107%	**80%**		94%	113%
1000	**4%**	**0%**	137%	**73%**		125%	99%
Mancozeb							
0	100%		100%	100%	100%		
50	**79%**		85%	110%	107%		
100	**73%**		79%	106%	119%		
150/100	**63%**		94%	102%	131%		

[#] Blood samples collected while animals were still being dosed.

Table 4. Behavioural effects in adult offspring from the three studies, compared to concurrent controls. Arrows represent significant increases (↑) or reductions (↓) in the performed behavioural and auditory tests. (–) represents non-significant effects, while blanks represent tests that were not performed.

	activity levels		**maze learning**		**hearing ability**	**Startle**
	female	male	female	male	fem. & male	fem. & male
PTU						
0.8	-	-		-	-	
1.6	-	-		↓	-	
2.4	↑	↑		↓	↓	
OMC						
500	-	-	-	↑	-	-
750	↓	-	-	-	-	-
1000	↓	-	-	↑	-	-
Mancozeb						
50	-	-	-	-		-
100	-	-	-	-		-
150/100	-	-	-	-		-

As can be seen in Table 3 the severity of T_4 reduction differed substantially depending on which compound was investigated and whether dam or offspring T_4 levels were studied. This was probably due to the fact that PTU, OMC and mancozeb affect the thyroid system by somewhat different mechanisms and because they most likely differ in their ability to cross over the placenta and/or into the breast milk.

In the PTU study, the offspring appeared more sensitive to thyroid disruption than the dams, as their T_4 levels on PND 16 were lowered by 84% in the highest dose group, compared to controls, while the highest PTU dose reduced T_4 levels in dams by around 60%. This effect in the dams was seen after only 8 days of dosing but as can be seen from the OMC data (where T_4 levels were measured both on GD15 and PND 15), 20 more days of dosing did not seem to reduce dam T_4 levels further. It is of course not possible to conclude that this was also the case in the PTU study, as the two compounds have different modes of action. Measurements of dam T_4 levels on PND 15 would therefore have been nice to have in the PTU study. The high sensitivity in the offspring indicates that PTU must have been readily transferred to the offspring through placental and/or lactational transfer. As can be seen in table 4, motor activity levels were increased in both male and female adult offspring, male maze learning was impaired and hearing function in both sexes was reduced after the PTU exposure. These effects were all expected after developmental PTU exposure (Brosvic *et al.,* 2002; Noda *et al.,* 2005; Goldey *et al.,* 1995b; Kobayashi *et al.,* 2005). As many previous studies have investigated the effects of perinatal PTU exposure on neurological development, one of the aims of this PTU study was to compare the degree of hypothyroidism caused by PTU, with

the severity of the observed behavioural changes. This was done in order to contribute to a clearer characterization of the relationship between disruption of THs and adverse effects on the brain, in the same way that it has previously been done for hearing ability after PCB exposure (Crofton, 2004). In the present study significant correlations between developmental T_4 levels and adverse effects on adult activity levels, learning and auditory function were seen, and as described in paper I, between 15 and 25 % of the variation in these endpoints could be explained by developmental T_4 levels. So in the case of PTU, developmental T_4 concentrations could be used to predict altered behaviour and hearing function in adult rats.

A large difference between the results from the PTU and OMC studies was seen in the severity of effects on the thyroid system in both dams and offspring. This difference may have been caused by the differing modes of action for the two chemicals. PTU inhibits TPO, thereby blocking formation of new THs, and furthermore inhibits peripheral deiodination of T_4 to T_3, thereby reducing the supply of active hormone needed for neural proliferation and differentiation in the brain. OMC's mode of action is still not fully understood, but may be a direct effect on the anterior pituitary, causing reduced secretion of TSH, which again reduces the synthesis of T_3 and T_4 in the thyroid. While reduced deiodinase activity has also been reported after OMC exposure, the authors suggested that this may rather be a consequence of reduced TH levels, than a mode of action for OMC (Klammer *et al.,* 2007). It is therefore possible that the differing mechanisms of TH disruption caused by the two compounds, could account for the observed differences. In the OMC study, offspring T_4 levels on PND 16 were only moderately affected, with a decrease of around 27% in the highest dose group, compared to the 84% reduction seen in the PTU study (Table 3). Increases in offspring thyroid weights on PND 16 were also smaller than those seen in the PTU study (table 2 in paper I and table 3 in paper II), and while thyroid histopathology in the PTU study showed follicular hyperplasia on PND 16, it was unaffected in the OMC offspring. The dams from the OMC study, on the other hand, showed a quite unexpected and very marked reduction in serum T_4 levels on both GD 15 and PND 15 (Table 3). As discussed in paper II, possible explanations for the moderate thyroid hormone disrupting effects in the offspring, compared to the marked effects seen in dams, could be that OMC was only transferred to the offspring in very low concentrations due to low placental or lactational transfer. Alternatively, the offspring were for some reason less susceptible to the T_4-diminishing effects of OMC than the dams.

With regards to behavioural effects, no correlations between developmental T_4 levels and behavioural outcomes were seen in the OMC study (described in paper II). This was a quite surprising finding, because of the very severe T_4 reductions observed in the dams. If maternal hypothyroxinemia was the determining factor for brain development in rat offspring, adverse effects on learning and memory, hyperactivity and hearing deficits in the offspring, would have been expected. However, none of these effects were seen and in fact completely opposite

effects on female activity levels and male maze learning behaviour were observed (Table 4). While it cannot be excluded that these atypical behavioural effects were in some way related to the severe maternal pre- and postnatal T_4 reductions, it seems probable that the observed behavioural effects were not mediated by developmental hypothyroidism, as they differed substantially from the effects seen in all other hypothyroidism studies. As discussed in paper II, the behavioural changes may rather be explained by the estrogenic properties of OMC, causing a masculinisation of the female activity behaviour, and a further polarization/ masculinisation of male spatial learning ability.

In the mancozeb study a significant reduction in maternal T_4 levels was observed on GD 15 in all three dose groups (Table 3). Interestingly, offspring levels of T_4 were not significantly affected in any dose group compared to controls, when measured on PND 16. Motor activity levels, maze learning and startle behaviour (Table 4) as well as reproductive development in the offspring, were also unaffected by the treatment. As discussed in paper III it is, however, unclear whether the thyroid hormone system of the offspring was unaffected by mancozeb during the entire dosing period. Like with OMC, it is possible that toxicokinetic factors may have affected maternal transfer of mancozeb and thereby limited exposure to the pups.

Based on the different thyroid effects seen in the 16 day old offspring, differences in lactation transfer of the three chemicals seem very plausible. But differences in placental transfer may also have influenced the differing results. If for example, PTU was transferred readily through the placenta while the two other compounds were not, then the foetal TH synthesis from GD 17.5 and onwards may have been completely blocked in the PTU study, while this may not have been the case in the two latter studies. Therefore TH levels in the PTU exposed offspring may have been too low during the critical periods of brain maturation, to lead to normal neurobehavioural development. In all three studies it would have been interesting to include measurements of offspring T_4 levels either during late gestation or shortly after birth, as this would have helped the interpretation of the results. Based on literature reviews we expected all three compounds to pass readily from dosed dams to the offspring, and since including an early postnatal section would have meant that fewer animals could be kept for the postnatal investigations, sections around the time of birth were unfortunately not included in the study design.

The time point for measuring T_4 levels in pregnant dams, GD 15, was chosen because at this time the dams had been dosed for 8 days, which we expected to be enough to cause a significant effect on T_4 levels, but still almost two weeks before the day of birth, to reduce the risk of miscarriage. Furthermore, at GD 15 the offspring were not producing TH themselves, and were therefore totally dependent on maternal supply. Therefore maternal T_4 levels at this point could have been determining for the behavioural outcome in the offspring.

6.2 Can T_4 levels be used as predictors for developmental neurotoxicity in the rat?

The second aim of the study was to assess whether reduced TH levels were good predictors for developmental neurotoxicity. Even though significant correlations between developmental T_4 levels and behavioral effects were seen in the PTU study, no correlations were found in the two studies of environmentally relevant xenobiotics. Therefore the hypothesis that reduced T_4 levels in pregnant dams can be used as predictors for DNT is probably not suitable for all compounds that adversely affect the thyroid hormone system.

Previously a paper has shown a clear and significant correlation between postnatal pup T_4 reductions, induced by PCB exposure, and hearing deficits (Crofton, 2004). This means that for some environmentally relevant xenobiotics it is possible to make such a connection in the postnatal period. However, based on the results presented in this thesis, maternal T_4 reductions in rat dams during gestation are not accurate predictors for altered behaviour or hearing deficits the offspring, and a clear relationship between the magnitude of maternal thyroidal dysfunction and developmental neurotoxicity could not be established.

It furthermore seems that some TDCs may affect behaviour differently than expected based on their thyroid effect, as seen with OMC. Therefore, the need for developmental neurotoxicity studies still exists, and risk assessment of TDCs cannot be based solely on TH measurement in adult animals, when evaluating the risk for developmental neurotoxicity posed by TDCs.

6.3 Is maternal hypothyroxinemia determining for altered behaviour in rat offspring?

In order to assess whether maternal hypothyroxinemia in rats is determining for altered behavioural development, it is necessary to take into account both the results presented in this thesis and all other published literature on this subject. In most of the rat studies dealing with effects on the developing brain, hypothyroidism has been induced during both gestation and lactation, and like in paper I offspring have therefore been hypothyroid during the whole period of brain formation and maturation. As mentioned in the background chapter, there are a few reports in the literature where hypothyroidism has been induced during gestation only and behavioural effects in the offspring have been reported. These studies are discussed in more detail below.

In a study by Hendrich *et al.* (1984) pregnant rat dams were rendered moderately hypothyroid by radio-thyroidectomy and subsequent T_4 replacement during the gestation period, in a way as to reduce their serum T_4 levels by approximately 50 % at the end of gestation but keep them at normal levels during lactation. The effects in the offspring's neurological development were quite severe as significant hyperactivity and deficits in learning and memory abilities were present in adulthood. Actual TH levels were not measured, but the thyroidectomy method had been used previously by the authors. Here it was shown that besides reducing dam TH levels, thyroidectomy also resulted in significant changes in TH levels in the neonatal offspring, as T_3 levels were significantly reduced at both PND 5 and 30 (Porterfield and Hendrich, 1981). So even though maternal hypothyroidism was only induced during pregnancy in this study, behavioural effects may also have been affected by reduced postnatal TH levels in the offspring.

Another study investigating the effects of maternal thyroidectomy during gestation on offspring neurodevelopment was conducted by Friedhoff *et al.* (2000). Here dams were surgically thyroidectomised before conception, no T_4 replacement was used, and after birth the offspring were cross-fostered and nursed by untreated dams. In this study THs were measured shortly after birth in the dams, while offspring TH levels were only measured at 120 days of age. Because of the thyroidectomy T_4 levels in the ThX dams after birth were undetectable, while no effects on offspring TH levels at PND 120 were seen. The adult offspring showed significant deficits in maze learning and higher activity levels. The authors concluded that their results showed that foetal deprivation of THs, prior to foetal thyroid hormone synthesis, results in neurobehavioral dysfunction. But since the offspring may have been hypothyroid neonatally as well, as seen in the other study investigating TH levels in offspring from ThX dams (Porterfield and Hendrich, 1981) this conclusion may need to be somewhat modified.

In a more recent study by Liu *et al.*, (2010) two groups of dams were also thyroidectomised prior to mating. One group received T_4 infusion at doses which resulted in normal T_4 levels but elevated TSH levels during pregnancy and lactation, while the other did not receive hormone replacement. In the offspring on PND 40 there were no significant differences in TSH and total T_4 levels between the control animals and the replacement group, however the animals from the replacement group showed impaired spatial learning abilities. The offspring from the ThX group without replacement treatment had higher TSH and lower T_4 levels than was seen in both of the other groups on PND 40, and performed even worse in the spatial learning test. The authors concluded that prenatal hypothyroxinemia caused impaired maze learning. However the replacement group mothers did not have low T_4 levels at any time during the study. Therefore hypothyroxinemia during gestation could not explain the decreased learning abilities seen in the offspring from the replacement group. And as seen in previous studies, the offspring from the ThX mothers without replacement were hypothyroid on PND40. This indicates that they were probably hypothyroid during the whole postnatal period, which may explain why their learning abilities were affected. So this study cannot be used to show that maternal hypothyroxinemia causes behavioural effects in the offspring.

Opazo *et al.* (2008) investigated how MMI in the drinking water of pregnant rats from GD12-15, affected molecular parameters in brain cell differentiation and spatial learning behaviour in the adult male offspring. The treatment caused T_4 levels to decrease significantly in dams when measured on GD15 but unfortunately no other TH measurements were performed in this study, in either dams or offspring. The authors found many effects in molecular and functional parameters in the neurons from the telencephalon, and reported significantly increased escape latencies in a water maze test. Based on this, they concluded that maternal hypothyroxinemia can significantly reduce the offspring's brain capacity for spatial learning. However they only used six dams in the control and treatment groups, and even though they tested 16-17 animals from each group in the water maze, litter effects were not included in the statistical analysis. And since the effects on spatial learning were not very marked, this methodological shortcoming could have affected the significance of their findings.

To summarize, there are no studies in the published literature to clearly show that prenatal T_4 reductions alone are enough to affect behavioural endpoints in rat offspring. This is in contrast to the ample number of human studies showing that maternal hypothyroxinemia causes adverse effects on cognitive development in children (Pop *et al.,* 1999; Haddow *et al.,* 1999; Kooistra *et al.,* 2006; Li *et al.,* 2010), as well as the numerous animal studies showing that prenatal hypothyroxinemia may significantly affect nerve cell migration and other molecular aspects of brain development (Sharlin *et al.,* 2008; Berbel *et al.,* 2010; Auso *et al.,* 2004; Opazo *et al.,* 2008; Gilbert and Sui, 2008).

Our studies indicate that maternal hypothyroxinemia is not necessarily correlated to adverse effects in learning, hearing and motor skills in the offspring, and that prenatal T_4 decreases in dams alone are therefore probably not determining for adverse behavioural development. However, presently very little is known about the consequences of TH deficiency at specific time points during development in rats, and since brain development happens during discrete time windows, the right hormone levels at these exact time points may be very determining for the behavioural outcome. In rats some of these windows probably occur in the postnatal period, and it is therefore plausible that T_4 levels in the offspring also need to be severely reduced postnatally for behavioural effects to occur. This hypothesis is based on the results from the three performed studies, and on the fact that postnatal TH insufficiency has been present in almost all studies showing behavioural effects after developmental hypothyroidism (Brosvic *et al.,* 2002; Noda *et al.,* 2005, Akaike *et al.,* 1991; Provost *et al.,* 1999).

Furthermore, a recent animal study investigating the behavioural effects of developmental exposure to perchlorate seems to corroborate this hypothesis. Here dams were dosed with perchlorate from GD 6-PND 30, which caused maternal T_4 levels to decrease markedly when measured on PND 30. Unfortunately, maternal T_4 levels were not measured at any other time point during the dosing period but based on the large postnatal T_4 reductions it seems probable that hypothyroxinemia was also present during the prenatal period. The offspring T_4 levels were unaffected on PND 4 and 14, which was probably due to limited offspring exposure. This point was however not discussed in the paper. The effects in the offspring included reduced synaptic transmissions in the hippocampus but no effects on spatial learning or motor activity. This surprised the authors, but based on the results presented in this thesis, behavioural abnormalities in the perchlorate treated offspring would not be expected.

It is furthermore possible that some compensatory mechanisms are taking place to protect the rodent offspring's brain development from maternal hypothyroxinemia. By providing radioactively labelled T_4 to pregnant rat dams from GD 11-21, Morreale de Escobar *et al.* (1990) showed that approximately 18% of a healthy foetus' T_4 at birth is from the mother. If however the foetus's own TH production was inhibited, the proportion of radioactive substance in the embryo was much higher. This indicates that in order to keep the damage to the foetal development as low as possible, more T_4 was transferred from the mother (Morreale de Escobar *et al.,* 1990). It is therefore possible that other compensatory mechanisms in rodents could protect the offspring from maternal hypothyroxinemia.

6.4 A regulatory point of view

In the present OECD guidelines for testing of chemicals, measurements of TH levels are optional in the repeated dose 28-day oral toxicity study in rodents (TG 407) (OECD 2008). They are, however, mandatory in the draft of the new reproductive toxicity test guideline, the extended one-generation reproductive toxicity study (OECD 2010). This study also includes a cohort of offspring which can be used for developmental neurotoxicity testing. When the guideline becomes implemented in testing of new and existing chemicals, these data will hopefully provide more insight into the relationship between postnatal TH levels and adverse behavioural effects.

Further testing of new and existing environmental chemicals for their effects on the TH system is very important, because at present almost all data on hypothyroidism come from animal studies where TH levels have been severely depleted. While these studies provide knowledge about the role of THs in nervous system development, they do not prove very useful in estimating risks from environmental TDCs. Studies of the relationships between low and moderate TH hormone perturbations and adverse outcomes are therefore crucial for correctly modelling outcomes in humans, where xenobiotics are likely to only modestly perturb TH homeostasis (Crofton, 2008). Consequently more studies, investigating the relationship between developmental exposure to environmental xenobiotics, offspring TH levels and developmental neurotoxicity, are planned in our laboratory. If, based on these and other investigations, some sort of relationship between timing of TH insufficiency and adverse behavioural effects can be established, this knowledge may be useful for regulatory purposes.

In all three studies performed in the present thesis, behavioral changes were less sensitive than changes in TH levels. Therefore, setting No Adverse Effect Levels (NOAELs) for these three TDCs, based on reductions in maternal TH levels would be protective against behavioral effects in the offspring, if these were caused by developmental hypothyroxinemia. Incorporation of such an approach would, however, not be advisable at present as we still know too little about the relationship between lowered TH levels and developmental neurotoxicity, as well as of the species differences between humans and rodents in relation to timing of TH insufficiency. Furthermore, our results show that behavioral testing of thyroid disruptors with multiple modes of action provides useful complementary information and contributes to a broader understanding of the toxicity of the tested chemicals than studies that only take hormonal measurements and development of reproductive organs into account. It is also important to remember that a number of compounds can cause developmental neurotoxicity without affecting the TH system, and therefore negative conclusions on potential developmental neurotoxicity should not be drawn based on lack of TH effects.

Another issue which should be considered is the method of offspring exposure. Because a large part of brain maturation in rodents happens after birth, the early postnatal period in rats mimics the last trimester in humans. If the entire period of human brain development is to be represented in a rat DNT study, then it is imperative to make sure that the animals are also exposed in the postnatal period. This makes testing of thyroid disrupters for DNT somewhat more complicated, because knowledge of the kinetics of a given compound is necessary in order to know whether offspring will be exposed to the chemical through lactation transfer, or if the offspring should be dosed directly in the postnatal period to ensure exposure during this period of brain development and maturation.

When dealing with regulation of chemicals, there is also the question of mixtures. This issue has not been addressed in the present Ph.D. project and based on the behavioural effects seen in the three performed studies we would not be able to perform cumulative risk assessment for the three tested chemicals, as we still know too little about their modes of action in regard to DNT. As reviewed in the background section of this thesis, there is however a large number of xenobiotics that disrupt the TH system and some of these have been shown to act in a dose-additive manner (Flippin *et al.,* 2009). The potential for concurrent exposure to many of these compounds therefore makes it imperative to develop an understanding of the potential impact of exposures to mixtures of TDCs. There are a number of challenges when dealing with the question of these mixtures, as presented in a recent review paper by Crofton (2008). A major question for modelling TDC mixtures is the choice of which biomarker to use as these can range from adverse clinical measures to molecular interactions at key target sites. Another challenge in predicting the effects of TDC mixtures is our lack of understanding of the complex array of feedback mechanisms, and compensatory processes that strive to maintain normal thyroid status. For example, increased brain deiodinase activity can compensate for xenobiotically induced decreases in circulating TH concentrations (Morse *et al.,* 1993), while lowered iodine concentration can result in increased expression of the sodium-iodine transporter (Spitzweg *et al.,* 1999; Opitz *et al.,* 2006). According to Crofton (2008), another major uncertainty that also hampers the risk assessment of mixtures of TDCs, as well as the effects of single TDC, is the lack of a clear characterization of the dose–response relationship between the degree of change in hormone concentrations and adverse consequences. So even if accurate predictions of the effects of TDC mixtures on circulating or tissue levels of TH are possible, there is still a concern as to what degree of change leads to adverse outcomes and whether the mechanisms responsible for the changes found in animal models are similar in humans (Crofton, 2008). These questions have also been addressed in the present thesis, and the same conclusions have been reached, as no clear dose-response relationship between the degree of TH reductions and adverse consequences was found, and major differences between rats and humans with regard to timing of TH insufficiency and behaviour were identified.

6.5 Recommendations for future studies

Based on the three performed studies, some methodological issues should be considered when planning future studies of thyroid disrupters. Generally, the rat seems to be a suitable model for investigating effects of TDCs on TH levels and neurobehavioural development. One advantage is that much data already exists on T_4 reductions after exposure to many different TDCs. Furthermore, a large number of studies exist, showing behavioural effects after developmental hypothyroidism. This shows that this mechanism is also present in the rat. There are, however, some issues one should keep in mind when using the rat as a model for this type of testing. In humans, moderate and transient maternal hypothyroxinemia during pregnancy seems to be determining for adverse neurological development. Our studies, on the other hand, indicate that in rats even severe maternal hypothyroxinemia during the entire period of gestation did not cause the behavioural effects usually seen after developmental hypothyroidism. This species difference between rats and humans has not previously been reported, but should be kept in mind when designing new DNT studies. Before testing a new chemical, it is therefore crucial to try to gather all available information about the compound's toxicokinetic properties. If the animal model is to mimic human exposure during all three trimesters of pregnancy, it is important to ensure that the rat offspring are exposed during their entire period of brain development, i.e. postnatally as well. For xenobiotics that are transferred readily through maternal milk, dosing of dams in the postnatal period should be sufficient to ensure this. If, however, the chemical is not transferred through the milk, then direct postnatal exposure of the pups should be considered.

The behavioural tests used to investigate DNT and the method used to determine auditory thresholds generally seemed suitable, as activity, learning and hearing were significantly affected in the reference study with PTU. Therefore, the fact that no behavioural or auditory endpoints were affected in the mancozeb study, and that the behavioural effects seen in the OMC study were different than expected, was probably not caused by methodological shortcomings of the test methods. The sensitivity of the used tests could, however, have contributed to the negative results, and it is plausible that other behavioural tests may have yielded other results. Furthermore, including additional developmental neurotoxicity endpoints e.g. measurements of neurotransmitters or weight and histopathology of specific brain regions, could have contributed with additional information about the investigated xenobiotics. This was, however, not practically possible in the three performed studies.
The tested group sizes were generally relatively large, and using about 17 litters per dose group should be sufficient to identify significant effects of exposure, if these were present. Behavioural studies performed with markedly smaller group sizes may not have detected significant group differences.

The hormonal measurements in the present studies only included assessment of T_4 levels. Additional information about the modes of action of the investigated compounds would have been obtained by including measurements of T_3 and TSH. However, from many of the collected blood samples there was only enough blood to include measurements of one hormone, and since T_4 levels are the most sensitive to thyroid perturbations, it was correct to prioritize this measurement. It is therefore also recommended that measurements of T_4 levels should be prioritised over T_3 or TSH measurements in future studies.

As previously mentioned, a further recommendation for future studies of this kind would be to include more measurements of offspring TH levels just before the time of birth and in the early postnatal period. This would help the interpretation of the results and would probably also increase the chances of finding significant correlations between TH levels and behaviour.

7. Conclusions

It is a well established fact that in humans, maternal hypothyroxinemia during pregnancy can be determining for altered behavioural development of the child (Pop *et al.,* 1999; Kooistra *et al.*, 2006; Li *et al.,* 2010. Furthermore, many animal studies have shown that maternal hypothyroxinemia leads to structural changes in the rat brain (Gilbert and Sui, 2006; Berbel *et al*., 2010; Lavado-Autric *et al.,* 2003; Sharlin *et al.,* 2008; Cuevas *et al.,* 2005). It was therefore expected that maternal T_4 levels in rats during early gestation, i.e. before the foetuses start producing their own THs, would also be determining for the neurological development in the rat offspring. In order to assess whether the severity of maternal hypothyroxinemia could be used to predict the degree of adverse behavioural effects in the offspring, three animal studies where rat dams were exposed to different TDCs during gestation and lactation, were performed. The obtained knowledge was meant to enable regulatory authorities to easier identify the risks of developmental neurotoxicity after exposure to TDCs.

Quite unexpectedly, reduced learning ability, hyperactivity and hearing loss were only observed in the study where dams were exposed to the known thyrotoxic drug PTU, even though exposure to all three compounds lead to decreased maternal T_4 levels during gestation. Furthermore PTU was the only compound to markedly affect the thyroid weights, histology and T_4 levels in the offspring. Mancozeb exposure caused moderate maternal T_4 reductions, but no behavioural effects in the offspring and in the OMC study maternal T_4 levels were severely decreased during the entire dosing period, but the behavioural effects in the offspring were opposite those normally seen after developmental hypothyroidism. From these results it was concluded that if a TDC causes both maternal and offspring T_4 reductions, then the expected behavioural changes will probably occur. If, however, maternal T_4 levels are correlated to behavioural changes in the offspring, a key step - i.e. the offspring's TH levels during the critical periods of brain maturation- is probably missing.

The fact that prenatal T_4 decreases in rat dams alone were not determining for adverse behavioural development can possibly be explained by the fact that a large part of the brain maturation occurs postnatally in rats (Howdeshell, 2002). At present, however, little is known about the specific time points, or discrete time windows, during rat brain development when correct TH levels are needed the most. The right hormone levels at these exact time points are probably very determining for the behavioural outcome, and since some of these windows occur postnatally in the rat, it is plausible that T_4 levels in the offspring also need to be severely reduced postnatally for behavioural effects to occur. This hypothesis is based on the results from the three performed studies, and on the fact that postnatal TH insufficiency has

been present in almost all studies showing behavioural effects after developmental hypothyroidism.

Our results furthermore showed that severity of T_4 reduction differed substantially depending on which compound was investigated and whether dam or offspring T_4 levels were studied. This was probably due to the fact that PTU, OMC and mancozeb affect the thyroid system by somewhat different mechanisms and because they most likely differed in their ability to cross over the placenta and into the breast milk. A better understanding of the modes of action by which different TDCs exert their effect on the TH system, and more knowledge on kinetics of different TDCs, which could help determine if the chemicals are being transferred through placenta and milk, will improve the understanding of the consequences of human exposure to thyroid disrupters and facilitate regulation of these chemicals.

Finally, it is important to keep in mind that the thyroid system has a very complex array of feedback mechanisms and compensatory processes that strive to maintain normal thyroid status. Therefore the mechanism by which different TDCs exert their action on the TH system may be determining for the outcome, and for the ability to link chemical exposure to TH reductions and eventually brain damage. It is for example plausible that if exposure to an environmental xenobiotic causes an up-regulation of hepatic metabolism, then the TH levels will be reduced in the beginning of the dosing period, but the thyroid will compensate and after some time produce more TH, in order to re-establish normal hormonal status. Therefore it is also possible that even if many more DNT studies of thyroid disrupting chemicals are performed, clear correlations between postnatal T_4 levels and behavioural outcomes may still be difficult to establish.

In conclusion, too little is presently known about many aspects of the thyroid hormone system in rats, and about the toxicokinetic properties of different TDCs, to use developmental T_4 measurements as predictors for adverse behavioural effects. Only when more knowledge on the transfer of the xenobiotic chemicals through the placenta and the milk, as well as more information about the effects of pre- and postnatal T_4 reduction on offspring brain development is present, the need for developmental neurotoxicity studies of thyroid disrupting chemicals may be reduced.

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Appendix - Papers I, II and III

Paper I

Axelstad, M., Hansen, PR., Boberg, J., Bonnichsen, M., Nellemann, C., Lund, S.P., Hougaard, K.S., Hass, U. (2008). Developmental neurotoxicity of Propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes. *Toxicol Appl Pharmacol* **232**, 1-13.

Paper II.

Axelstad, M., Boberg, J., Hougaard, K.S., Christiansen, S., Jacobsen, P.R., Mandrup, K.R., Nellemann, C., Lund, S.P., Hass, U. (2011). Effects of pre- and postnatal exposure to the UV-filter Octyl Methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring. *Toxicol Appl Pharmacol* **250**, 278-90.

Paper III

Axelstad, M., Boberg, J., Nellemann, C., Kiersgaard, M., Jacobsen, P.R., Christiansen, S., Hougaard K.S., Hass, U. (2011). Exposure to the widely used fungicide Mancozeb causes thyroid hormone disruption in rat dams but no developmental neurotoxicity in the offspring. *Toxicol Sci* **120,** 439–446.

Paper I

Axelstad, M., Hansen, PR., Boberg, J., Bonnichsen, M., Nellemann, C., Lund, S.P., Hougaard, K.S., Hass, U. (2008). **Developmental neurotoxicity of Propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes.** *Toxicol Appl Pharmacol* **232**, 1-13.

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Developmental neurotoxicity of Propylthiouracil (PTU) in rats: Relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes

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ABSTRACT

Markedly lowered thyroid hormone levels during development may influence a child's behaviour, intellect, and auditory function. Recent studies, indicating that even small changes in the mother's thyroid hormone status early in pregnancy may cause adverse effects on her child, have lead to increased concern for thyroid hormone disrupting chemicals in the environment.

The overall aim of the study was therefore to provide a detailed knowledge on the relationship between thyroid hormone levels during development and long-lasting effects on behaviour and hearing. Groups of 16–17 pregnant rats (HanTac:WH) were dosed with PTU (0, 0.8, 1.6 or 2.4 mg/kg/day) from gestation day (GD) 7 to postnatal day (PND) 17, and the physiological and behavioural development of rat offspring was assessed. Both dams and pups in the higher dose groups had markedly decreased thyroxine (T_4) levels during the dosing period, and the weight and histology of the thyroid glands were severely affected. PTU exposure caused motor activity levels to decrease on PND 14, and to increase on PND 23 and in adulthood. In the adult offspring, learning and memory was impaired in the two highest dose groups when tested in the radial arm maze, and auditory function was impaired in the highest dose group. Generally, the results showed that PTU-induced hypothyroxinemia influenced the developing rat brain, and that all effects on behaviour and loss of hearing in the adult offspring were significantly correlated to reductions in T_4 during development.

This supports the hypothesis that decreased T_4 may be a relevant predictor for long-lasting developmental neurotoxicity.

Introduction

Normal thyroid hormone status is essential for a child's neurological development, and even small disruptions in the mother's thyroid status early in gestation, may cause intellectual and behavioural abnormalities in her children. Early in gestation, thyroid hormones are necessary for correct neural development, but during the first few months of pregnancy the human foetus does not produce thyroid hormones itself and therefore depends totally on the supply of thyroid hormones from the mother (Morreale de Escobar et al., 2000). Recent studies, using cohorts of healthy pregnant women not suffering from hypothyroidism and without dietary iodine deficiency, have shown that both low maternal thyroxine (T_4) levels and high levels of thyroid-stimulating hormone (TSH) early in pregnancy correlate significantly with impaired psychomotor development of the children (Pop et al., 1999; Haddow et al., 1999). These findings have contributed to increased concern as to the presence of thyroid hormone disrupting chemicals (TDC) in the environment.

The relationship between adverse neurobehavioral development and low thyroid hormone levels during development has been studied in laboratory animals for many years. In most cases Propylthiouracil (PTU) has been used as an anti-thyroid agent, because of its specific and well-characterized mode of action. PTU is an anti-thyroid drug which inhibits both the synthesis of thyroid hormones in the thyroid gland, and the conversion of thyroxine (T_4) to its active form, triiodothyronine (T_3), in peripheral tissues. For a detailed description of the many modes of action of PTU and the observed neurological abnormalities resulting from PTU exposure, see the review by Zoeller and Crofton (2005). In early rat studies developmental hypothyroidism was often induced by dosing pregnant dams with high doses of PTU in the drinking water. The observed effects included delayed development and reduced growth in the young offspring and persistent neurobehavioral effects, e.g. impaired learning and memory abilities and increased spontaneous activity (Davenport et al., 1976a,b, Davenport and Dorcey 1972; Shalock et al., 1979). In more recent PTU

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studies, in which more moderate changes in thyroid hormone homeostasis have been investigated, similar developmental and behavioural effects have been seen (Akaike et al., 1991; Gilbert and Sui 2006; Goldey et al., 1995b; Kobayashi et al., 2005; Noda et al., 2005). Hearing loss has also been demonstrated after both pre- and postnatal PTU treatment (Uziel et al., 1985; Goldey et al., 1995b; Henley and Rybak 1995). Crofton and co-workers have shown that hypothyroidism during the early postnatal period in the rat leads to impaired hearing and permanent damage in the cochlea (Crofton et al., 2000a,b). Furthermore, the hearing loss correlates significantly with decreases in early postnatal T_4 levels, seen after e.g. prenatal exposure to polychlorinated biphenyls (PCBs) (Crofton, 2004; Crofton and Zoeller, 2005).

Like PCB, several other industrial chemicals and pesticides have been shown to affect thyroid function and/or thyroid hormone levels (Brucker-Davis, 1998). Independently, some of these substances have also been shown to cause developmental neurotoxicity (DNT) in experimental animals (Schantz and Widholm, 2001). However, correlations between the degree of hypothyroidism and the severity of behavioural changes have not previously been established. Consequently, a major uncertainty in assessing the risk of developmental exposure to TDCs, is the lack of a clear characterization of the relationship between disruption of thyroid hormones and adverse effects on the brain. Thus, the main aim of the present study was to investigate how developmental thyroid hormone reduction is associated with adverse effects on the developing nervous system.

Furthermore, studies like this may in the future reduce the need for large DNT studies. If a clear and consistent connection can be established between thyroid hormone disruption and adverse neurobehavioral effects, then chemicals known to disrupt the thyroid hormone axis may in the future be evaluated and classified for developmental neurotoxicity based solely on the effect on thyroid hormones. This will decrease the need for costly DNT studies and also reduce the use of experimental animals.

The study was designed based on the newly approved OECD Test Guideline for Developmental Neurotoxicity testing TG426 (OECD, 2007) and the dams were dosed with PTU during both pregnancy and lactation. Since the rat brain and auditory system undergo substantial development postnatally, induction of hypothyroidism during the pre- and postnatal period in the rat corresponds to the thyroid sensitive stages of the nervous and auditory system development during prenatal life in humans (Goldey et al., 1995b). The study included measurements of thyroid hormone levels in dams and offspring during the gestation and lactation period, assessment of postnatal growth and physical development in the offspring, and histology of the thyroid gland. To test the effects of hypothyroxinemia on the development of the brain and the organ of hearing, the animals were tested in a battery of behavioural and physiological tests, including tests of activity, learning and memory, and auditory function.

Materials and methods

Test compound

The test compound was 6-propyl-2-thiouracil, PTU. CAS no. 51-52-5, product number P3755, purity >99.0% (Sigma-Aldrich, Brøndby, Denmark). Corn oil (Bie & Berntsen, Herlev, Denmark) was used as vehicle.

Animals and treatment

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the Inhouse Animal Welfare Committee. Eighty eight (88) time-mated, nulliparous, young adult Wistar rats (HanTac: WH, Taconic Europe, Ejby, Denmark) were supplied at day 3 of pregnancy. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions: semitransparent plastic cages (15×27×43 cm) with Aspen bedding (Tapvei, Gentofte, Denmark) situated in an animal room with controlled environmental conditions (12h reverse light–dark cycles with light starting at 9 p.m., light intensity 500lx, temperature 22±1 °C, humidity 55±5%, ventilation 10 air changes per hour). Food (Altromin Standard Diet 1314) and acidified tap water were provided ad libitum.

The day after arrival, i.e. gestation day (GD) 4, the animals were weighed and assigned to four groups of 22 animals, with similar weight distributions. They were given 4 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. The study was run in three blocks, with 2 weeks in between each block and an equal representation of each dose group in each block. Dams in the four experimental groups were gavaged once a day at approximately the same time, from GD 7 to postnatal day (PND) 17 with 0, 0.8, 1.6, or 2.4 mg/kg PTU. The vehicle control and the PTU solutions were continuously stirred during the dosing period, and prepared anew for each of the three study blocks. The dams were treated at a constant volume of 2ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing. The dams were pair-housed until GD 17 and individually hereafter. They were observed daily for signs of toxicity, and body weights were recorded on GD 4 and during the entire dosing period.

Delivery and postnatal development

After delivery, weights of dams and individual pups were recorded. The pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 22, was designated PND 0 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as the animals gave birth on GD 22–23. Body weight of offspring was recorded on PND 6, 14, 17, 23, 27, after weaning on PND 66 and at the age of 4, 5 and 7 months.

At PND 16, litter size was standardized to 3 males and 3 females, when possible. From these offspring, 1–3 males and 1–2 females from each litter were weaned on PND 27, and kept for later behavioural testing. The weaned offspring was housed in pairs of the same sex and exposure status. After weaning each of the four dose groups consisted of two subgroups of animals. One had 18–20 male and 18–20 female pups from 16–17 different litters per group. These animals were used for motor activity tests, Morris water maze test and assessment of hearing. The other subgroup consisted of 18–20 male rats per dose group (from 17 litters per group), and these animals were tested in the radial arm maze.

Thyroid weights and histopathology PND 16 and 27

On PND 16, 1–9 pups in each litter were sacrificed depending on the original size of the litter. All sacrificed pups were weighed and decapitated, and trunk blood was collected for T_4 analysis (pooled for all males and all females within each litter). Thyroid glands from two males and two females per litter were used for histopathological investigations.

Dams and offspring (one male and one or two females in each litter) that were not kept for behavioural and functional studies, were sacrificed on PND 27. All animals were weighed and decapitated after CO_2/O_2 anesthesia, and trunk blood was collected for measurement of thyroxine in serum. The uteri of the dams were excised, and the number of implantation scars was registered. The thyroid glands were dissected, weighed, and used for histopathological investigation. In the offspring, the thyroid gland from one male and one female per litter were excised, weighed, and used for histopathology. The glands were fixed in formalin, embedded in paraffin, and examined by light microscopy after staining with haematoxylin and eosin.

The histological evaluation included scoring of thyroids as being "normal" or having "moderate" or "marked" effects. In PND 16 pups, thyroids were classified as having "marked" effects, when hyperplasia and hypertrophy of epithelial cells were observed along with papillary projections into the follicle lumen. A score for "moderate" changes required one of the following: slightly irregular follicular lumen, increased cellularity, and few papillary structures in the follicle lumen. In PND 27 pups, histological changes were generally milder, and a score for "moderate" changes required the presence of enlarged colloid-filled follicles, an increased cellularity and/or pseudostratified epithelium projecting into the lumen. In dams, thyroid effects at PND 27 were generally more marked than in the PND 27 pups, and a score for "marked" changes required the presence of irregular follicles, pseudostratified epithelium, and papillary projections into the follicle lumen. In adulthood, effects were mild, and a score for "moderate" effects was given when an increased number of large follicles with flattened epithelium were observed.

Thyroid hormone analysis

On GD 15 dams were anesthesized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam) and blood was drawn from the tail vein. From PND 16 pups, PND 27 pups, dams on day 27 and from 7 months old offspring, trunk blood was used for the hormone analysis. The plasma level of the thyroid hormone thyroxine (T_4) was analyzed using a modified Delfia T_4 (cat. no. 1244-030) time-resolved fluoroimmunoassay from Perkin Elmer (Wallac Oy, Turku, Finland). Instead of the T_4 standards and the T_4 antibody supplied in the Delfia kits, T_4 standards in T_4-free rat serum (cat. no.30042 and 30041, respectively), as well as biotinylated T_4 (30039) antibody from Biovian Ltd., Finland were used. The assay was run as outlined in the protocol supplied by Biovian Ltd. using streptavidin microtitration strips 8×12 wells (4009–0010) and T_4 assay buffer (1244-029 or 1244-111) from Perkin Elmer. The measurements were performed by use of a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences, Turku, Finland).

Behavioral testing

The investigations were performed between 9 a.m. and 4 p.m. during the animals' dark cycle, i.e. their active period. The experimenter was kept unaware as to which group an individual rat belonged. Exposed and control animals were tested alternately and so were females and males.

Motor activity and habituation capability. Motor activity in the rat offspring was measured four times in the study, using the same animals in all tests. One to two male and female pups were randomly selected from each litter, and tested for the first time on PND 14, and again on PND 17 and 23. After testing the animals were tail-marked so it was possible to distinguish the tested animals from their littermates. All animals that were tested in pre-puberty were kept for the activity testing in adulthood, and tested again at 16 weeks of age. At testing, the animals were placed individually in clean plastic cages without bedding, and the cages were placed in activity boxes with photocells, which measured horizontal activity for 30 min. The position of the cages in the activity boxes was adjusted in height, depending on the age of the offspring. Neither food nor water was supplied during the measurement period. A computer in an adjoining room automatically recorded the output of the photocells and collected data for each of 10 three-minute intervals. The total activity during the 30 min observation period was used as a measure of general activity. In order to assess habituation, the 30 min were divided into three periods, period 1 (1–9 min), period 2 (10–21 min), and period 3 (22–30 min). As there were no sex differences in motor activity levels in the prepubescent animals, litter means were calculated, and used for calculation of group mean values.

Learning and memory (Morris water maze). All animals that had previously been tested for activity in pre-puberty, were tested at the age of 8 and 9 weeks in a Morris water maze, as described earlier by Hass et al. (1995, 1999) with minor modifications. The pool had a diameter of 220 cm, and a circular transparent platform was situated on a solid support and submerged 1 cm below the water surface, and thus invisible from water level. The animals were tested in four daily trials using four different starting points. When the rats swam to and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60s, it was led to the platform. A video-tracking device (Viewpoint video-tracking system, Sandown Scientific, Middlesex, England) tracked the route of the animals, and the latencies to find the platform, the path lengths, and swimming speeds were used as endpoints. The following scheme was used:

Learning. With the platform situated at the same place, the animals were trained for 5+2 days (they were only tested Monday–Friday), until a stable performance was established.

New platform position (reversal learning). The day after the last learning test, the animals were tested in a reversal procedure with the platform placed opposite the original location. The animals were tested for two consecutive days.

Radial arm maze. At the age of 5–6 months, the second subset of weaned animals (not previously tested males), were tested in a standard 8-arm Radial Arm Maze (RAM) with photoelectric cells. The maze was elevated 90 cm above the floor, and built of transparent plexiglass with 8 arms (55 cm long) radiating from an octagonal central area (50 cm wide). Two photocells were mounted on each maze-arm, one close to the central area, for the registration of the rat's entry to the arm, and one at the end of each arm, for registration of movement at the end of the arm, where the reward was placed. A computer located in an adjoining room automatically recorded the output of the photocells. A video camera was mounted above the maze, so the rat's performance could be viewed on a monitor placed in the adjoining room.

One week before testing, the animals were housed one per cage and given a restricted amount of food (15 g/day). This restriction was expected to lead to a decrease

Table 1
Pregnancy and litter data, including body weight (BW) of dams and offspring exposed to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Dams and litters				
No. of dams (litters)	*n*=22 (17)	*n*=22 (16)	*n*=22 (16)	*n*=22 (17)
Dam BW-gain, GD7–GD21	83.2±4.3	78.9±3.8	83.7±3.9	76.5±3.2
Dam BW-gain, GD7–PND 1	17.4±1.6	20.6±1.8	17.1±1.5	14.8±2.3
Dam BW-gain PND 1–PND 17	34.6±2.6	31.6±4.0	36.9±2.4	44.9±3.3 *
Dam BW-gain PND 17-PND 27	−20.1±2.7	−19.8±2.4	−22.0±3.3	−20.7±3.1
Gestation length	23.0±0.2	22.8±0.1	22.7±0.1	23.0±0.1
% postimplantation loss	10.2±3.0	15.5±6.6	13.9±3.3	8.6±3.5
% perinatal loss	14.8±5.7	18.2±6.6	15.0±3.4	10.4±3.4
Litter size	10.8±0.8	9.6±0.8	11.1±0.7	10.9±0.8
% perinatal deaths	0.8±0.5	3.4±1.4	1.3±0.9	1.8±0.9
% males	48.1±4.7	51.8±4.8	50.4±2.6	48.6±3.9
Offspring				
Mean birth weight	5.9±0.1	6.2±0.1	5.9±0.1	5.9±0.1
Mean BW-gain 1–6	6.7±0.3	7.4±0.3	7.1±6.5	6.5±0.3
Mean BW-gain 6–17	21.0±0.4	22.6±0.8	21.2±0.8	21.0±0.7
Mean BW day 17	33.7±0.8	36.8±1.3	34.1±1.1	33.6±1.1
Mean female BW day 23	53.7±1.1	56.7±1.3	52.9±1.0	50.0±1.2**
Mean male BW day 23	56.5±1.3	59.1±1.2	54.9±1.2	53.4±1.6**
Mean female BW day 27	71.9±1.4	75.2±1.5	70.3±1.2	67.2±1.4**
Mean male BW day 27	78.0±1.6	79.8±1.4	74.3±1.4*	73.5±2.1**
Female BW-gain 17–27	38.7±0.8	39.4±0.7	36.7±0.7	34.0±0.5**
Male BW-gain 17–27	43.8±0.9	43.2±0.7	39.8±0.6**	39.7±1.4**
Female BW-gain 27–66	120.0±2.6	128.8±6.5	116.6±2.6	120.6±2.8
Male BW-gain 27–66	221.6±4.4	221.3±5.7	208.0±3.3	221.6±3.5

Data represent group means based on litter means±SE. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

Table 2
Terminal body weights, thyroid gland weights, and T_4 levels in rat offspring sacrificed at PND 16, PND 27 or in adulthood, and from dams sacrificed PND 27, after exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Pups PND 16				
No. of litters (male/female pups)	17 (18/21)	13 (18/18)	15 (20/19)	15 (20/20)
Body weight (g)	29.80±0.56	31.34±0.55	29.12±0.63	28.87±0.44
Thyroid gland weight	4.38±0.13	6.43±0.22**	7.97±0.23**	9.41±0.38**
T_4 levels (nM)	30.33±0.8	15.22±0.8**	6.89±0.5**	4.79±0.4**
Pups PND 27				
No. of litters (male/female pups)	18 (10/17)	16 (12/15)	17 (13/17)	15 (13/15)
Female body weight (g)	71.5±1.5	73.9±2.1	69.1±1.0	68.2±1.5
Male body weight (g)	78.3±2.3	80.3±1.3	73.6±1.7	69.6±1.9**
Thyroid gland weight	8.08±0.3	10.1±0.4*	11.1±0.3**	11.9±0.3**
T_4 levels (nM)	18.28±1.8	21.11±1.3	19.71±1.1	16.20±1.1
Dams				
No. of dams	17	16	17	17
T_4 levels in dams GD 15 (nM)	28.05±1.6	26.24±1.1	15.38±1.3**	11.38±1.4**
T_4 levels in dams PND 27 (nM)	17.66±2.1	14.56±1.7	18.24±1.6	24.04±2.2
Body weight PND 27 (g)	259.9±3.6	255.6±6.1	257.7±5.3	261.4±3.9
Thyroid gland weight PND 27 (mg)	15.32±0.54	16.18±0.75	18.14±0.57**	20.24±0.7**
Adult female offspring				
No. of females	20	18	20	16
Body weight (g)	251.8±5.3	261.67±6.4	249.5±4.3	249.94±4.7
Thyroid gland weight	17.23±1.05	18.81±1.31	21.24±1.81	21.84±1.98
T_4 level (nM)	36.49±2.6	32.97±2.9	39.20±2.6	27.90±2.0**
Adult male offspring				
No. of males	20	18	20	18
Body weight (g)	429.15±9.4	35.39±11.5	421.42±9.6	442.56±12.1
Thyroid gland weight (mg)	15.25±0.92	18.88±1.44	24.23±2.05**	23.62±1.37**
T_4 level (nM)	25.18±1.1	21.50±1.7	21.52±1.0	23.15±1.3

Data represent group means based on litter means±SE. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

Table 3
Histopathological changes in the thyroid gland of rats exposed perinatally to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17

	Control	0.8 mg PTU	1.6 mg PTU	2.4 mg PTU
Male and female pups PND 16				
Normal	38/39	18/33**	2/36**	0/ 34**
Moderate	1/39	14/33**	6/36	0/ 34
Marked	0/39	1/33	28/36**	34/ 34**
Male and female pups PND 27				
Normal	26/26	21/24	13/26**	4/26**
Moderate	0/26	3/24	13/26**	22/26**
Marked	0/26	0/24	0/26	0/26
Dams PND 27				
Normal	13/17	10/16	2/16**	2/17**
Moderate	4/17	4/16	5/16	0/17
Marked	0/17	2/16	9/16**	15/17**
Adult offspring				
Normal	31/34	21/34**	12/34**	15/33**
Moderate	3/34	13/34**	22/34**	18/33**
Marked	0/34	0/34	0/34	0/33

The cellular changes have been categorized into 3 groups, normal, moderate, and marked. See Materials and methods section for further description of the histological alterations, as the character and severity of the changes varies between the age groups. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

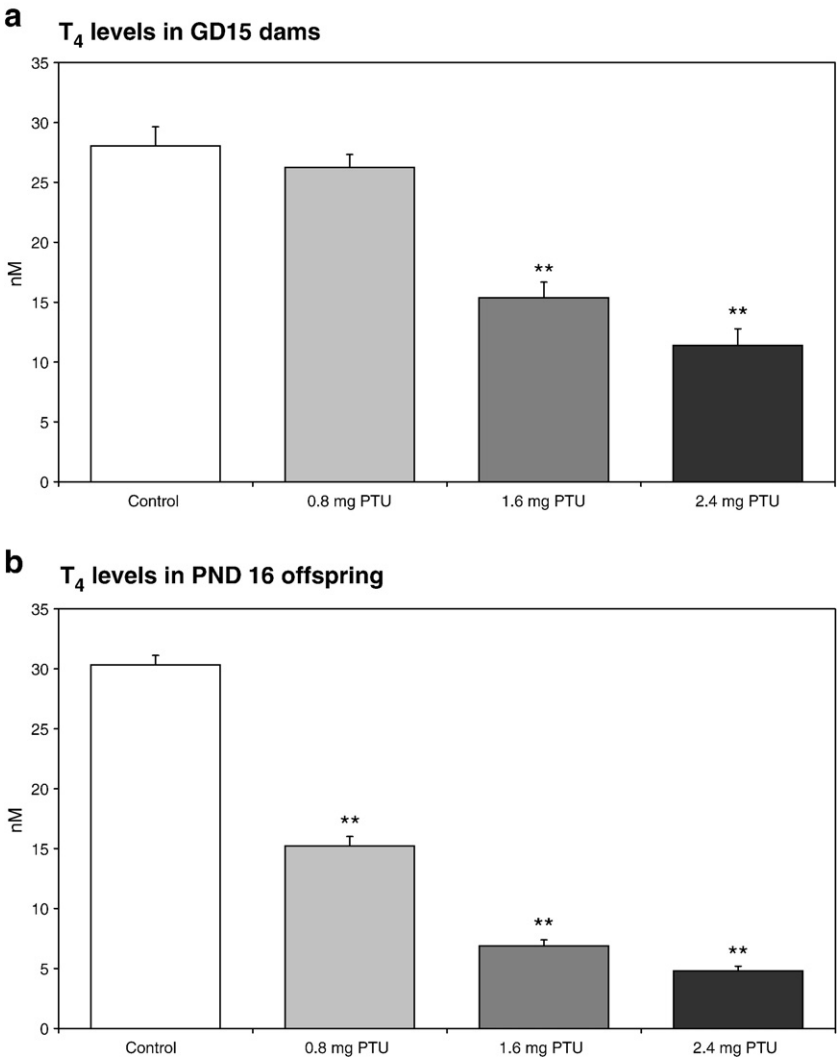

Fig. 1. Thyroxine (T_4) levels (nM) in dams on GD 15 (a) and offspring on PND 16 (b) after exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. Data represent group means, based on litter means + SE, $n = 16–17$. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$.

in body weight of approximately 10–15%, at the end of the testing period (based on previous studies). Once a day, the animals were also given a few small pieces of peanut (Brogaarden, Gentofte, Denmark) that were to be used as reward in the RAM. The food restriction continued throughout the testing period. The animals were tested in one daily session in a total of 15 sessions during 3 consecutive weeks (5 trials per week). All eight arms in the maze were baited with small pieces of peanut. In the daily test session,

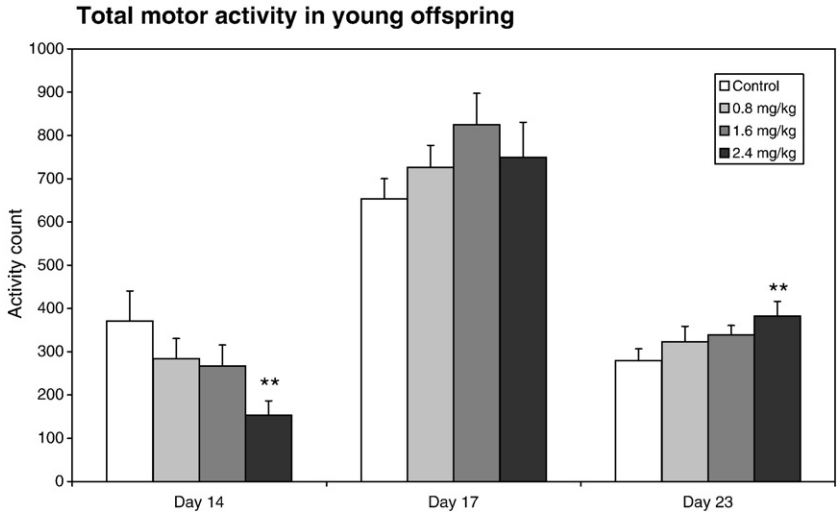

Fig. 2. Total motor activity level during 30 min of testing, on PND 14, 17 and 23 in rat offspring exposed to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. Data represent group means, based on litter means + SE, $n = 16–17$. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$.

each rat was placed in the central maze area in a plexiglass tube. The experimenter left the room, and from the adjoining room the tube was elevated, giving the rats access to the arms of the maze. The rats were allowed to explore the maze until all arms were visited, or 10 min had elapsed. Latency to pass all the distally placed photoelectric cells and the choice of arms was registered by computer. The number of errors, defined as visiting an arm that had already been visited, was calculated from the data.

Test of hearing

Hearing was assessed with the animals in general anesthesia (Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam)), by measuring distortion product oto-acoustic emissions (DPOAE). Using the same stimulus source (probe assembly), hearing thresholds (HT) at 4kHz were also assessed by auditory brain stem response (ABR). Twelve males and twelve females per group, from the subset of animals earlier tested in the Morris Maze, fulfilled the experimental protocol.

Measurement of DPOAE in rats and the equipment used have been described recently (Rasmussen et al., 2005; Hougaard et al., 2007). The measured DPOAE was the amplitude of the cubic distortion product (CDP, i.e. 2f1 − f2), having a fixed ratio of the first and the second primary tones (f1 and f2) of f2/f1 = 16/13 = 1.23, and always at a level of f1 (L1) 10dB higher than the level of f2 (L2 = L1 − 10dB). The primary tones were generated with a two-channel tone generator with phase control (HP 8904), and the output from the probe microphone was fed to an FFT spectrum analyzer (HP 35670A). The distortion product diagrams (DP-grams) across frequencies were obtained by measuring the CDP at fixed levels of primary tones (L1 = 60 and L2 = 50dB SPL), and each spectrum was based on 64 time-averaged recordings. DPOAE input/output curves (I/O curves) were made at f2 = 4096Hz, 8192Hz, 16384Hz, 32384Hz, and 65536Hz by measuring the amplitude of the CDP at varying levels of primary tones in 5dB steps. The number of time-averaged recordings for each point of the I/O curves were based on calculations of the signal to noise ratio (S/N; N = mean of 8 bins, 4 on either side of the CDP), but were not allowed to exceed 512, and the amplitudes obtained on the I/O curves always had S/N better than 3dB. All test procedures and equipment were controlled by a computer and were programmed in the visual programming language Agilent VEE (formerly HP VEE).

Recordings of ABR were modified from Lund et al. (2001). The active electrode was a silver wire inserted subcutaneously at the back of the head, a small roll of silver wire in the mouth served as the reference electrode, and a stainless steel needle in the tail as the ground electrode. The hearing thresholds were tested at 4kHz by pure tone stimuli, generated with a repetition rate of $19.9s^{-1}$ by a programmable function generator (Hameg 8130) as symmetrical tapered 1.4ms tone-pips. The response was amplified 50,000 times, filtered through an analogue band-pass filter (10Hz to 10kHz), and 15ms were sampled at a rate of 51.2kHz by a 16-bit data acquisition board. The ABR of each stimulus level consisted of 256 artifact-free recordings that were averaged and stored on hard disk for later analysis. After further digital filtering (2kHz cut-off frequency and 4kHz stop band) of the stored ABRs, the hearing thresholds were determined manually as the lowest stimulus level, where both the first wave and the first trough of the ABR could be clearly identified.

Thyroid weights and histopathology in adult animals

Seven months old male and female offspring (18–20 of each sex per group) were anesthetized by CO_2/O_2 and decapitated. Trunk blood was collected and analysed for T_4. Thyroid glands from all animals, were excised, weighed, fixed in formalin, embedded in paraffin and examined by light microscopy after staining with haematoxylin and eosin.

Statistical analysis

For all analyses, the alpha level was set at 0.05. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Statistical analyses of the effects on macroscopic lesions and histopathology, were done using Fisher's Exact Test.

Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). Litter size was included as a covariate in the analyses of body weight and body weight gain of the pups. Body weight was included as a covariate in the analyses when relevant, e.g. when testing terminal organ weights. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA (i.e. for data on body and organ weights, activity levels, radial arm maze performance and Morris maze performance). Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means.

For correlation analysis between T_4 levels and behavioural and auditory endpoints, simple linear regression analysis was run using litter means. Additionally for the auditory endpoints curvilinear regressions were included, assuming an exponential fit. For activity levels at PND 14, 17 and 23, and for the auditory endpoints, results from male and female offspring were combined into litter means, when analysing the correlation with T_4 levels. For endpoints where data were analysed separately for males and females (errors in the RAM, and activity levels in adult animals) litter means were also used, when more than one pup per litter per sex had been tested. Asterisks in tables and figures, indicate a statistically significant difference compared to controls *: $p \leq 0.05$; **: $p < 0.01$. All analyses were performed using SAS Enterprise Guide 3 (2004), SAS Institute Inc., Cary, NC, USA.

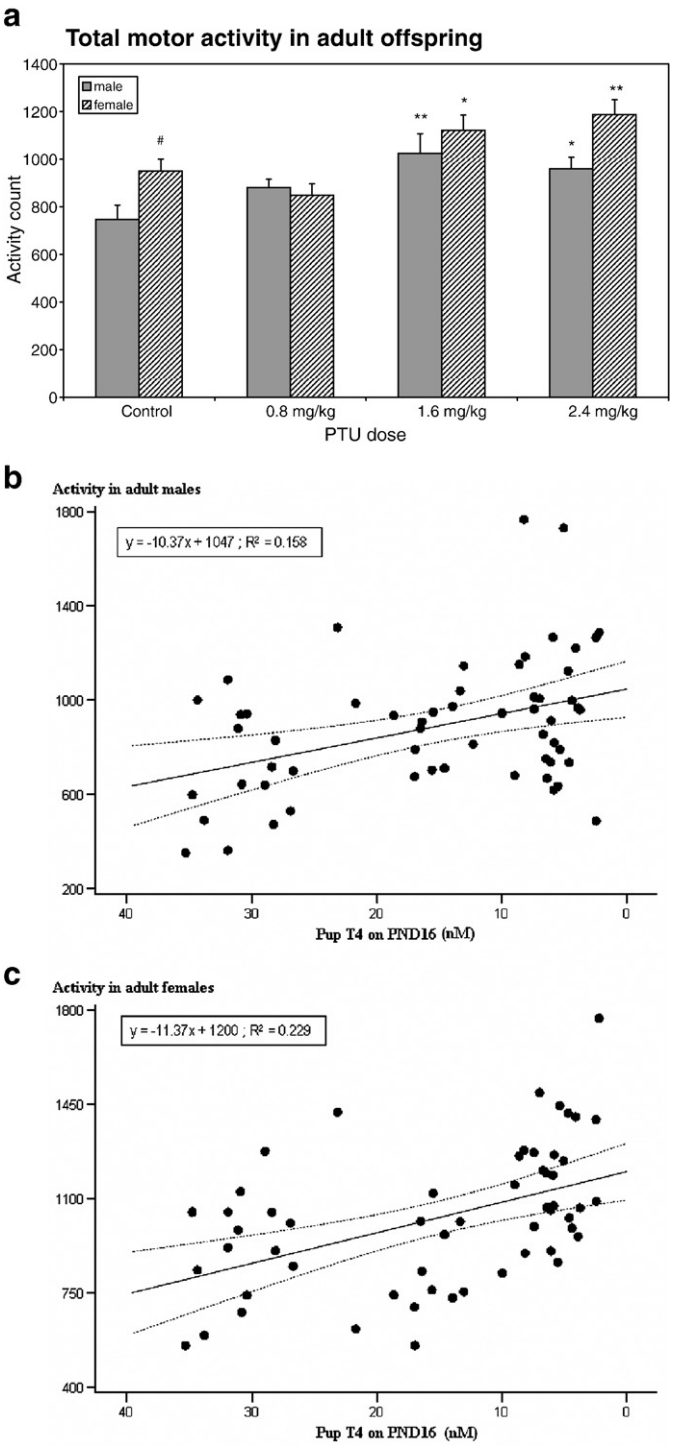

Fig. 3. Total motor activity level during 30 min of testing of male and female offspring at 16 weeks of age, after perinatal exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. a) Data represent group means ± SE, n = 16–17. Asterisks indicate a statistically significant difference compared to same sex controls *: $p<0.05$; **: $p<0.01$. #) Difference between male and female control animals is statistically significant, $p<0.01$. Below, correlation figures between pup T_4 levels (nM) measured on PND 16, and activity levels in the offspring. Each individual point represents a litter, and shows the mean activity level of either males (b) or females (c) from that litter, and mean T_4 level measured on PND 16 in pups from that litter. The solid line represents the correlation, while the dotted lines represent the 95% confidence intervals.

Results

Pregnancy data and postnatal growth

Maternal body weight gain during pregnancy, gestation length, litter size, postimplantation loss, neonatal death, and pup birth weight were similar in the four groups (Table 1). During lactation (PND 1–17) dams receiving 2.4 mg/kg PTU gained more weight than controls. Pup body weights were unaffected by PTU treatment during this period, and no significant differences in body weights between controls and dosed offspring were seen on PND 17 or earlier (Table 1). When measured on PND 23 and PND 27 both female and male pup body

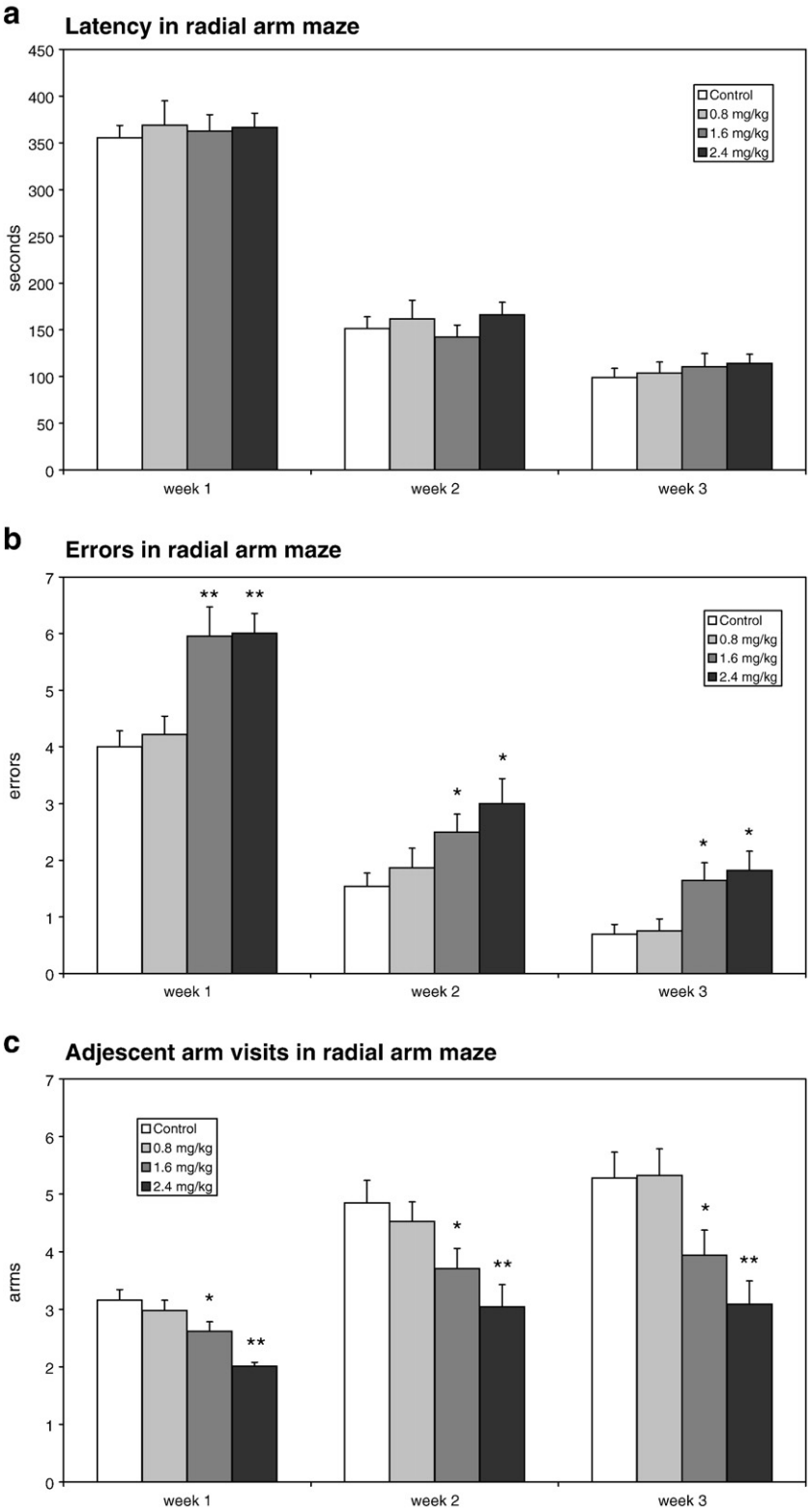

Fig. 4. Radial arm maze results of adult male rats, after perinatal exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. Shown for each of the three test weeks are latencies in seconds to visit all 8 arms (a), mean number of errors made (b), and mean number of adjacent arms visited (c). Data represent group means+SE, $n=17$. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$.

weights were significantly decreased in the 2.4 mg/kg group (PND 23; $p = 0.002$, $p = 0.004$ — PND 27; $p = 0.002$, $p = 0.001$). On PND 27, male body weights were also significantly affected in the 1.6 mg/kg group ($p = 0.019$). Similar results were seen when body weight gains PND 17–27 were calculated (Table 1). The analyses of body weights PND 27 were based on data from all offspring, both those that were kept for weaning, and those sacrificed on PND 27. After weaning body weights were recorded on PND 66, and at this point there were no longer any differences in body weight or weight gains among groups (Table 1). This was also the case during the remaining period of the study (data not shown).

Autopsy, thyroid weight and histopathology PND 16 and PND 27

Body and thyroid gland weights of male and female offspring sacrificed at PND 16 and PND 27 are shown in Table 1 and Table 2. On PND 16, pup body weights of the sacrificed animals did not differ between males and females, and were unaffected by PTU exposure. The weight of the thyroid gland in offspring of both sexes, was significantly higher in all PTU-dosed groups ($p < 0.0001$), and displayed a clear dose effect relationship (Table 2). In PTU-dosed offspring, diffuse hyperplasia and hypertrophy of thyroid follicular cells were observed in all treatment groups, along with a reduced lumen size and papillary enfolding of the epithelium (Table 3).

On PND 27, body weights of the dams were unaffected but weights of the thyroid glands were significantly elevated in the two highest dose groups ($p = 0.0024$ and $p < 0.0001$ respectively). In the offspring sacrificed at PND 27, female body weights were not significantly affected in any of the treatment groups, whereas male body weights in the high dose offspring were significantly reduced ($p = 0.0027$). The thyroid gland weights on PND 27 were clearly elevated compared to controls. This was statistically significant for all three PTU-dosed

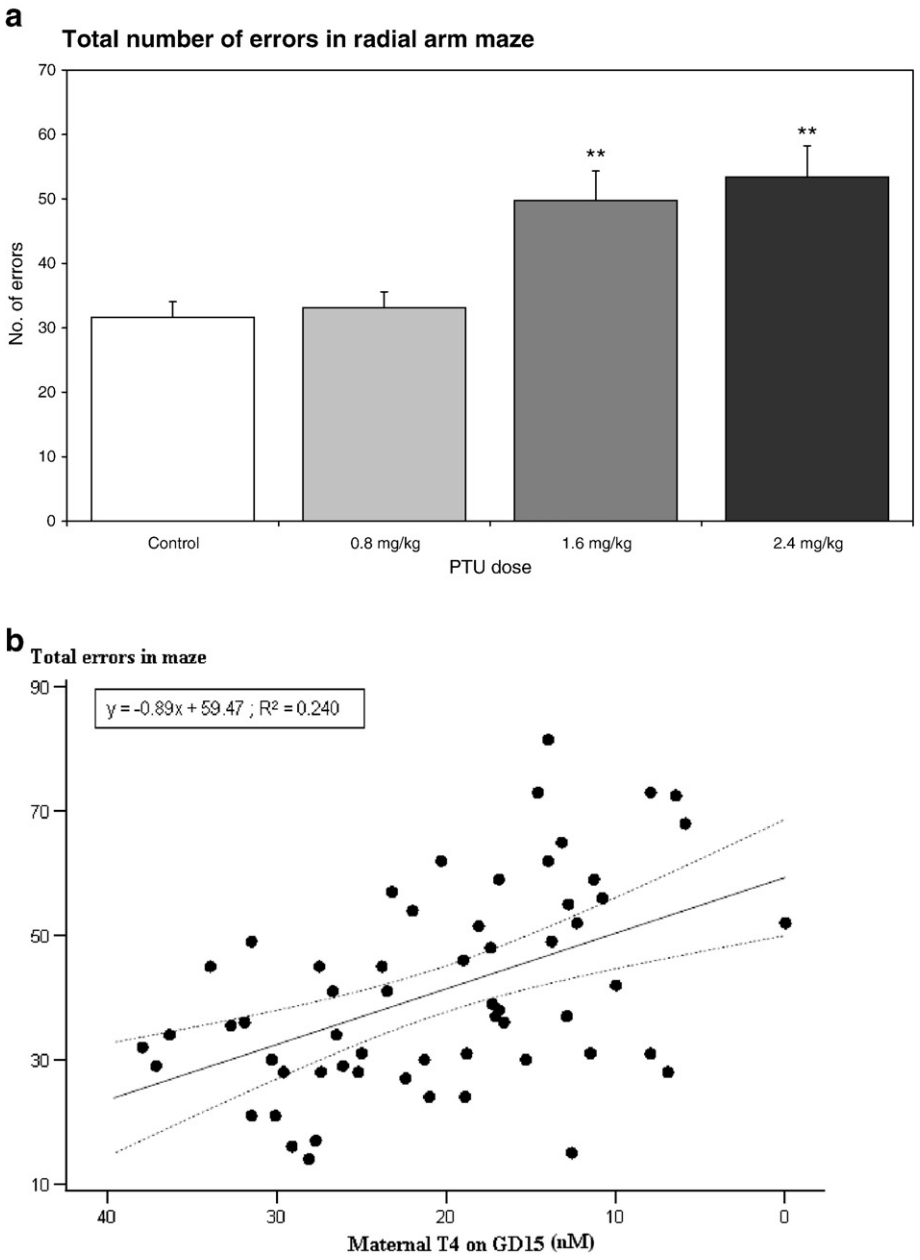

Fig. 5. The total number of errors made in the radial arm maze during 3 weeks of testing, in adult male rats, perinatally exposed to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. a) Data represent group means + SE, $n = 17$. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$. b) The correlation between maternal T_4 levels (nM) measured on GD 15, and the total number of errors in the radial arm maze. Each individual point represents a litter, and shows the mean number of errors made by males in that litter, and the maternal T_4 levels (GD 15) in that litter. The solid line represents the correlation, while the dotted lines represent the 95% confidence intervals.

groups ($p = 0.016$ for the 0.8 mg/kg group, and $p < 0.0001$ for the two highest dosed groups). Histological examination of the thyroid glands from dams and pups on PND 27 showed irregular follicles and increased cellularity in PTU-dosed animals, although the effects were less prominent than on PND 16 (Table 3).

Hormone levels GD 15, PND 16 and PND 27

Thyroxine (T_4) levels were measured in blood from dams on GD 15 and PND 27, and from the offspring on PND 16 and 27. Results are shown in Fig. 1 and Table 2. The T_4 levels decreased with higher doses of PTU in both dams on GD 15 and pups on PND 16, as expected. The difference was significant for the two highest doses in GD 15 dams, and for all three PTU doses in pups on PND 16 ($p < 0.0001$). On PND 27, 10 days after dosing had stopped, the T_4 levels in exposed dams and pups had normalized, and compared to values in control animals (Table 2).

Motor activity levels in pups PND 14, 17 and 23

Total motor activity levels of young pups are shown in Fig. 2. On PND 14, PTU exposure lowered the offspring's activity levels significantly in the highest dose group compared to controls ($p = 0.0002$). On PND 17, the pups in all dosed groups exhibited very high activity levels compared to the other days, but no significant differences between control and PTU-dosed animals were revealed in the statistical analysis. By day 23, the overall activity levels had decreased, and animals in all groups displayed habituation (data not shown). The PTU exposure caused activity levels on PND 23 to rise, and the difference was statistically significant in the 2.4 mg/kg PTU group compared to control ($p = 0.004$).

To investigate the relationship between T_4 levels during development and performance in behavioral tests, linear regression analyses between motor activity levels on PND 14, 17 and 23, and T_4 levels during development was calculated. The analyses were performed on a litter basis, for both maternal (GD 15) and pup T_4 levels (PND 16). Correlation coefficients (R^2) were calculated to estimate the proportion of variation in the dependent variable (activity) that was accounted for by the predictors (T_4). None of the correlations with maternal T_4 proved significant, and even though the correlation analyses between activity counts on PND 14 and 23 and pup T_4 levels PND 16 were significant (data not shown) the correlation coefficient values were only 0.088 and 0.087 for PND 14 and 23, respectively. Thus only 8–9% of

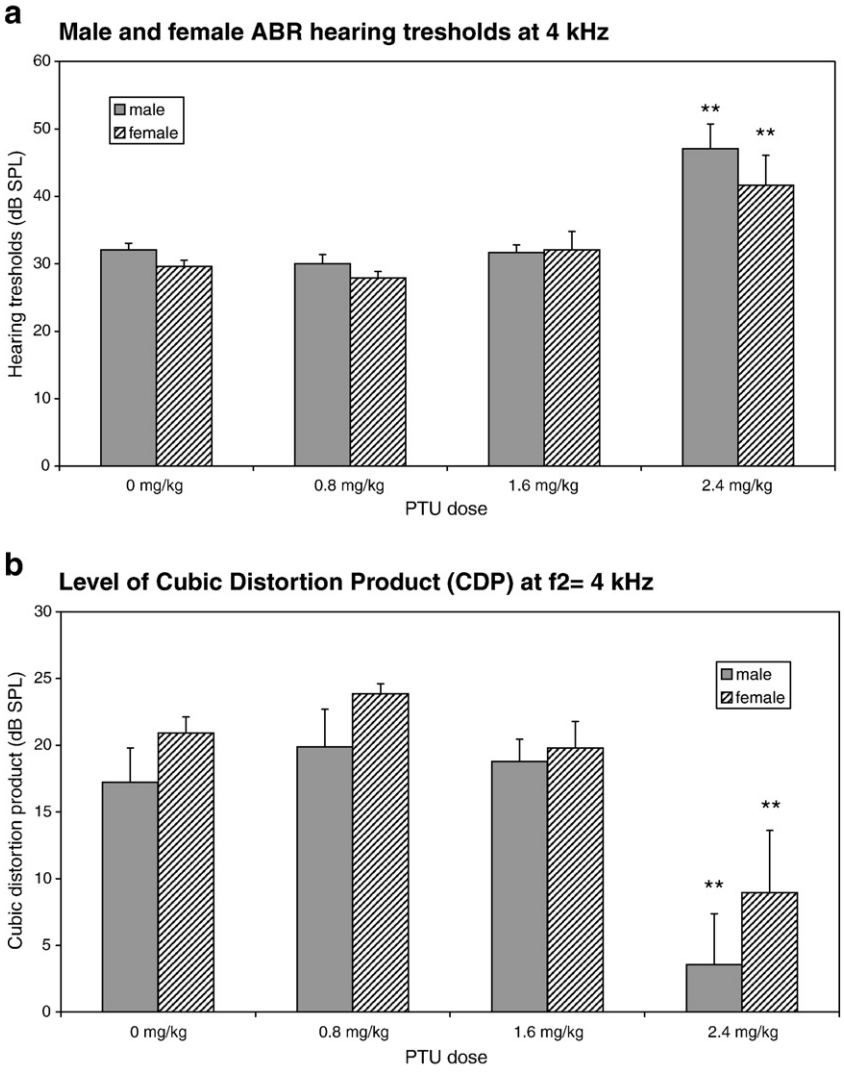

Fig. 6. Hearing in adult male and female rats after perinatal exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. (a) Hearing thresholds, assessed by auditory brain stem response (ABR), and (b) level of cubic distortion product. Data represent group means ± SE, $n = 12$. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$. Below, the straight dotted line shows the linear regression between maternal T_4 levels (nM) measured on GD 15, and hearing threshold (c) or cubic distortion product (d) in adult rats, while the thick line shows the best curvilinear regressions obtained by assuming an exponential fit. Each individual point represents a litter, and shows the mean hearing threshold in that litter, and the maternal T_4 levels (GD 15) in that litter. The solid line represents the regression, while the fine dotted lines represent the 95% confidence intervals.

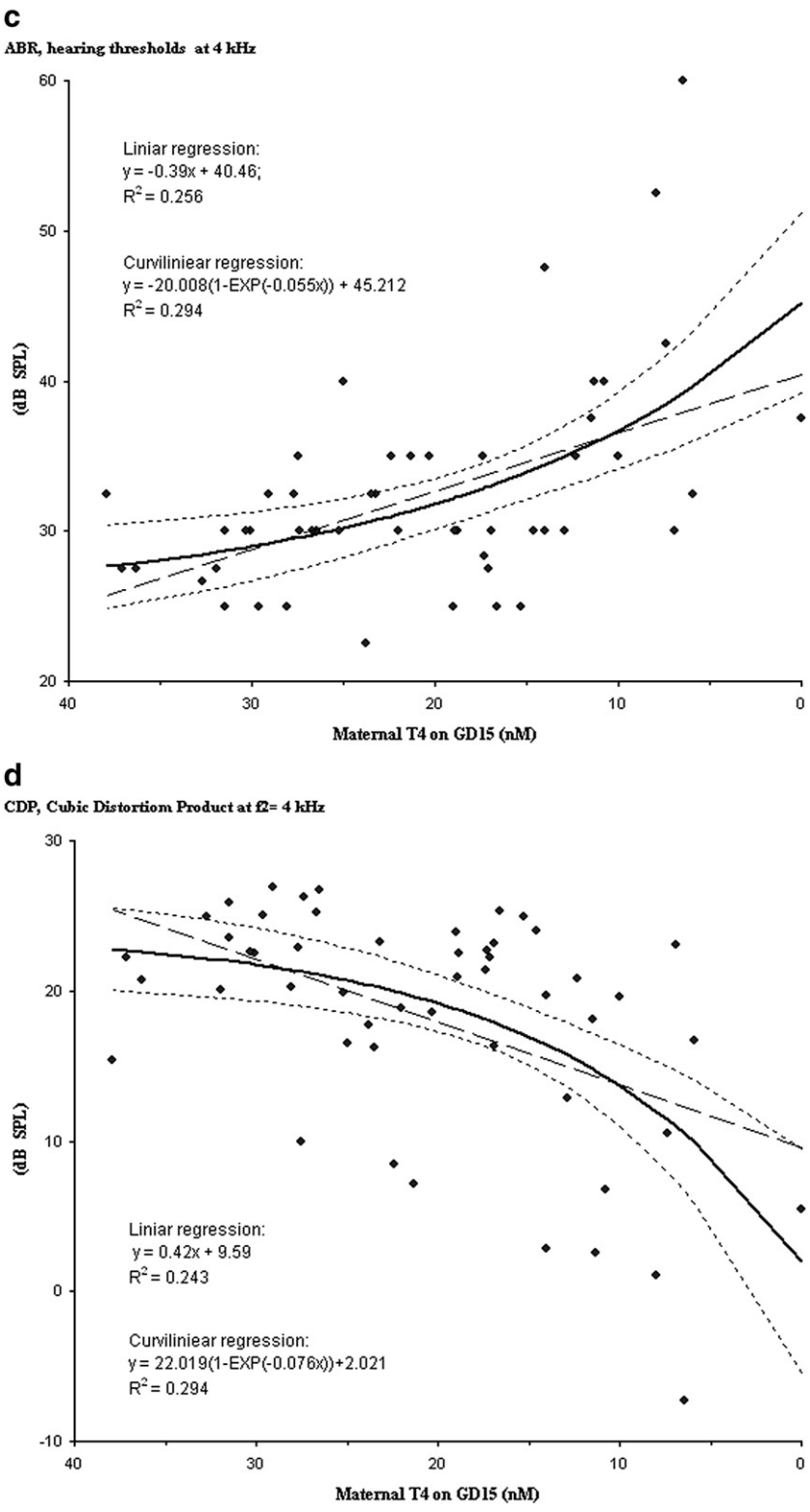

Fig. 6 (*continued*).

the variation in the activity of young offspring could be explained in terms of the T_4 levels assessed on PND 16.

Motor activity levels in adult male and female offspring

Results from the activity test in 16week old male and female offspring are shown in Fig. 3. Fig. 3a shows the total activity levels during 30 min of testing. As expected, female control animals showed significantly higher activity levels than males ($p = 0.0065$). Both male and female animals exposed to PTU were hyperactive compared to controls, significantly so in the 1.6 and 2.4 mg/kg PTU groups, in both males ($p = 0.0019$ and $p = 0.0156$) and females ($p = 0.038$ and $p = 0.003$). When the 30-minute test period was divided into 3 time periods, both male and female animals in all dosed groups displayed

distinctly higher activity levels in the beginning compared to the end of the test (data not shown). In both genders, the difference between control and high dose animals was most pronounced during the first period of the test while there were no differences between PTU-dosed and control animals during the last time period, indicating that the generally higher activity levels in PTU animals were not owing to effects on habituation. Regression analyses showed that adult activity levels correlated significantly with developmental T_4 levels — the lower the T_4, the higher the activity levels (Figs. 3b,c). T_4 levels on PND 16 correlated significantly with activity levels in adult males (p = 0.002) and females (p = 0.0002), and the correlation coefficients (R^2) were 0.158 and 0.229 respectively, indicating that between 16 and 23% of the variation in adult activity could be explained by T_4 levels on PND 16. The correlations between adult activity levels and maternal T_4 levels (GD 15) seamed less strong but were still highly significant (Males: R^2=0.098; p = 0.0143, Females: R^2=0.140; p = 0.0035) (data not shown).

Morris water maze

Both during the initial 7-day learning period, and during the 2 days of reversal learning, no significant exposure related differences were observed in either male or female offspring. Path lengths, latencies, swimming speeds, and percentage of animals finding the platform in the new position did not differ among groups or gender (data not shown).

Radial arm maze

Results from the male offspring that were tested in the RAM for three consecutive weeks are shown in Figs. 4 and 5 (females not tested). The food restriction caused a small reduction in animal body weights. After 1 week, the mean body weights were reduced by 7%, and at the end of the testing period, the reduction was around 12%.

In the maze, a general decrease in latencies and in the mean number of errors was seen for all groups during the testing period (Fig. 4). There were no significant differences in latencies between the PTU exposed animals and the controls (Fig. 4a), however the males exposed to 1.6 and 2.4 mg/kg PTU made markedly more errors than controls during all 3 weeks of testing (1.6 mg/kg; p = 0.0018, p = 0.046, p = 0.048—2.4 mg/kg; p = 0.0005, p = 0.0123 and p = 0.017 for each week, respectively) (Fig. 4b). The PTU exposed males also exhibited a lower frequency of choosing adjacent arms in the maze than control males (Fig. 4c), and the difference was statistically significant in all 3 weeks for both 1.6 mg/kg (p = 0.028, p = 0.025 and p = 0.040) and 2.4 mg/kg PTU males (p < 0.0001, p = 0.0008 and p = 0.0008).

The total number of errors made in the maze during all 3weeks of testing was significantly elevated for both the 1.6 mg/kg (p = 0.0006) and the 2.4 mg/kg males (p < 0.0001) (Fig. 5), and it correlated very well with developmental levels of T_4. The lower the levels of T_4, the more errors were made. Both the correlation with maternal T_4 (GD 15) (Fig. 5b) (p < 0.0001; R^2=0.240) and pup T_4 (p < 0.0001; R^2=0.275) was significant (data not shown). Thus, between 24–28% of the variation in the number of errors could be explained by developmental T_4 levels.

Auditory function

The hearing thresholds for all groups at 4kHz are shown in Fig. 6a. ABR (auditory brain stem response) thresholds were increased by 12–15dB, in both males and females in the 2.4 mg/kg PTU group as compared to controls. Correspondingly, the Cubic Distortion Products (CDP) at f2=4kHz were decreased by 12–13dB (Fig. 6b). This difference between the control and the high dose group was highly statistically significant (p < 0.001 and p < 0.01, for males and females, respectively). The HT as well as the loss of CDP at 4kHz in the highest dose group were slightly higher in the males than in the females, but the difference was not statistically significant. Both the hearing thresholds (R^2=0.256; p = 0.0002) and the CDP (R^2=0.243; p = 0.0002) at this frequency correlated well with the maternal blood levels of T_4 at GD 15 (Figs. 6c, d). The goodness of fit increased by assuming a curvilinear fit between maternal T_4 and both HT (R^2=0.284; p = 0.00005) and CDP (R^2=0.296; p = 0.0004). A significant correlation between both hearing thresholds (R^2=0.139; p = 0.0076) and CDP (R^2=0.114; p = 0.017) was also found for T_4 levels in PND 16 pups (data not shown), however the R^2 coefficients were lower, indicating that maternal T_4 levels on GD 15 could explain a larger part of the variation in hearing ability, than pup T_4 levels on PND 16.

There seems little doubt that the PTU treatment affected the activity of the outer hair cells (OHCs) of the cochlea. Furthermore, the decrease in OHC activity was not localized to specific areas and thereby frequencies of the Basilar membrane, but spread more or less equally along the whole frequency range of the membrane. This is visualized in the DP-gram in Fig. 7, which shows the loss of CDP across the frequency range in the different dosage groups of both sexes combined. The mean decrease in CDP across frequencies in the highest dose group compared to controls was 10dB (p < 0.001). Although the mean difference across the frequencies appeared slightly higher at 4–8kHz than at the

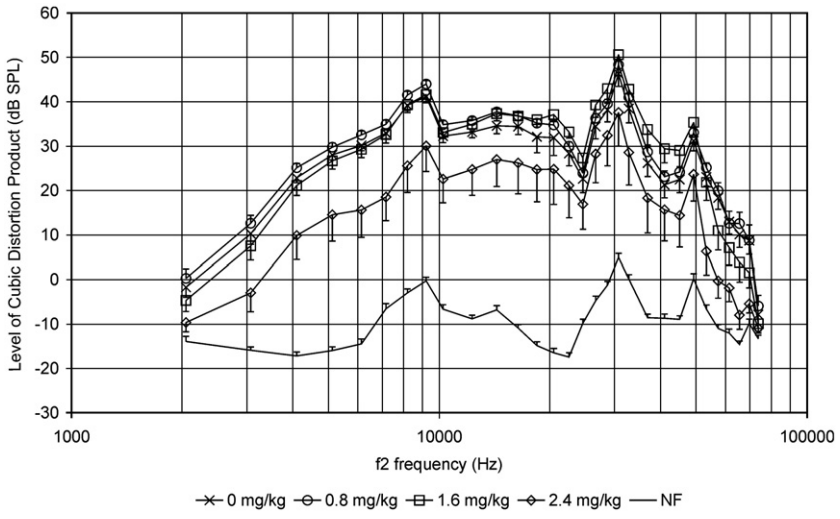

Fig. 7. Level of cubic distortion product at all tested frequencies in adult male and female offspring after perinatal exposure to 0, 0.8, 1.6 or 2.4 mg/kg/day PTU from GD7 to PND 17. NF denotes the noise floor, determined as mean of values in the frequency bins next to the CDP. Data represent group means ± 95% confident interval, n = 12.

frequencies above 20kHz, the differences could be traced at all the frequencies between 3 and 69kHz. The reduction in CDP was observed only at the highest dose level. Actually, both the 0.8 mg/kg and the 1.6 mg/kg PTU groups displayed slightly higher mean CDP across frequencies than did controls, statistically significant in the 0.8 mg/kg PTU group ($p < 0.05$) but not in the 1.6 mg/kg PTU group ($p = 0.44$).

T_4 levels and thyroid weights in adult animals

Body and organ weights and T_4 levels in male and female rat offspring at 7 months of age are shown in Table 2. Both male and female adult body weights were unaffected by PTU exposure.

The weight of the thyroid gland was still elevated in the PTU exposed animals in adulthood, but the difference from controls was only statistically significant in the males in the 1.6 and 2.4 mg/kg PTU groups ($p = 0.0015$ and $p = 0.0039$, respectively). T_4 levels in the PTU exposed adults were significantly lowered in the high dose females ($p = 0.0018$) but not in the males. Histological examination of the thyroid glands in adult males and females showed an increased number of large colloid-filled follicles with flattened epithelium in the high dose PTU group compared to controls (Table 3).

Discussion

Human data indicate that even small reductions in maternal thyroxine levels during critical periods in early pregnancy, can have severe consequences for the neurological development of the child. The objective of our study was to investigate this relationship in an animal model, and thereby contribute to a clearer characterization of the relationship between disruption of thyroid hormones and adverse effects on the brain.

Studies like this, in which both thyroid hormone levels and behavioural effects are measured, are necessary in order to conclude more generally on the risks of exposure to environmental TDCs during critical periods of nervous system differentiation. Furthermore, information from this study, in combination with more data on the subject, may reduce the future need for large toxicological studies of some potentially neurotoxic chemicals, as it may support the possibility for evaluating and classifying some TDCs for developmental neurotoxicity, based solely on their effects on the thyroid hormone system. Indeed, we were successful in demonstrating clear correlations between T_4 levels during development and adverse effects on rat neurobehaviour and hearing. In the adult offspring, diminished learning and memory abilities in the RAM, impaired hearing, and elevated motor activity levels correlated well with developmental reductions in T_4, indicating that the developmental hypothyroxinemia contributed markedly to the observed effects.

One of the aims of the study was to dose pregnant dams with doses of PTU that would induce transient developmental hypothyroxinemia, without causing general maternal toxicity effects. When the T_4 levels were measured in dams after 7 days of dosing (GD 15), T_4 levels in the two highest dose groups were significantly reduced compared to controls, while pups from all three dose groups had significantly lowered T_4 levels at the end of the 30-day dosing period (PND 16). This marked decrease was however only temporary since T_4 levels were not significantly affected in dams and offspring when measured 10 days after dosing had stopped. Unlike T_4 levels, thyroid gland size and histology had not normalized within 10 days of cessation of treatment. Thyroid weights were still significantly elevated on PND 27 in offspring from all dose groups, and the histopathological examination showed marked effects, including hyperplasia, hypertrophy, and the presence of papillary structures. In adulthood, the weight of the thyroid gland was still elevated in PTU animals, although the difference was only statistically significant in the males. T_4 levels in adulthood were significantly lowered in the high dose females, but not in the males. For a male as well as female offspring, the thyroids were thus large compared to their T_4 production, indicating persistent dysfunction and goitre. This slight dysfunction was also evident histologically, as follicles were enlarged and displayed flattened epithelium in PTU exposed animals compared to controls.

These effects were accomplished with no general maternal toxicity or any effects on litter size or pup mortality. In most animal studies of developmental PTU exposure the weight of the dosed offspring has been markedly affected (Akaike et al., 1991; Kobayashi et al., 2005; Gilbert and Sui 2006; Noda et al., 2005). In the highest dose group in our study we did find significant body weight reductions on PND 23 and 27, but the decrease was only around 10%. Furthermore, the PTU-dosed offspring caught up, and the difference in body weight no longer existed after puberty, during behavioural testing, or in adulthood. Based on these results, it would seem that the developmental hypothyroxinemia induced by the applied doses of PTU, was relatively mild compared to other studies. This is noteworthy, as behavioural and auditory effects of PTU were rather marked in the current study.

The activity levels in prepubescent rats were clearly affected by the perinatal PTU exposure, as it caused hypoactivity on PND 14, while it led to hyperactivity on PND 23. Our results were quite similar to those described by others. Goldey et al. (1995b) found that offspring from a PTU dose group (5ppm in drinking water) that caused T_4 levels to fall markedly, but did not affect pup body weight, were hypoactive on PND 15, while their activity levels were elevated on PND 19 and 21 (although not significantly). Kobayashi et al. (2005) observed that perinatal dosing with 2.5 mg/kg PTU reduced pup activity levels slightly on day 16 (although not significant), while activity levels were significantly elevated on PND 22 and in 9week old animals. In our study, activity levels on PND 14 and 23 only correlated weakly with developmental T_4 levels, which indicates that other factors than the developmental hypothyroxinemia contributed to observed effects. Kobayashi et al. (2005) explained the observed hypoactivity on PND 16 in their study with blindness, because of retarded eye-lid opening in the PTU animals. We did not investigate this parameter, but since this effect of hypothyroidism has also been seen in other studies (Comer and Norton 1982; Davenport et al., 1976a,b) delayed eye-opening might also explain our results.

In the adult offspring in our study, we found the expected sex difference, with females in the control group being significantly more active than the males. Both male and female adult rats were similarly affected by the perinatal PTU exposure, which caused activity levels to rise by approximately 25% in the two highest dose groups. In both sexes a significant correlation with developmental T_4 levels was seen.

In the radial arm maze (RAM), the perinatal PTU exposure was associated with impaired spatial learning and memory in a dose related manner. Maternal levels of T_4 at GD 15 correlated well with the number of errors made by the offspring. In the two highest PTU dose groups the number of errors rose by 63% and 75% respectively, compared to controls, and the PTU-dosed animals chose significantly less adjacent arms, indicating a change in strategy. Based on findings in the literature, we had expected PTU to impair spatial learning in the rats in both the RAM and the Morris Water Maze. In other PTU studies, many different types of mazes have been used to show the effects of developmental hypothyroidism, including the radial arm maze (Akaike et al., 1991), the Biel-type water T-maze (Noda et al., 2005), the E-maze swimming test (Kobayashi et al., 2005), and the Morris Water Maze (Gilbert and Sui 2006). In our study, the RAM turned out to be much more sensitive than the Morris Water Maze, as no significant effects were seen in the latter. A possible explanation for this could be that the subset of animals used in the RAM had not previously been tested in any other behavioral test, and had therefore not been handled very much for a long period of time. On the other hand, the rat offspring used for the Morris Maze had been activity tested and weaned only 4 weeks prior to their test in the maze, which might have reduced the sensitivity of this test.

Maternal treatment with PTU had a clear effect on the hearing ability of the offspring in the highest dose groups. Maybe more

importantly, the maternal level of T_4 at GD 15 correlated with the hearing thresholds as well as the reduction of CDP at f2 = 4kHz in the offspring. The reduction in CDP was observed over the whole frequency range as assessed by DPOAE. This finding was similar to observations reported by Goldey et al. (1995b), who emphasized that PTU is unique, due to its dual action, i.e. PTU inhibits both the formation of the thyroid hormones as well as the peripheral deiodination of T_4 to T_3 (based on Braverman and Ingbar, 1963; Geffner et al., 1975; Oppenheimer et al., 1972). In studies of the thyroid-disrupting effects of other chemicals, e.g. PCBs, some observe primarily effects on hearing at the low frequencies in the offspring of perinatally treated rats (Lasky et al., 2002; Crofton et al., 2000a,b; Goldey et al., 1995a; Herr et al., 2001; Crofton and Rice 1999), but effects over the whole frequency range are also observed (Powers et al., 2006). Following perinatal exposure to a commercial PCB mixture, Crofton et al. (2000a) observed low-frequency hearing loss associated with a loss of OHCs in the apical part of the cochlea, but it is quite possible that hearing impairment in offspring from rats due to thyroid-disrupting chemicals is caused by a range of different developmental insults to the cochlea. Administration of PTU to pregnant or lactating rats has been shown to induce several structural changes in the offspring, with different critical periods of maximal vulnerability for each cochlear structure, corresponding to the time of their maturation (Uziel et al., 1983, 1985; Gabrion et al., 1984; Uziel 1986). In the rat, inner hair cells and their afferent and efferent innervation develop both pre- and neonatally, whereas the epithelium filling the inner sulcus (Köllikers organ) and the afferents beneath the OHCs primarily transform postnatally, and the pillar cells and the efferent innervation of the OHCs primarily develop after birth. According to (Uziel et al., 1985), the transformation of Köllikers organ and the formation of the tectorial membrane from the inner sulcus cells appear very sensitive to the action of thyroid hormone over a prolonged period of postnatal development. A lack of contact between the tectorial membrane and the OHC all along the cochlea due to maternal exposure to PTU has also been observed (cf. the conference discussion in Uziel, 1986), which may be crucial in relation to the suggested key role of the tectorial membrane in the cochlear amplifier (Ghaffari et al., 2007; Gummer et al., 1996). Although development of the tectorial membrane or the interaction between the OHC and the tectorial membrane may explain the reduction in OHC activity throughout the whole frequency range, alternative mechanisms cannot be ruled out.

The aim of this paper was to compare the degree of hypothyroidism with the severity of behavioural changes to learn more about the relationship between disruption of thyroid hormones and adverse effects on the brain. In the adult offspring, the higher activity levels, the impaired spatial learning in the radial arm maze, and the impairments of hearing were significantly correlated with developmental reductions in T_4, and between 15 and 25% of the variation could be explained by developmental T_4 levels. Compared to the correlation coefficient of 0.85 between T_4 levels and hearing loss found in the study by Crofton (2004), our correlations seem less strong, but there is a very important difference in the way the correlations have been calculated. Our calculations are based on litter means, while group mean data were used in the analysis by Crofton (2004), which naturally eliminates a very large part of the variation in data. Since we are not comparing results from many different studies, and therefore at any given time only have four different T_4 levels and four corresponding mean values for each behavioural or auditory endpoint, depicting the group means in a correlation analyses with T_4 levels would not make much sense. For argument's sake, we have also calculated correlation coefficients based on the group mean values, and achieved very high R^2 values, e.g. 0.99 for errors in the RAM. These calculations have, however, not been included in the result section, as we find the correlations based on litter means to be biologically much more relevant, as they include information about variation between litters that is otherwise lost.

Humans are exposed to many different thyroid-disrupting chemicals through the environment, including polybrominated diphenyl ethers (PBDEs), PCBs, perchlorate and pesticides like the dithiocarbamates. Exposure to these and possibly many other compounds may contribute to subclinical hypothyroidism in part of the population. When pregnant women are hypothyroid this can impair the neurological development of their children, and result in an increase of children born with learning disabilities. The observation of clear correlation between T_4 and neurological effects in this study has important implications for assessing the risk of thyroid related developmental neurotoxicity. This correlation clearly demonstrates that pre- and postnatal T_4 concentration is a good and predictive biomarker of altered behaviour and hearing loss in adulthood. In this study, the adverse effects of exposure to PTU were most pronounced at PND 16 as both offspring thyroid gland weights and T_4 levels were significantly affected at the lowest dose level (0.8 mg/kg). At 1.6 mg/kg, PTU also caused significant effects on dam T_4 levels. At the same dose level, behavioural changes in the adult offspring such as hyperactivity and deficiencies in maze learning, were also observed. This indicates that setting a NOAEL (No Observed Adverse Effect Level) for behavioural effects based on NOAELs for effects on T_4 levels in both dams and offspring is reasonable.

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Effects of pre- and postnatal exposure to the UV-filter Octyl Methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring

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ABSTRACT

Octyl Methoxycinnamate (OMC) is a frequently used UV-filter in sunscreens and other cosmetics. The aim of the present study was to address the potential endocrine disrupting properties of OMC, and to investigate how OMC induced changes in thyroid hormone levels would be related to the neurological development of treated offspring.

Groups of 14–18 pregnant Wistar rats were dosed with 0, 500, 750 or 1000 mg OMC/kg bw/day during gestation and lactation. Serum thyroxine (T_4), testosterone, estradiol and progesterone levels were measured in dams and offspring. Anogenital distance, nipple retention, postnatal growth and timing of sexual maturation were assessed. On postnatal day 16, gene expression in prostate and testes, and weight and histopathology of the thyroid gland, liver, adrenals, prostate, testes, epididymis and ovaries were measured. After weaning, offspring were evaluated in a battery of behavioral and neurophysiological tests, including tests of activity, startle response, cognitive and auditory function. In adult animals, reproductive organ weights and semen quality were investigated.

Thyroxine (T_4) levels showed a very marked decrease during the dosing period in all dosed dams, but were less severely affected in the offspring. On postnatal day 16, high dose male offspring showed reduced relative prostate and testis weights, and a dose-dependent decrease in testosterone levels. In OMC exposed female offspring, motor activity levels were decreased, while low and high dose males showed improved spatial learning abilities. The observed behavioral changes were probably not mediated solely by early T_4 deficiencies, as the observed effects differed from those seen in other studies of developmental hypothyroxinemia. At eight months of age, sperm counts were reduced in all three OMC-dosed groups, and prostate weights were reduced in the highest dose group. Taken together, these results indicate that perinatal OMC-exposure can affect both the reproductive and neurological development of rat offspring, which may be a cause of concern, as humans are systematically exposed to the compound through usage of sunscreens and other cosmetics.

Introduction

The UV-filter Octyl Methoxycinnamate (OMC), also known as Ethylhexyl Methoxycinnamate, is a very frequently used chemical in sunscreens and cosmetics worldwide. OMC is absorbed though the skin and is detectable in human blood and urine samples after topical application (Janjua et al., 2004). OMC has also been found in milk samples of women who have used OMC containing products (Schlumpf et al., 2008a), which indicates that humans are systemically exposed to this compound. Findings in several studies indicate that the substance acts as an endocrine disruptor (Schlumpf et al., 2001, 2008b; Schmutzler et al., 2004; Seidlova-Wuttke et al., 2006; Klammer et al., 2007).

Some *in vitro* studies have shown OMC to act as an estrogenic compound, as it enhanced proliferation of breast cancer cells (Schlumpf et al., 2001) and activated transcription in human cell lines via the estrogen receptor (Schreurs et al., 2002; Gomez et al., 2005). In an ecotoxicology study, Inui et al. (2003) demonstrated that in male medaka, OMC caused increased production of vitellogenin, a classical marker of estrogenic action in fish. In several studies in rats, OMC exposure has lead to increased uterine weight. This has been observed in an uterotropic test with immature female rats after 4 days of dosing with 1035 mg OMC/kg bw/day (Schlumpf et al., 2001), in ovariectomized (OVX) adult rats treated for 5 days with 1000 mg

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OMC/kg bw/day (Klammer et al., 2005), and in OVX rats fed OMC for 12 weeks, at a dose of approximately 1100 mg/kg bw/day (Seidlova-Wuttke et al., 2006). These results indicate that OMC possesses estrogenic activity, as estradiol causes similar effects on uterine weight. However, OMC also significantly increased serum LH levels (Seidlova-Wuttke et al., 2006) and upregulated expression of the estrogen receptor beta (Klammer et al., 2005) which are effects opposite those observed after estradiol treatment. Thus, OMC seems to exert both estrogenic and estrogen-independent activities *in vivo*.

In a large two-generation study, pregnant dams were treated with OMC in doses of 150, 450 or 1000 mg/kg bw/day in the feed. Exposure began before mating and continued throughout gestation, lactation, adolescence, mating of the F1 generation and until weaning of the F2 generation. Exposure caused no adverse effects on reproduction and development. The authors concluded that OMC displayed no estrogenic potential *in vivo* (Schneider et al., 2005), but no behavioral or thyroid endpoints were included in this study.

There is however good reason to assume that OMC does interfere with the hypothalamo-pituitary-thyroid (HPT) axis, and therefore could also affect brain development. Schmutzler et al. (2004) found that treatment of OVX rats for 12 weeks with 1100 mg OMC/kg bw/day, reduced thyroxine (T_4) levels in the blood as well as activity of 5′-deiodinase (the enzyme that converts T_4 to triiodothyronine (T_3)) in the liver. Reduction of deiodinase activity in peripheral tissues is also one of the mechanisms by which the potent anti-thyroid drug Propylthiouracil (PTU) exerts its effects (Cavalieri and Pitt-Rivers, 1981; Leonard and Rosenberg, 1978; Visser et al., 1979). Decreased T_4 levels were also observed in OVX rats treated with 200 and 1100 mg OMC/kg bw/day for 12 weeks, although a statistically significant effect was only observed in the low dose group (Seidlova-Wuttke et al., 2006). In OVX females treated with 10, 33, 100, 333 or 1000 mg OMC/kg/day for 5 days, the two highest doses also reduced T_4 levels in serum and deiodinase activity in the liver (Klammer et al., 2007).

Altogether, many studies indicate that OMC possesses endocrine disrupting properties, and especially the reductions in T_4 levels seem consistent across studies. The aim of the present study was to address the potential endocrine disrupting properties of OMC on the developing reproductive and thyroid hormone systems, and to investigate how OMC induced changes in thyroid hormone levels would be related to the neurological development of the offspring. Altered hormone levels during shorter periods of adulthood may not lead to adverse effects, but during early pre- and postnatal development, such alterations can affect both the physiological and neurological development of the offspring, as developmental hypothyroidism can cause delayed development, reduced growth, hearing loss and persistent neurobehavioral effects, in both experimental animals and humans (Zoeller and Crofton 2005; Miller et al., 2009; Pop et al., 1999; Haddow et al., 1999). Previously our group has shown that hearing loss, hyperactivity and impaired maze learning in rats could be directly correlated to decreases in early developmental T_4 levels—when these decreases were induced by developmental exposure to the anti-thyroid drug PTU (Axelstad et al., 2008). In order to gain more knowledge on how hypothyroxinemia in the pre- and postnatal period is related to subsequent neurotoxicity in rats, when caused by an environmentally relevant chemical with more possible modes of action, we wanted to examine if correlations between reduced T_4 levels and altered behavior would also exist after developmental OMC exposure.

Materials and methods

Test compound

The test compound was 2 Ethylhexyl 3-(4-Methoxyphenyl)-2 Propenoate, also called 2-ethylhexyl-4-methoxycinnamate or simply Octyl Methoxycinnamate (OMC). CAS no 5466-77-3, product number ACR291160250, purity 98.0% (VWR Bie & Berntsen, Herlev, Denmark). Corn oil (VWR Bie & Berntsen, Herlev, Denmark) was used as vehicle.

Animals and treatment

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the Inhouse Animal Welfare Committee. Seventy-two time-mated, nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at day 3 of pregnancy. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions: semitransparent plastic cages ($15 \times 27 \times 43$ cm) with Aspen bedding (Tapvei, Gentofte, Denmark) situated in an animal room with controlled environmental conditions (12 h reverse light–dark cycles with light starting at 9 p.m., temperature 22 ± 1 °C, humidity 55 ± 5%, ventilation 10 air changes per hour). Food (Altromin Standard Diet 1314) and acidified tap water were provided ad libitum.

The day after arrival, i.e. gestation day (GD) 4, the animals were weighed and assigned to four groups of 18 animals each, with similar weight distributions. They were given 3 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. The study was run in two blocks, with 2 weeks in between blocks and an equal representation of each dose group in each block. Dams in the four experimental groups were weighed and gavaged once daily at approximately the same time, from GD 7 to postnatal day (PND) 17 (day of delivery excluded) with 0 (vehicle), 500, 750, or 1000 mg OMC/kg bw/day. Vehicle control and OMC solutions were continuously stirred during the dosing period, and fresh solutions were prepared for each of the two study blocks. The dams were treated at a constant volume of 2 ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing. The dams were pair-housed until GD 17 and individually hereafter. They were observed daily for signs of toxicity, and body weights were recorded daily from GD 4 and during the entire dosing period.

Delivery, postnatal development and weaning

The day after delivery, weights of dams and individual pups were recorded. The pups were counted, sexed, checked for anomalies, and anogenital distance (AGD) was measured using a stereomicroscope. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 22, was designated PND 0 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as the animals gave birth on GD 22–23.

At PND 7 and 13 all pups were weighed and on PND 13 they were also examined for the presence of nipples/areolas, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Body weight of offspring was recorded again on PND 21, 28, 36 and 50. After PND 50 body weights were recorded when behavioral and physiological tests were performed in postnatal weeks (PNW) 9, 13–16 (females), 17, 22–25 (males), 31 and 35 (necropsy). At PND 16, litter size was standardized to 1–2 males and 3 females, when possible. From these offspring, 1–2 males and 1–2 females from each litter were weaned on PND 28, and kept for assessment of onset of puberty and for later behavioral testing. Remaining pups were euthanized on PND 28 (see the following discussion). Of the 72 time-mated dams, 57 gave birth to viable litters, which after weaning resulted in four groups of 14–18 male and 14–18 female pups, representing 12–18 litters per group. The weaned offspring was housed in pairs of the same sex and exposure status.

Onset of puberty

Onset of puberty was assessed by determining day of vaginal opening (VO) in female offspring and preputial separation (PPS) in males. All weaned females were scored on PND 29, 31 and daily from PND 34–38, while all weaned males were scored on PND 41, 43, 45, 48 and 49. The weight of the animals was measured when VO and PPS was observed.

Organ weights, histopathology and gene expression analysis PND 16 and 28

On PND 16, 1–10 pups in each litter were euthanized depending on the original size of the litter. All euthanized pups were weighed and decapitated. Trunk blood was collected for analysis of T_4, testosterone, progesterone and estradiol levels (blood samples were pooled for all males and all females within each litter). From 3 males and 2 females per litter the liver, adrenals and thyroid gland were excised, weighed and used for histopatological examinations. Adrenals from two pups per litter were placed in RNAlater for gene expression analysis. Thyroid glands intended for histopathology (from one male and one female per litter) were not weighed, but were excised on the thyroid cartilage in order to obtain optimal histological preservation. In all euthanized offspring the reproductive organs were examined macroscopically for anomalies. From 3 male and 2 female offspring per litter the testes, epididymides, ventral prostates and ovaries were excised, weighed, fixed in formalin and processed for paraffin embedding. From one male per litter prostate and testes were placed in RNAlater for gene expression analysis.

Dams and one female in each litter were euthanized on PND 28. The animals were weighed and decapitated after CO_2/O_2 anesthesia, and trunk blood was collected for measurement of T_4, progesterone and estradiol in serum. The uteri of the dams were excised, and the number of implantation scars was registered. The thyroid glands were dissected, weighed, and prepared for histopathological investigation. In the female pups, the thyroid gland, the uterus and ovaries were excised, weighed, and prepared for histopathology.

All organs intended for histopathological examinations were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histological evaluation was made of maternal thyroids and of all organs in which statistically significant changes in organ weights were seen.

All organs for gene expression analysis were kept in RNAlater (Qiagen) for later RNA purification and cDNA production according to the procedure from the manufacturer and as described in Laier et al. (2006). Expressions of the genes: P450scc, P450c17 and StAR in the adrenals, StAR, P450c17, P450scc, PEM, Bzrp and Scarb 1 in testis and TRPM-2 and PBP C3 in the ventral prostate were all taken relative to the endogenous control 18S rRNA and evaluated by real-time RT-PCR on a Taqman 7900 HT (Applied Biosystems) as described in Laier et al. (2006) where all abbreviated gene names are explained and primers and probes are listed as well.

Hormone analysis

On GD 15 and PND 15 dams were anesthetized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam) and blood was drawn from the tail vein. From offspring on PND 16 and PND 28, dams on day 28 and from eight months old offspring, trunk blood was collected for the hormone analysis. T_4 was examined in all blood samples (except offspring PND 28), while testosterone was measured in males on PND 16, and estradiol and progesterone were measured in dams on GD 15, PND 15 and 28 and in female offspring on PND 16 and 28. Plasma level of T_4 was analyzed using a modified Delfia T_4 (as described in Axelstad et al., 2008). Testosterone, estradiol and progesterone were extracted from the serum as previously described (Vinggaard et al., 2005) and the hormones were measured by time-resolved fluorescence using commercially available fluoroimmunoassay (FIA) kits (PerkinElmer Life Sciences, Turku, Finland).

Behavioral testing

The investigations were performed between 9.00 a.m. and 4 p.m. during the animals' dark cycle, i.e. their active period. The behavioral studies and hearing tests were recorded by experimenters who were blinded with respect to exposure groups. Exposed and control animals were tested alternately and except from tests in the radial arm maze, so were females and males.

Motor activity and habituation capability. Motor activity was measured twice in all weaned rat offspring—in postnatal week (PNW) 9 and again in PNW 17 (4 months). At testing, the animals were placed individually in clean plastic cages without bedding, food or water, and the cages were placed in activity boxes with photocells which measured activity for 30 min (as described in Axelstad et al., 2008). A computer automatically recorded output from photocells and collected data. Total activity during the 30 min observation period was used as a measure of general activity. In order to assess habituation, the 30 minute period was divided into shorter time intervals (3×10 min and 2×15 min) when analyzing the data.

Radial arm maze. At the age of 3 months for females (PNW 13–16), and 5 months for males (PNW 22–25) the weaned animals were tested in a standard 8-arm Radial Arm Maze (RAM) from Viewpoint (Sandown Scientific, Middlesex, England). The maze was built of transparent plexiglas with 8 arms (55 cm long) radiating from an octagonal central area (50 cm wide), and was situated on a light-sensitive board which was elevated 68 cm above the floor. The walls in the room were decorated with a number of spatial cues (geometric shapes and posters), in order for the rats to triangulate their position. At the end of each arm a reward (peanut) was placed. Through the light-sensitive board, a computer automatically recorded movements of the rats in the maze and performance could be viewed on a monitor placed in the adjoining room.

One week before testing, the animals were housed one per cage and given a restricted amount of food (12 g of food/day for females and 15 g/day for males) and one peanut to get accustomed to the taste. The food restriction was expected to lead to a decrease in body weight of approximately 10%, at the end of the testing period (based on previous studies). The animals were tested in one daily session in a total of 15 sessions during 3 consecutive weeks (5 trials per week), as described in Axelstad et al. (2008). In the daily test session, each rat was placed in the central maze area, and at this moment the monitoring system was started through a remote control. The experimenter left the room and the rat was allowed to explore the maze until all arms were visited, or 10 min had elapsed. Latency to visit the end of all 8 arms and the choice of arms was registered by the computer. The number of errors, defined as visiting an arm that had already been visited, was calculated from the data.

Acoustic startle reaction. Acoustic startle reaction (ASR) and prepulse inhibition (PPI) were tested in 56 female and 57 male offspring (representing all litters), at the age of 6½ months (PNW 28), as earlier described (Hougaard et al., 2005) in two chambers (San Diego Instruments, San Diego, USA) with 70 dB(A) white background noise. Each chamber contained a Plexiglas test tube (diameter 8.2 cm and length 25 cm), mounted on a platform with a piezoelectric accelerometer attached beneath. The accelerometer detected and transduced displacement of the tube, in response to movements of the rat. Animals were acclimatized 5 min in the test tube before sessions started and ended with 5 startle trials of 40 ms 120 dB(A) bursts of white noise. In between, 35 trials were delivered in semi-randomized order (10 trials of 120 dB(A); 5 each of 4 prepulse + startle trials (20 ms prepulses of 72, 74, 78, and 86 dB(A); 5 trials with only background noise)). Tube

movements were averaged over 100 ms following onset of the startle stimulus (AVG). For each prepulse intensity, the five AVGs were averaged and used in calculation of PPI, which was expressed as percent reduction compared to the average of the 10 middle startle trials: %PPI = 100 − ((AVG at prepulse + startle trial)/(AVG at 10 middle startle trials))*100%.

Test of hearing

At seven months of age (PNW 31), hearing function was evaluated in most of the animals previously tested in the acoustic startle reaction test. 48 male and 48 female rats were tested (12 males and 12 females per group, representing 11–12 litters per group). Hearing was assessed by measurements of distortion product oto-acoustic emissions (DPOAE) and by determination of hearing thresholds (HT) at 4 kHz, assessed by measurements of auditory brain stem response (ABR), with the animals in general anesthesia (Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam)), as described in Axelstad et al. (2008).

Organ weights, histopathology and semen quality analysis in adult animals

At eight months of age (PNW 35–36), all male and female offspring were anesthetised by CO_2/O_2 and decapitated. Trunk blood was collected and analyzed for testosterone and T_4 levels. Thyroid gland, liver, prostate, testes, vesiculas, ovaries and uterus were excised, weighed, fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin. Organs displaying statistically significant weight changes were evaluated histologically.

From all males the epididymides were removed and the cauda of the right epididymis was used for sperm motility analysis. The cauda of the left epididymis was frozen in liquid nitrogen for later sperm count.

Sperm motility. Spermatozoa were obtained from the distal cauda and sperm samples were prepared and analyzed by computer assisted sperm analysis (CASA) as described in Jarfelt et al. (2005). The parameters evaluated in this study were: percent motile and percent progressive spermatozoa, curvilinear velocity, amplitude of lateral head displacement which describes the vigor of the spermatozoa, and some progressive parameters, i.e. average path velocity and straight line velocity.

Sperm count. Cauda of the left epididymis was thawed at room temperature and prepared for sperm count analysis as described by Jarfelt et al. (2005). Samples were analyzed using 10× UV fluorescent objective and IDENT OPTIONS set A. Ten fields were analyzed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda.

Statistical analysis

For all analyses, the alpha level was set at 0.05. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as

Table 1
Pregnancy and litter data, including body weight (bw) of dams and offspring exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Dams and litters	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of dams (litters)	n = 18 (18)	n = 18 (12)	n = 18 (14)	n = 18 (13)
Dam bw gain, GD 7–21	87.2 ± 3.3	80.4 ± 4.3	77.2 ± 3.4*	69.8 ± 2.1***
Dam bw gain, GD 7–PND 1	21.7 ± 1.6	12.7 ± 3.2**	12.5 ± 1.9**	5.23 ± 3.1***
Dam bw gain PND 1–PND 21	24.2 ± 2.3	35.5 ± 2.3*	33.6 ± 4.0*	37.9 ± 3.4**
Gestation length (days)	22.8 ± 0.1	23.0 ± 0.1	22.9 ± 0.1	23.1 ± 0.1
% postimplantation loss	10.2 ± 3.4	7.95 ± 4.2	5.82 ± 2.2	7.51 ± 1.8
% perinatal loss	13.7 ± 3.8	10.9 ± 4.2	10.2 ± 2.6	13.6 ± 3.2
Litter size	10.9 ± 0.8	11.3 ± 1.1	10.5 ± 0.7	11.9 ± 0.6
% perinatal deaths	3.97 ± 2.0	3.00 ± 1.8	4.65 ± 1.6	6.55 ± 2.9
% males	51.4 ± 3.5	57.1 ± 5.2	48.5 ± 4.4	48.2 ± 4.0
Offspring				
Mean birth weight	6.08 ± 0.1	6.04 ± 0.2	5.76 ± 0.1*	5.14 ± 0.1**
AGD males (mm)	3.50 ± 0.03	3.40 ± 0.04	3.47 ± 0.04	3.45 ± 0.05
AGI males (mm/cu. root bw)	1.90 ± 0.02	1.86 ± 0.02	1.92 ± 0.03	1.98 ± 0.03
AGD females (mm)	1.82 ± 0.04	1.70 ± 0.03	1.77 ± 0.02	1.72 ± 0.04
AGI fem. (mm/cu. root bw)	1.01 ± 0.02	0.95 ± 0.01	0.99 ± 0.01	1.00 ± 0.02
Nipples (areolas) males	0.03 ± 0.01	0.05 ± 0.04	0.10 ± 0.04	0.12 ± 0.05
Nipples (areolas) females	12.4 ± 0.1	12.6 ± 0.2	12.6 ± 0.1	12.7 ± 0.1
Mean bw PND 7	14.8 ± 0.4	13.2 ± 0.3*	13.5 ± 0.3*	11.6 ± 0.2**
Mean bw PND 14	28.7 ± 0.9	25.1 ± 0.8**	26.6 ± 0.7*	23.1 ± 0.6**
Mean bw PND 21	47.8 ± 1.2	44.4 ± 1.2	46.2 ± 0.7	41.7 ± 0.6**
Mean female bw PND 28	74.5 ± 1.7	69.2 ± 1.0*	71.3 ± 1.2	62.7 ± 1.2**
Mean male bw PND 28	81.2 ± 1.4	73.3 ± 1.6**	74.8 ± 1.1**	68.7 ± 1.5***
Mean female bw PND 36	110.4 ± 2.0	104.6 ± 1.3*	109.1 ± 2.0	98.8 ± 1.9**
Mean male bw PND 36	129.3 ± 2.5	118.3 ± 2.3**	121.6 ± 2.4*	114.4 ± 2.4***
Mean female bw PND 50	150.2 ± 3.0	147.4 ± 2.4	154.6 ± 2.9	139.8 ± 2.6*
Mean male bw PND 50	212.4 ± 3.8	195.0 ± 3.3**	203.8 ± 3.9	195.5 ± 3.6**
Mean age vag. opening (vo)	33.7 ± 1.5	34.5 ± 1.2	34.5 ± 1.0	34.6 ± 0.9
Mean weight at vo.	100.4 ± 10.1	98.4 ± 6.5	102.2 ± 7.5	93.7 ± 6.8*
Mean age, preputial sep. (pps)	43.1 ± 1.3	44.1 ± 1.9	44.1 ± 1.4	44.4 ± 2.4
Mean weight at pps.	171 ± 13	163 ± 10	168 ± 11	164 ± 11
Mean male bw PNW 9	257 ± 21	237 ± 13**	243 ± 17*	238 ± 12**
Mean male bw PNW 17	411 ± 42	370 ± 43**	383 ± 25*	377 ± 31*
Mean male bw PNW 22	447 ± 52	407 ± 30*	411 ± 34*	407 ± 33*
Mean male bw PNW 25	414 ± 34	382 ± 23**	388 ± 27*	380 ± 25**
Mean male bw PNW 35	499 ± 44	462 ± 36*	453 ± 32**	463 ± 34*

an independent, random and nested factor in ANOVA, or analysis was done on litter means. Litter size was included as a covariate in analyses of body weight of the pups until PND 16. Body weight was included as a covariate in analyses when relevant, e.g. for terminal organ weights. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskal–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Statistical analyses of the effects on macroscopic lesions and histopathology were done using Fisher's Exact Test. Startle data were analyzed by use of Systat Software Package v. 9. All other analyses were performed using SAS Enterprise Guide 3.0 (2004), SAS Institute Inc, Cary, NC, 274 USA.

Results

Pregnancy data and postnatal growth

Maternal body weight gain was significantly reduced during the gestation period in the OMC exposed groups (Table 1). Body weight gains from GD 7 to the day before birth (GD 21) were significantly lowered in the two highest dose groups compared to controls ($p = 0.032$ and $p = 0.0004$). When adjusted for weight and number of offspring (BW gain GD 7-PND 1), the effect on maternal weight gain was even more marked and statistically significantly different from controls in all three dose groups ($p = 0.0092$, $p = 0.0057$, and $p = 0.0001$). During the lactation period, dams in all three OMC groups gained significantly more weight than controls ($p = 0.010$, $p = 0.024$, and $p = 0.002$), which eliminated differences in body weights between dosed and control animals at the time of weaning. Gestation length, litter size, postimplantation loss, neonatal death, and gender distribution were similar in the four groups (Table 1). Neither anogenital distance at birth, nor nipple retention on PND 13 differed by exposure, in either gender.

Offspring body weights were significantly reduced by OMC treatment (Table 1). The body weight of male offspring was significantly reduced in the two highest dose groups at birth and in all three dose groups on PND 7, 14, 28 and 36. Body weights in female offspring were significantly reduced in the highest dose group at birth, in all three groups at PND 7 and 14, and in the lowest and highest dose groups on PND 28 and 36 (Table 1). After PND 50 the weights of OMC-dosed females no longer differed significantly from that of controls (data not shown). Male body weights remained significantly lower in all OMC dose groups compared to controls from PND 50 and throughout the rest of the study (Table 1).

Mean age and body weight at sexual maturation, measured as day of vaginal opening (VO) and preputial separation (PPS) are shown in Table 1. Both male and female offspring from the high dose OMC group reached sexual maturation a little later than control offspring, though the difference was not statistically significant. This tendency towards later sexual maturation may likely be due to lower body weights in this group.

Hormone levels in dams and juvenile offspring

T_4 levels measured in blood from dams and offspring are shown in Fig. 1 and Table 2. T_4 levels decreased markedly with higher doses of OMC in dams on both GD 15 and PND 15 and were significantly affected in all dose groups ($p < 0.0001$). Male pup T_4 levels on PND 16 were also significantly decreased in all three dose groups ($p < 0.0001$) (Fig. 1c), while female T_4 levels on PND 16 were unaffected by OMC exposure (Table 2). All control T_4 levels in dams and pups were comparable to historical control levels from our laboratory. Progesterone, estradiol and testosterone levels in serum from dams and offspring are shown in Table 2. No statistically significant differences in progesterone and estradiol levels were observed in the dams. In the female offspring on PND 16 estradiol levels were unaffected by OMC treatment. Progesterone levels appeared lower in all dosed groups, but the difference from controls was not statistically significant. On PND 28, progesterone levels were decreased in female offspring from all dosed groups ($p = 0.021$, $p = 0.002$, and $p = 0.017$), whereas estradiol levels were decreased in the two lower ($p = 0.008$ and $p = 0.012$) but not in the highest OMC dose group. In male pups,

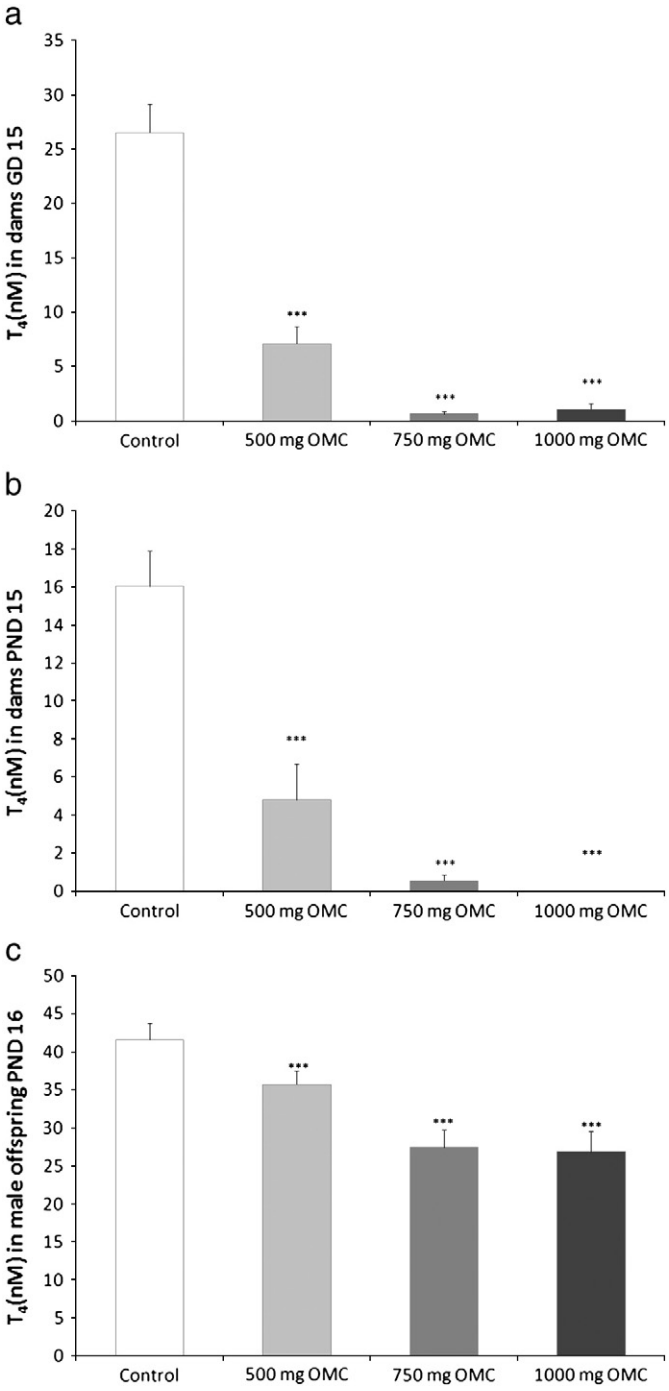

Fig. 1. Thyroxine (T_4) levels (nM) in dams on GD 15 (a) and PND 15 (b), and male offspring on PND 16 (c) after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means, based on litter means + SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$.

Table 2
Effect of perinatal OMC exposure on the reproductive and thyroid hormone systems. T_4 levels in all dams and offspring, progesterone and estradiol levels in dams and female offspring and testosterone levels in male offspring euthanized at GD 15, PND 15, 16, 28 or in adulthood, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. N = 11–18. Data represent group means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Hormone measurements (nM)	Control	500 mg OMC	750 mg OMC	1000 mg OMC
Dams GD 15				
Progesterone	192.1 ± 44	135.7 ± 69	174.1 ± 68	133.0 ± 61
Estradiol	0.030 ± 0.01	0.030 ± 0.01	0.033 ± 0.01	0.031 ± 0.01
T_4	26.5 ± 10.9	7.12 ± 5.6***	0.61 ± 1.0***	1.02 ± 1.9***
Dams PND 15				
Progesterone	64.47 ± 7.8	68.88 ± 6.0	70.33 ± 4.1	70.58 ± 4.9
Estradiol	0.0066 ± 0.001	0.0073 ± 0.001	0.0079 ± 0.003	0.0095 ± 0.003
T_4	16.03 ± 7.71	4.80 ± 6.66***	0.56 ± 1.17***	0.00 ± 0.00***
Dams PND 28				
Progesterone	29.87 ± 26.6	40.25 ± 38.2	48.95 ± 38.2	36.77 ± 28.8
Estradiol	0.051 ± 0.06	0.032 ± 0.03	0.031 ± 0.02	0.079 ± 0.08
T_4	26.57 ± 8.04	25.68 ± 8.09	28.47 ± 7.32	36.39 ± 10.73
Offspring PND 16				
Progesterone	10.48 ± 6.6	5.41 ± 2.8	6.33 ± 2.7	7.23 ± 1.6
Estradiol	0.035 ± 0.016	0.037 ± 0.009	0.035 ± 0.007	0.029 ± 0.007
Testosterone	0.843 ± 0.61	0.389 ± 0.24**	0.337 ± 0.25***	0.242 ± 0.19***
T_4 male	41.60 ± 9.3	35.72 ± 6.3***	27.44 ± 8.5***	26.85 ± 9.7***
T_4 female	35.79 ± 7.1	35.41 ± 7.9	34.75 ± 8.4	30.68 ± 7.9
T_4 litter mean	39.19 ± 7.5	35.56 ± 6.6	31.27 ± 6.2*	28.77 ± 7.2***
Offspring PND 28				
Progesterone	11.25 ± 8.2	6.56 ± 8.2*	3.74 ± 1.6**	4.82 ± 2.9*
Estradiol	0.011 ± 0.005	0.005 ± 0.002***	0.006 ± 0.003*	0.009 ± 0.006
Adult offspring				
T_4 male	28.3 ± 8.4	28.4 ± 4.9	31.9 ± 8.5	27.9 ± 9.3
T_4 female	33.2 ± 14.1	31.4 ± 8.1	31.3 ± 9.5	41.6 ± 13.9
Testosterone	1.98 ± 1.8	1.95 ± 2.5	3.40 ± 2.8	2.27 ± 1.7

testosterone levels were significantly decreased in all three dose groups on PND 16 ($p = 0.006$, $p = 0.0005$, and $p = 0.0001$) (Fig. 2), but were unaffected at termination of the study (Table 2).

Organ weights, histopathology and gene expression in juvenile animals

Body and organ weights from offspring euthanized on PND 16 and PND 28 are shown in Table 3. As body weights were lower in the OMC-dosed animals compared to controls, absolute organ weights that seemed similar between control and high dose groups, proved statistically different when body weight was included in the statistical analysis of the organ weights (as covariates). There was, however, great compliance between results from the statistical analysis of absolute organ weights (with body weight as a covariate) and relative organ weights, therefore significant differences in organ weights between control and OMC-dosed groups will only be referred to as "relative to body weight", in the remainder of the paper.

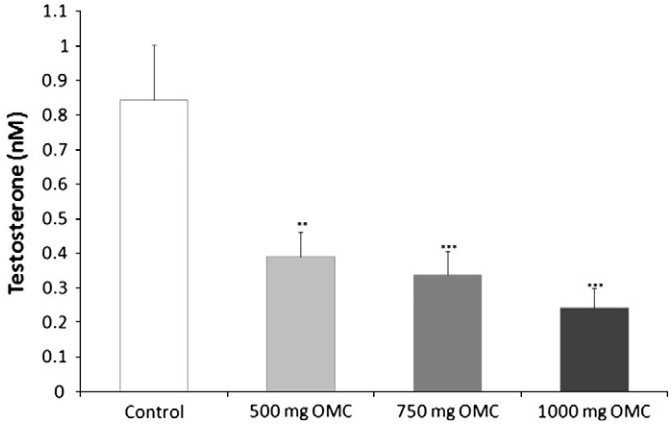

Fig. 2. Testosterone levels (nM) in male offspring on PND 16 after exposure to 0, 500, 750 or 1000 mg OMC /kg/day from GD 7 to PND 17. Data represent group means, based on litter means + SEM, n = 11–15. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$.

On PND 16, the relative weight of the thyroid gland was significantly higher in the two highest dose groups, when litter means including both male and female values were analyzed ($p<0.0001$). This increase was more pronounced in male than in female offspring, though not significantly different from controls in any dose group, when data from the two sexes were analyzed separately (data not shown). Histological examination of the thyroid glands from PND 16, showed no alterations by OMC treatment (Table 4).

On PND 16 the relative testis weights were significantly decreased in the two high dose groups ($p = 0.031$ and $p = 0.0007$), as can be seen in Table 3. The testes were evaluated for degree of lumen formation in the seminiferous tubules, as an indicator of the degree of testicular maturation. In all animals from the highest dose group, less than 1/3 of the tubular cross-sections had lumen formation, whereas animals with more than 1/3 and more than 2/3 tubules with lumen formation were frequently observed in the other dose groups (Table 4). In the highest dose group, 10 of 12 animals showed no tubule formation versus only 2 of 16 animals in the control group, indicating delayed testis development. Epididymal histopathology on PND 16 did not appear altered by OMC treatment.

Relative prostate weights were significantly decreased in the high dose group ($p = 0.0003$) (Table 3). Evaluation of prostate histopathology on PND 16 revealed dose-related changes in development. In the highest dose group, no animals had the large, fluid-filled acini,

Table 3
Effect of OMC on terminal body weights (bw) and organ weights in male and female rat offspring euthanized at PND 16 and in female offspring euthanized PND 28, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Liver, thyroid and adrenal weights are litter mean values from male and female offspring combined. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; ***: $p<0.0001$.

Organ weights PND 16	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of litters	16	11	14	13
Bw male (g)	31.8 ± 3.5	29.1 ± 2.1*	30.7 ± 4.0	26.6 ± 2.9***
Bw female (g)	29.8 ± 3.7	28.6 ± 2.0	28.8 ± 2.2	25.5 ± 2.5***
Adrenals (mg)	9.17 ± 1.5	8.80 ± 1.0	9.20 ± 1.1	7.61 ± 0.8
Adrenals (mg/100 g bw)	29.4 ± 3.3	30.5 ± 2.2	30.6 ± 3.1	29.1 ± 2.4
Liver (mg)	775 ± 91	734 ± 47	797 ± 89[a]	727 ± 87[a]
Liver (mg/100 g bw)	2484 ± 83	2539 ± 82	2640 ± 149***	2758 ± 95***
Thyroid gland (mg)	4.67 ± 0.8	4.18 ± 0.8	5.40 ± 1.8[a]	4.62 ± 0.6 [a]
Thyroid gland (mg/100 g bw)	15.1 ± 1.9	14.5 ± 2.5	18.0 ± 4.8***	17.6 ± 2.3***
Ovaries (mg)	11.8 ± 1.6	10.8 ± 1.5	11.1 ± 1.4	9.55 ± 0.8
Ovaries (mg/100 g bw)	39.1 ± 5.0	37.5 ± 5.1	39.0 ± 5.5	38.4 ± 4.2
Testes (mg)	111 ± 14	99.7 ± 7.5	101 ± 7.8[b]	85.1 ± 8.9[b]
Testes (mg/100 g bw)	349 ± 21	344 ± 26	331 ± 22*	320 ± 17***
Prostate (mg)	12.4 ± 2.0	10.4 ± 1.5	11.3 ± 2.4	8.49 ± 1.8[b]
Prostate (mg/100 g bw)	39.0 ± 4.5	36.0 ± 4.3	36.3 ± 5.7	31.8 ± 4.6***
Epididymides (mg)	23.6 ± 3.3	21.7 ± 1.7	23.1 ± 2.9	20. ± 2.1
Epididymides (mg/100 g bw)	74.2 ± 8.4	75.4 ± 8.7	75.8 ± 10	75.7 ± 9.0
Organ weights PND 28	**Control**	**500 mg OMC**	**750 mg OMC**	**1000 mg OMC**
No. of litters	15	11	13	13
Bw in female offspring	74.4 ± 8.89	68.9 ± 4.59	70.3 ± 3.47	63.5 ± 3.69
Thyroid gland (mg)	8.54 ± 2.34	8.54 ± 2.32	7.57 ± 1.77	7.36 ± 1.36
Thyroid gland (mg/100 g bw)	11.6 ± 3.09	12.3 ± 2.98	10.7 ± 2.43	11.6 ± 1.93
Ovaries (mg)	33.7 ± 3.19	33.6 ± 4.62	31.9 ± 4.18	31.1 ± 3.65
Ovaries (mg/100 g bw)	45.6 ± 4.96	48.8 ± 6.70	45.5 ± 6.74	49.2 ± 5.67
Uterus (mg)	46.5 ± 9.22	41.5 ± 11.1	43.8 ± 5.82	37.7 ± 8.31
Uterus (mg/100 g bw)	63.0 ± 12.	60.5 ± 16.9	61.8 ± 7.08	59.3 ± 12.2

[a] Indicates an absolute organ weight that was significantly larger than in the control group, when body weight was included in the statistical analysis as a covariate ($p<0.05$).
[b] Indicates an absolute organ weight that was significantly smaller than in the control group, when body weight was included in the statistical analysis as a covariate ($p<0.05$).

characteristic of control prostates (Table 4). Among the 750 mg OMC males, half the males had large acini, but these were not fluid-filled as seen in controls. These histological changes reflect the lower prostate weights seen in the highest dose group.

Relative liver weights were significantly increased in the two highest dose groups ($p<0.0001$), while the relative weights of the adrenals, the epididymides and the ovaries were unaffected by OMC treatment (Table 3). No differences between exposure groups were seen in the evaluation of liver histology (data not shown). Gene expression analysis was done on PND 16 testes, prostate and adrenal glands. No significant changes in expression were found for any of the genes in neither of these organs (data not shown).

Table 4
Histopathology of dams and offspring after exposure to 0, 500, 750, or 1000 mg OMC/kg bw/day from GD 7 to PND 17. Data are percentages of affected animals followed by the number of animals with the listed characteristics/total number of animals evaluated. Figures in bold are statistically significantly different from controls, *: $p<0.05$, ***: $p<0.001$ with a two-sided Fisher's exact test.

Histopathology	Control	500 mg OMC	750 mg OMC	1000 mg OMC
Dam thyroids PND 28				
No hyperplasia	53%, 9/17	38%, 3/8	31%, 4/13	45%, 5/11
Unilaterally hyperplasia	18%, 3/17	13%, 1/8	23%, 3/13	18%, 2/11
Bilaterally hyperplasia	29%, 5/17	50%, 4/8	46%, 6/13	36%, 4/11
Uni- or bilaterally hyperplasia	47%, 8/17	63%, 5/8	69%, 9/13	55%, 6/11
Pup thyroids PND 16 (female)				
No hyperplasia	85% (11/13)	70% (7/10)	80% (8/10)	64% (7/11)
Hyperplasia	15% (2/13)	30% (3/10)	20% (2/10)	36% (4/11)
Testis PND 16				
0% tubules with lumen	13%, 2/16	27%, 3/11	14%, 2/14	**83%, 10/12*** **
<1/3 tubules with lumen	62%, 10/16	73%, 8/11	64%, 9/14	**100%, 12/12***
1/3 to 2/3 tubules with lumen	19%, 3/16	27%, 3/11	29%, 4/14	0%, 0/12
>2/3 tubules with lumen	19%, 3/16	0%, 0/11	7%, 1/14	0%, 0/12
Cellular debris in a few tubules	13%, 2/16	27%, 3/11	14%, 2/14	8%, 1/12
Prostate PND 16				
Alveolar lumen size				
Presence of large, fluid-filled acini	50%, 8/16	50%, 4/8	**8%, 1/12***	**0%, 0/9***
Presence of large, flattened acini	6%, 1/16	13%, 1/8	**50%, 6/12***	0%, 0/9
Intermediate size acini	31%, 5/16	25%, 3/8	25%, 3/12	**78%, 7/9***
Small acini	13%, 2/16	13%, 1/8	17%, 2/12	22%, 2/9
Prostate adults				
General acinar atrophy	0%, 0/18	7%, 1/14	**27%, 4/15***	**50%, 7/14*** **

On PND 28, female offspring body weights were still significantly reduced in the 500 and 1000 mg OMC/kg bw/day groups ($p = 0.021$ and $p = 0.0001$), while relative weights of the uterus and ovaries showed no variation with OMC exposure. The relative weights of the thyroid glands were no longer significantly affected by the OMC exposure (Table 3). In the dams, body and thyroid weights were not affected by OMC exposure on PND 28, nor was the thyroid histology (data not shown).

Motor activity levels in young and adult male and female offspring

Results from the activity test in PNW 9 and PNW 17 are shown in Fig. 3. In the younger animals, female control animals showed significantly higher activity levels than control males, as expected ($p<0.0001$). Female offspring exposed to OMC were less active compared to controls, significantly so in the 750 and 1000 mg OMC/kg groups ($p = 0.0004$ and $p = 0.041$), while male activity levels seemed unaffected by OMC exposure. Fig. 3b shows the total activity levels from when the animals were tested in PNW 17. Again, female control animals showed significantly higher activity levels than control males ($p = 0.0006$). Female animals exposed to OMC were again less active compared to controls, but this time only significantly so in the highest dose group ($p = 0.05$). Male offspring from the 750 mg OMC/kg bw/day group displayed elevated activity levels compared to control males ($p = 0.014$). As this effect was not present in the 1000 mg OMC/kg bw/day males, it may be a chance finding. When the 30-minute test period was divided into shorter time periods, both male and female animals in all dose groups displayed distinctly higher activity levels in the beginning compared to the end of the test. Females were approximately 3 times more active in the first 10 min of the test compared with the last 10, while males were 4–5 times more active in the beginning of the test than in the end. This was the case for both control and OMC-dosed animals, indicating that habituation was not affected by OMC exposure. This pattern was seen both in PNW 9 and PNW 17 (data not shown).

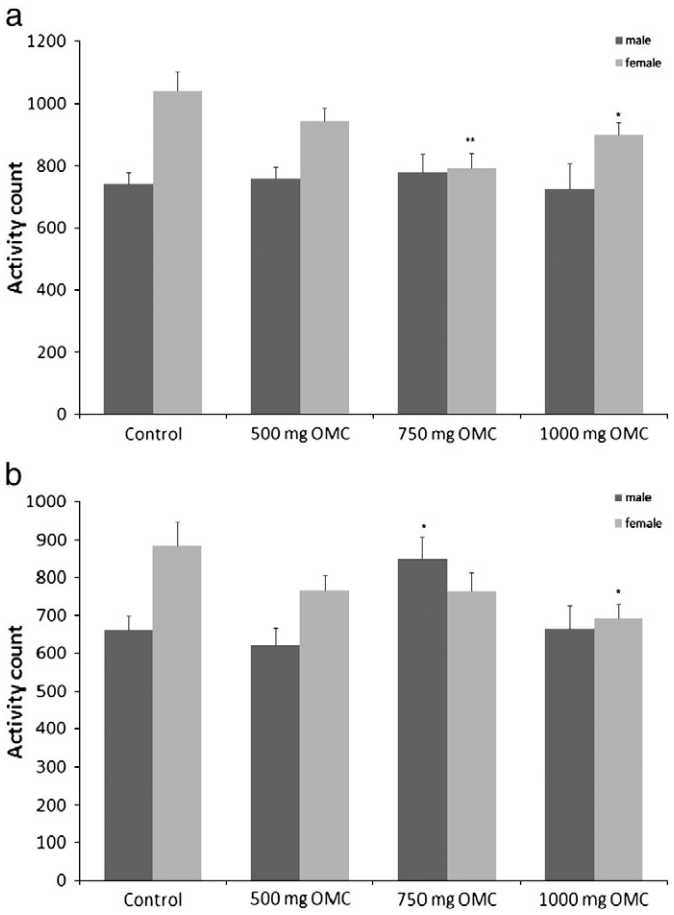

Fig. 3. Total motor activity level during 30 min of testing of male and female offspring at 9 (a) and 17 (b) weeks of age, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means, based on litter means ± SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to same sex controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$. Difference between male and female control animals is statistically significant, $p<0.01$.

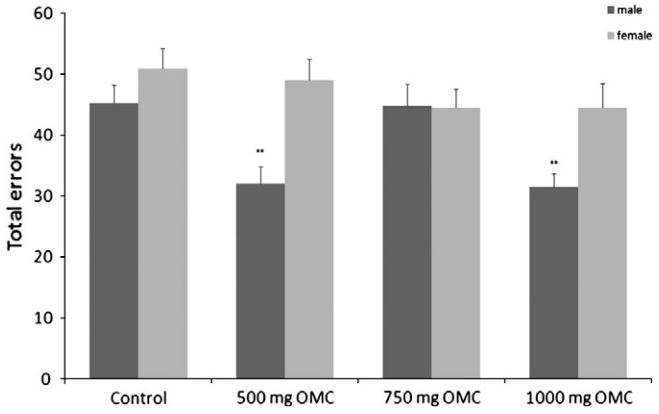

Fig. 4. Total number of errors in the radial arm maze during three weeks of testing, in adult male and female rats, exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means + SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to same sex controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$.

Radial arm maze

The total number of errors made during three weeks of testing in the RAM is presented in Fig. 4. In OMC treated males a reduction in the number of errors was registered for both the 500 and 1000 mg OMC/kg groups ($p = 0.0076$ and $p = 0.0024$) compared to controls, while performance in the 750 mg/kg bw/day males was not significantly different from controls. The effect in OMC-dosed males was only evident during the first week of testing (data not shown). At no time point did latency or frequency of choosing adjacent arms differ between dosed and control males. In females, behavior was similar in control and exposed offspring on all tested parameters. There was no significant difference between male and female control values for the total number of errors. Food restriction caused a small reduction in male body weights (at end of the testing period, the reduction was around 7%), while female body weights were unaffected.

Acoustic startle reaction

When the acoustic startle reaction was tested, control and exposed males startled at comparable levels. Control females generally displayed a lower level of startle than did exposed females. However, statistical analysis indicated no significant differences in exposed compared to dosed offspring for basal reactivity, habituation to the initial 5 noise pulses, or PPI, in either males or females (data not shown).

Auditory function

Hearing as assessed by both oto-acoustic emissions and auditory brainstem response at 4 kHz was closely similar in all groups, and

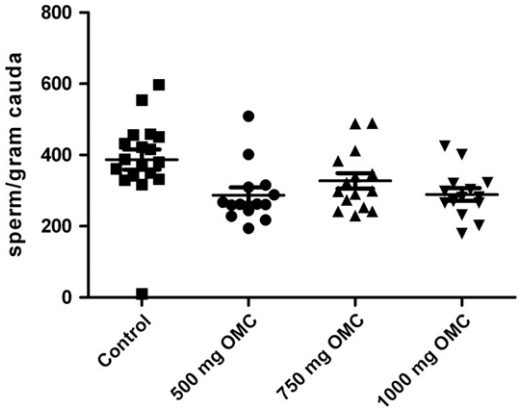

Fig. 5. Number of sperm per gram cauda in adult male rats exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Individual male values are shown, together with group mean and SEM, n = 14–18. If the outlier in the control group is included in the statistical analysis, the 500 and 1000 mg OMC/kg groups are significantly different from control, while all three OMC dose groups are significantly lower than controls if the outlier is excluded from the statistical analysis.

there were no differences between dosed and control groups in either males or females (data not shown).

Hormone levels, organ weights and semen quality in adult offspring

No effect was seen on T_4 or testosterone levels, when measured in blood from 35 week old animals (Table 2). In Table 6, body and organ weights in adult rat offspring are shown. Male body weights were lower in all OMC groups ($p = 0.022$, $p = 0.0021$, and $p = 0.013$), while female body weights were unaffected by exposure. Relative weight of thyroid gland, liver, ovaries, uterus, testes and vesicula was unaffected by OMC treatment. In the control group, one male had very low testis weight, and histological evaluation revealed general degeneration of seminiferous chords in this animal. Testes weights were analyzed both with and without the outlier, but in both cases no treatment-related effects on testes weights were seen. Relative prostate weights were significantly reduced in the 1000 mg OMC/kg bw/day group ($p = 0.0073$), and histological examination showed a significantly increased number of animals with general acinar atrophy in the two highest dose groups, 27% and 50% respectively, while none was seen in the control group.

The sperm count was significantly lowered in the OMC exposed groups compared to controls (Fig. 5 and Table 5). The sperm count results are shown as number of sperm per gram cauda epididymis, and the observed dose-dependent reduction was not due to effects on the weight of the epididymis, as this was unaffected by OMC exposure (Table 5). The one male from the control group having low testes weights, also had almost no sperm cells in the epididymis. When this outlier value was included in the statistical analysis, the reduced sperm count was statistically significant in the 500 and 1000 mg/kg bw/day groups ($p = 0.009$ and $p = 0.0039$ respectively), whereas all three dosed groups differed significantly from the control group ($p = 0.0002$, $p = 0.0085$ and $p < 0.0001$) if this outlier was excluded from the results. Mean values and standard deviation with and without the outlier from the control group are shown in Table 5. None of the investigated sperm motility parameters was significantly affected by OMC exposure (Table 5).

Table 5
Effect of OMC on terminal body weights (bw), organ weights and semen quality in male and female rat offspring euthanized at PNW 35 (8 months), after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Organ weights adult males	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of males	18	14	15	14
BW (g)	499 ± 43	462 ± 36*	453 ± 32**	462 ± 34*
Liver (g)	13.93 ± 1.33	12.69 ± 1.23	12.74 ± 1.01	13.10 ± 1.15
Liver (g/100 g bw)	2.79 ± 0.19	2.74 ± 0.18	2.81 ± 0.12	2.83 ± 0.16
Thyroid gland (mg)	24.81 ± 4.4	24.11 ± 4.6	21.63 ± 3.1	23.32 ± 5.3
Thyroid gland (mg/100 g bw)	5.04 ± 0.9	5.25 ± 0.9	4.77 ± 0.6	5.04 ± 1.1
Testes (mg)[a]	3917 ± 662	3945 ± 285	3960 ± 272	3795 ± 413
Testes (mg/100 g bw)[a]	790 ± 141	857 ± 77.5	877 ± 76	821 ± 69
Prostate (mg)	963 ± 146	728 ± 192	694 ± 160	603 ± 104**
Prostate (mg/100 g bw)	168 ± 28	158 ± 42	153 ± 35	131 ± 28**
Vesicula (mg)	1790 ± 286	1702 ± 294	1791 ± 243	1887 ± 283
Vesicula (mg/100 g bw)	360 ± 64	368 ± 61	395 ± 54	408 ± 54
% motile sperm	46.2 ± 17.9	46.5 ± 14.4	42.5 ± 12.8	47.4 ± 13.5
% progressive sperm	27.1 ± 11.0	27.7 ± 8.9	25.3 ± 8.2	28.1 ± 11.0
Curvilinear velocity	344.3 ± 37.3	356.9 ± 23.5	357.6 ± 34.3	341.4 ± 31.7
Ampl. of lateral head displ.	17.9 ± 1.7	17.9 ± 1.7	18.6 ± 1.8	18.1 ± 1.6
Average path velocity	186.9 ± 21.3	194.0 ± 11.3	189.5 ± 17.9	180.3 ± 15.8
Straight line velocity	122.0 ± 17.9	127.2 ± 10.9	125.2 ± 14.07	114.8 ± 12.4
Cauda epididymis (mg)	282.3 ± 46.1	269.4 ± 32.3	276.4 ± 30.1	269.6 ± 29.9
Sperm/g cauda (all data)	387.3 ± 119	287.3 ± 80.3**	327.4 ± 84.1	289.2 ± 66.5**
Sperm/g cauda (without outlier)	409.5 ± 78.3	287.3 ± 80.3***	327.4 ± 84.1**	289.2 ± 66.5***
Organ weights adult females	**Control**	**500 mg OMC**	**750 mg OMC**	**1000 mg OMC**
No. of females	18	14	16	14
BW	268 ± 25	262 ± 21	273 ± 18	256 ± 23
Liver (mg)	7316 ± 840	7170 ± 939	7542 ± 666	7070 ± 815
Liver (mg/100 g bw)	2727 ± 152	2733 ± 265	2768 ± 178	2760 ± 277
Thyroid gland (mg)	17.9 ± 2.5	22.3 ± 10	18.0 ± 5.9	19.4 ± 6.4
Thyroid gland (mg/100 g bw)	6.70 ± 1.1	8.63 ± 4.0	6.56 ± 2.0	7.30 ± 2.2
Uterus (mg)	631 ± 182	615 ± 209	553 ± 144	784 ± 428
Uterus (mg/100 g bw)	237 ± 72	238 ± 93	203 ± 51	313 ± 183
Ovaries (mg)	82.2 ± 13	87.7 ± 24	94.9 ± 20	89.4 ± 17.4
Ovaries (mg/100 g bw)	31.2 ± 5.0	33.5 ± 9.0	35.0 ± 6.7	35.1 ± 7.5

[a] Testes weight means are shown for all data. Excluding the outlier in the control group from the data analysis, gave mean values for testes weight of 4043 ± 405 mg, and 816 ± 901 mg/100 g bw respectively, but did not alter the fact that no significant effect of OMC exposure was seen on adult testes weights.

Discussion

In the present study, pre- and postnatal OMC exposure was associated with changes in both reproductive and thyroid hormone levels, and altered development of some reproductive organs, semen quality as well as some behavioral endpoints.

Table 6 summarizes the endpoints that were significantly affected by OMC treatment. Serum T_4 levels in dams were almost completely reduced on both GD 15 and PND 15, while T_4 levels in the offspring were only reduced about 30% in the males, and were unaffected in the females on PND 16. In a study of PTU, using a similar study design (Axelstad et al., 2008), rather different effects were seen. Here, offspring levels of T_4 decreased severely on PND 16 (75% reduction in the high dose group) and furthermore a marked increase in thyroid weights and adverse effects on thyroid histology were seen. At the same time, reductions in dam T_4 levels on GD 15 were much less marked than in the present study. These results indicate that the mechanisms by which the two chemicals affect the thyroid and reduce T_4 levels probably differ substantially, even though both have been shown to reduce deiodinase activity (Cavalieri and Pitt-Rivers, 1981; Visser et al., 1979; Schmutzler et al., 2004; Klammer et al., 2007). The striking difference in OMC's effects on T_4 levels in dams and offspring respectively, was surprising. A possible explanation could be that OMC was only transferred to the offspring in very low concentrations, and therefore only affected T_4 levels slightly. Alternatively, the offspring for some reason were less susceptible to the thyroxine diminishing effects of OMC than the dams. No published rat studies have investigated the internal OMC levels in milk or serum after experimental exposure. However, studies in rats dosed with two other UV-filters, 4-methylbenzylidenecamphor (4-MBC) and 3-benzylidene camphor (3-BC), which have been shown to have similar effects on the developing reproductive and thyroid hormone systems as OMC, are transferred to rat breast milk in a dose-dependent manner (Schlumpf et al., 2008a). Furthermore, an epidemiological study of UV-filter exposure has shown that several UV-filters, including OMC, are transferred to human breast milk, as a clear correlation between exposure to OMC containing cosmetics and presence of OMC in human breast milk samples was found (Schlumpf et al., 2008a). It is therefore plausible that OMC is also transferred to breast milk in rats. Furthermore, a clear and dose-dependent reduction in testosterone level was seen in male offspring on PND 16, which also indicates that OMC did reach the offspring. Taken together, these results indicate that OMC probably affected the thyroid status of adult female rats more than seen in the offspring. In future studies, measurements of T_4 levels soon after birth would be useful for investigating if placental OMC transfer alone impacts on neonatal thyroid status. Furthermore, investigating effects of OMC given by gavage directly to neonatal rats rather than through the dams, would further elaborate on the mechanisms by with OMC causes thyroid hormone disruption. In order to compare experimental doses with human exposure more efficiently, including measurements of OMC concentrations in rat milk would be helpful.

Table 6
Endpoints that in the present study show association with OMC treatment. The LOAEL is given in mg OMC/kg/day.

Endpoint	OMC dose
T_4 levels in dams GD 15 and PND 15	Reduced at 500
T_4 levels male offspring PND 16	Reduced at 500
Testosterone levels male offspring PND 16	Reduced at 500
Progesterone levels female offspring PND 28	Reduced at 500
Estradiol levels female offspring PND 28	Reduced at 500 (but not 1000)
Body weight female offspring PND 7–14	Reduced at 500
Body weight female offspring PND 21–50	Reduced at 1000
Body weight male offspring (all study)	Reduced at 500
Testes weight PND 16	Reduced at 750
Testes histology PND 16	Affected at 1000
Prostate weight PND 16	Reduced at 1000
Prostate histology PND 16	Affected at 750
Thyroid gland weight PND 16	Increased at 750
Liver weight PND 16	Increased at 750
Motor activity PNW 9 female	Reduced at 750
Motor activity PNW 17 female	Reduced at 1000
Motor activity adult male	Increased at 750 (but not 1000)
RAM performance male	Improved at 500 (but not 750)
Sperm count	Reduced at 500
Prostate weight adult	Reduced at 1000

Reduced growth is a well known consequence of developmental hypothyroidism (Akaike et al., 1991; Kobayashi et al., 2005; Gilbert and Sui, 2006; Noda et al., 2005). However in most studies of hypothyroidism, growth retardation is not observed until the offspring are about two weeks of age, and is primarily seen when offspring T_4 levels are very severely reduced. In the present study, female offspring body weights were diminished from birth until PND 50, while body weights in male OMC offspring were low throughout the study. These effects may therefore not have been a consequence of developmental hypothyroidism at all, but rather a direct effect of OMC exposure. This was probably also the case for the effects on liver weight, as alterations in liver weight generally suggest treatment-related induction of enzymes or peroxisome proliferation (Sellers et al., 2007). The increase in relative liver weight seen in the present study was comparable to the effects seen in a two-generation study (Schneider et al., 2005), where OMC exposure caused increased liver weights in adulthood in both the parental and the F1 generations. In that study the increased weights were accompanied by slight cytoplasmic eosinophilia, which was not seen in offspring on PND 16 in the current study.

The observed dose-dependent decrease in testosterone levels on PND 16 (Fig. 2) corresponded well with the reductions in relative testes and prostate weights, as well as with the histopathological changes seen in these organs. The gene expression data indicate that the lower prostate weights were not related to antagonism of the androgen receptor. This conclusion is reached as both PBP C3 and TRPM-2 are regulated by AR mediated pathways, and their expression did not change after exposure to OMC. The reduction in testosterone observed in the male pups at PND 16 was not due to effects on the expression of the investigated steroidogenic enzymes, as these were not altered by OMC.

In the adult animals, prostate weights remained low in the high dose group, and histopathological changes were seen in the two highest dose groups. The observed acinar atrophy, is a typical finding in rats exposed to estrogenic chemicals. Interestingly, histopathology of prostates was a more sensitive endpoint than prostate weight changes in the current study. Furthermore, OMC exposure lowered sperm counts in all the exposed groups (Fig. 5). In the Schneider et al. (2005) study, the total number of spermatids/g cauda epididymis in the F1 generation was also significantly reduced in animals receiving 1000 mg/kg bw/day. The authors inferred that the effects were caused by anomalously high control values, exceeding historical controls. In the present study, control values did not differ from historic controls and our findings therefore indicate that the lowered sperm counts are caused by developmental OMC exposure. This is a quite alarming result in relation to the low sperm counts and declining sperm quality in humans reported during the last decades (Carlsen et al., 1992; Jørgensen et al., 2006). No effect on sperm motility parameters was seen in the OMC exposed animals in the present study or in the Schneider et al. (2005) study. This indicates that OMC affects the number but not the function of the sperm cells.

Developmental exposure to another UV-filter 4-MBC, has also caused adverse effects on the male reproductive system, as decreased

testes weights in young animals and decreased prostate weights in adulthood have been seen (Schlumpf et al., 2008b). So far no investigations of sperm counts have been performed in rats developmentally exposed to 4-MBC or other UV-filters, so it is not clear whether they exert similar effects on sperm production.

The reproductive system of the female offspring was also affected by OMC exposure, as both estradiol and progesterone levels were significantly lowered in PND 28 offspring. We did not see any effects on uterine weight, an endpoint that has been affected in some previous studies (Schlumpf et al., 2001; Klammer et al., 2005; Seidlova-Wuttke et al., 2006). However in these studies the effects were observed in females still undergoing exposure to OMC. Whether OMC affected uterine weight in the present study would have been better assessed in the female offspring on PND 16 rather than on PND 28.

In the two-generation study by Schneider et al. (2005) no effect on uterine weight on PND 21 was reported. However, in this paper only results from significantly affected organs were shown, and these were defined as statistically significant changes in both absolute and relative weights, showing a dose–response relationship. If this was not the case with uterine weight, this might explain the differing results. Another explanation could be the use of different rat strains, as Schneider et al. (2005) used Wistar rats, while Sprague–Dawley and Long–Evans rats were used in the studies where an effect was seen (Schlumpf et al., 2001; Klammer et al., 2005; Seidlova-Wuttke et al., 2006) and furthermore studies of OVX females could be more sensitive to changes in uterine weight than studies in intact animals.

Endocrine disruption can also affect timing of sexual maturation, however this endpoint was not affected in the present study. Pre- and postnatal exposure to estrogenic chemicals usually delays sexual maturation in male offspring, and causes precocious sexual maturation in female offspring. These effects have been seen after estradiol treatment (Biegel et al., 1998; Rodriguez et al., 1993), and have also been seen in males exposed to the UV-filters 4-MBC and 3-BC. The UV-filter exposure did however not affect sexual maturation in the female offspring, even though it caused significant alterations in uterine and ovary weights (Schlumpf et al., 2008b).

OMC exposure also affected the neural development in the present study. A number of animal studies have shown that transient thyroid hormone insufficiency during development is associated with structural abnormalities in the brain (Auso et al., 2004; Sharlin et al., 2008; Gilbert and Sui, 2008). We have previously hypothesized that the degree of hypothyroxinemia can be directly correlated to auditory function, learning abilities and motor activity levels in the offspring (Axelstad et al., 2008). However, this was not the case in the present study. T_4 levels were significantly decreased in both dams and male offspring during OMC dosing, but most of the behavioral endpoints were affected differently than would have been expected after developmental hypothyroxinemia, and did not correlate to T_4 levels in dams or offspring. This can probably be explained by the fact that OMC is a chemical with many properties, including estrogenic, anti-thyroid and possibly more modes of action.

Based on previous studies, we expected to see hyperactivity in the adult offspring that suffered from hypothyroxinemia during early brain development (Axelstad et al., 2008; Kobayashi et al., 2005). Instead, motor activity levels were reduced in females, and no consistent effects were evident in males. Based on these results we would hypothesize that in rats, only severe postnatal reductions in T_4 levels lead to hyperactivity, while reductions in maternal T_4 levels are of less importance. Furthermore, a possible explanation for the observed effects on female activity levels could be a masculinisation of this sexually dimorphic behavior. Male rats are usually less active than females and in the present study activity levels in the high dose females resembled those of the control males. Perinatal exposure to exogenous estrogens can induce male-specific behaviors in females (Sharpe, 2010), and a masculinising effect on female behavior after developmental exposure to estrogenic compounds has been reported in a number of recent publications. Developmental estradiol exposure altered female saccharin preference and mating behavior in the male direction (Ryan et al., 2010) and developmental exposure to the UV-filters 4-MBC and 3-BC strongly impaired female sexual behavior (Schlumpf et al., 2008b). However, motor activity levels in general can also be interpreted much more globally than as a type of sexually dimorphic behavior, and since the changes in female activity levels did not correlate with the observed reductions in estradiol, a direct effect of OMC on female activity levels may be a more plausible explanation of the results.

In the radial arm maze we had expected to see impaired spatial learning abilities (Axelstad et al., 2008; Akaike et al., 1991; Noda et al., 2005). The observed improvements in learning and memory ability in the males are therefore difficult to explain based on the anti-thyroid properties of OMC. However, this type of polarization of behavior (i.e. a further masculinisation of male behavior) has been seen in other studies from our lab where males have been treated neonatally with endocrine disrupting chemicals such as flutamide, procymidone or vinclozolin (Hass et al., 2001; Christiansen, 2009). These chemicals are anti-androgens, but apparently result in effects on male maze learning which resemble the ones observed in the present OMC study.

The acoustic startle reaction was unaffected by OMC exposure. This is in accordance with findings after perinatal exposure to the PCB-mixture Arochlor 1254, which also reduced circulating levels of T_4 in dams, fetuses and offspring. However, startle testing on both PND 28 and 70 did not reveal treatment-related effects on the amplitude of the startle response, nor were any effects on habituation seen (Crofton et al., 2000). Two studies of PTU do report increased startle amplitude in adult animals neonatally exposed to PTU (Goldey et al., 1995; Kobayashi et al., 2005). In both studies, exposure reduced levels of T_4 massively and to a greater extent than Arochlor 1254. Provided that hypothyroxinemia is the cause of change in startle amplitude, postnatal levels of T_4 probably need to be severely reduced for effects on startle amplitude to appear.

In the hearing test, no differences were present between dosed and control animals, and again our data indicate that postnatal T_4 levels in the offspring are the determining factor. This is much in line with findings in the cross fostering study of Arochlor 1254 (Crofton et al., 2000). In this study, rat offspring were exposed to Arochlor via the mother either during gestation or during lactation. Offspring that were exposed solely during gestation did not present with hearing deficits, but so did lactationally exposed offspring. This is also consistent with the fact that the greater part of development of the hearing organ takes place during the first postnatal weeks in the rat (Crofton et al., 2000).

In summary, the present study addressed the potential endocrine disrupting properties of OMC on the developing reproductive and thyroid hormone systems, and investigated how changes in thyroid hormone levels would affect the neurological development of the offspring. We have shown that OMC possesses endocrine disrupting properties, as severely decreased T_4 levels were seen in exposed dams from all three dose groups. These changes did however not cause the expected behavioral effects in the offspring and no correlations with T_4 levels were seen. Our results indicate that in rats, only severe postnatal T_4 decreases are determining for adverse brain development. Furthermore, our working hypothesis that the degree of pre- and postnatal hypothyroxinemia correlates directly with reduced auditory function, decreased learning abilities and increased motor activity (Axelstad et al., 2008) was not corroborated in the present study, and is probably not suitable for all compounds that adversely affect the thyroid hormone system, especially if these have several modes of action. The same

conclusions were drawn by Miller et al. (2009) who investigated the effect of developmental PCB exposure on the thyroid hormone system, and found that if the compounds causing T_4 reductions also have other mechanisms of action, then changes in thyroid hormone levels are not necessarily causative of downstream neurotoxic outcomes (Miller et al., 2009).

Based on the present study only a LOAEL could be set for OMC, as adverse effects were seen on male body weights and sperm counts at all dose levels. Behavioral changes were less sensitive than changes in thyroid hormone levels, which indicate that setting a NOAEL for OMC based on reductions in thyroid hormone levels, will also be protective against behavioral effects caused by developmental hypothyroxinemia. Furthermore, our results show that behavioral testing of thyroid disruptors with multiple modes of action provides useful complementary information and contributes to a broader understanding of the toxicity of the tested chemicals, than studies that only take hormonal measurements and development of reproductive organs into account.

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Paper III

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Exposure to the Widely Used Fungicide Mancozeb Causes Thyroid Hormone Disruption in Rat Dams but No Behavioral Effects in the Offspring

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The widely used fungicide mancozeb has been shown to cause hypothyroxinemia and other adverse effects on the thyroid hormone system in adult experimental animals. In humans, hypothyroxinemia early in pregnancy is associated with adverse effects on the developing nervous system and can lead to impaired cognitive function and motor development in children. The aim of the present study was therefore to assess whether perinatal mancozeb exposure would cause developmental neurotoxicity in rats. Groups of 9–21 time-mated Wistar rats were dosed with 0, 50, 100, or 150 mg mancozeb/kg body weight (bw)/day by gavage from gestation day (GD) 7 to postnatal day (PND) 16, and total thyroxine (T_4) levels were measured in dams during gestation. On PND 16, hormone levels and several organ weights were measured in the offspring, whereas motor activity, startle response, and cognitive function were assessed in the adult offspring. The dose of 150 mg/kg/day caused neurotoxicity in the pregnant dams and was therefore reduced to 100 mg/kg bw/day in mid study. T_4 levels showed a dose-dependent and significant decrease in dams from all three dose groups on GD 15, whereas offspring T_4 levels, thyroid weights, and histology were unaffected on PND 16. No effects on reproductive organ weights were seen, and no behavioral changes were observed. Taken together, these results indicate that in rats, moderate maternal hypothyroxinemia during gestation does not necessarily lead to hyperactivity or reduced special learning abilities in the offspring. Mancozeb exposure did, however, reduce T_4 levels in dams and may therefore still be a potential contributor to thyroid disruption in humans and in result adversely affects the developing brain.

Key Words: **mancozeb; developmental neurotoxicity; behavior; rats; thyroid-disrupting chemicals.**

Even mild changes in human thyroidal function in early prenatal life can have severe consequences for a child's neurological development (Haddow *et al.*, 1999; Henrichs *et al.*, 2010;Kooistra *et al.*, 2006; Li *et al.*, 2010; Pop *et al.*, 1999, 2003). This knowledge has prompted much focus on the importance of thyroid-disrupting chemicals (TDCs) in relation to neurological development (Howdeshell, 2002; Porterfield, 2000; Zoeller, 2007; Zoeller and Crofton, 2000), as numerous chemicals have been shown to adversely affect thyroid function (Brucker-Davis, 1998; Hurley *et al.*, 1998). One group of TDCs are the fungicides from the dithiocarbamate family, which are widely used for protection of fruits, vegetables, and field crops from fungal diseases (WHO, 1988). The main degradation product of many of the dithiocarbamates is ethylene thiourea (ETU), a compound that exerts various toxic effects in rats, including thyroid neoplasms, developmental toxicity, and teratogenicity (NTP, 1992). The present study investigated the dithiocarbamate mancozeb, which is the most commonly sold fungicide in, e.g., Denmark (Danish EPA, 2010), Norway (Nordby *et al.*, 2005), and the United States (Acquavella *et al.*, 2003).

Mancozeb has been shown to cause adverse health effects in both humans and experimental animals. In a recent American agricultural health study, mancozeb exposure was strongly associated with increased incidence of thyroid disease in female spouses of pesticide applicators (Goldner *et al.*, 2010), and a Norwegian study has shown a moderate association between mancozeb exposure and neural tube defects in newborns from farmer families (Nordby *et al.*, 2005). In most of the published literature on the toxicological effects of mancozeb in rats, adult animals have been exposed to doses between 500 and 1500 mg/kg/day for longer periods of time. In these studies, mancozeb exerts numerous effects related to the function of the thyroid gland, including decreases in serum thyroxine (T_4) levels, thyroid peroxidase activity and iodine uptake, increased production of thyroid stimulating hormone (TSH) and thyroid weight, hyperplasia and hypotropy of follicular cells in the thyroid, as well as thyroid cancer (Hurley *et al.*, 1998; JMPR, 1993; Kackar *et al.*, 1997b; Trivedi *et al.*, 1993; WHO, 1988). Besides the thyrotoxic effects, reproductive effects in rats have been observed. These include decreased

ovary, testes, and epididymis weights, disrupted estrus cycles, and histopathological changes in the reproductive organs (Baligar and Kaliwal, 2001; Kackar *et al*., 1997a; Mahadevaswami *et al*., 2000). Furthermore, higher dose levels of mancozeb have caused general toxicity as well as neurotoxic effects in rats, including dyspnea, salivation, diarrhea, and paralysis of the hind limbs, followed by death of the animals (Kackar *et al*., 1997b; Trivedi *et al*., 1993; WHO, 1988).

In humans, risk of acute intoxication by high doses of mancozeb is small and is mainly a concern for agricultural and industrial workers, but the population at large can be exposed to mancozeb and other dithiocarbamates through residues in food (Rossi *et al*., 2006). Together with exposure from other TDCs, this could potentially contribute to disruption of the thyroid hormone system. In pregnant women, such a disruption could have severe consequences for the neural development of unborn children, as hypothyroxinemia in the first part of pregnancy is associated with adverse effects on cognitive function and motor development in infants, toddlers, and young children (Henrichs *et al*., 2010; Li *et al*., 2010; Pop *et al*., 1999, 2003). The aim of the present study was therefore to investigate if mancozeb exposure during the pre- and postnatal period would affect any reproductive, thyroid, or behavioral endpoints in rat offspring.

MATERIALS AND METHODS

Two separate mancozeb studies were performed. In both studies, time-mated Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were gavaged once daily from gestation day (GD) 7 to postnatal day (PND) 16 (day of delivery excluded). The dams were treated at a constant volume of 2 ml/kg body weight (bw)/day, with individual doses based on the bw of the animal on the day of dosing. The first study was a range-finding study with 24 time-mated dams ($n = 6$) and the second a larger dose-response study with 88 time-mated dams ($n = 22$). The studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee. In both studies, the animals received a complete rodent diet (Altromin Standard Diet 1314) and acidified tap water *ad libitum* and were housed under standard conditions as described in Axelstad *et al*. (2008).

The vehicle used was corn oil (Sigma-Aldrich, Brøndby, Denmark). Mancozeb (CAS no. 8018-01-7, Lot.no. 371-131c) was from VWR—Bie & Berntsen, Herlev, Denmark. The supplier did not have information about the compound's purity, only that it was a technical quality. The mancozeb solutions were kept dark, at room temperature, and continuously stirred during the dosing period, as the product was not fully soluble in corn oil. Fresh solutions were prepared for each of the three study blocks. No verification of dose concentrations was performed.

In the range-finding study, which was run in one block, the doses were 0, 200, 350, or 500 mg/kg bw/day. After a few days of dosing, severe weight loss and signs of neurotoxicity (paralysis of the hind limbs) were observed in animals from all mancozeb groups. Consequently, all doses were halved on GD 12. However, the observed toxic effects continued, so on GD 14 all animals from the two highest dose groups and two animals in the lowest dose group were sacrificed. Two dams from the control group were sacrificed on GD 14 for comparison. Before sacrifice, each dam was scored for severity of hind limb paralysis on a scale from 0 to 3. The remaining animals in the lowest dose group continued receiving 100 mg/kg bw/day from GD 14 to PND 16, at which point the study was terminated. This dose level did not cause further clinical signs of toxicity.

In the dose-response study, the plan was to dose the dams with 0, 50, 100, or 150 mg mancozeb/kg bw/day from GD 7 to PND 16. The study was divided into three blocks, with 1 week in-between, and an equal representation of each dose group in the blocks. After the first week of dosing, indications of toxic effects were observed in the highest dose group. Two of the animals suffered from severe weight loss and mild hind leg paralysis and were sacrificed on GD 16, and the highest mancozeb dose was reduced from 150 to 100 mg/kg bw/day. In consequence, the high-dose animals from the first block received 150 mg/kg from GD 7–21 and 100 mg/kg bw/day for the rest of the dosing period, whereas the animals in the second block only received 150 mg/kg bw/day from GD 7 to 14 and 100 mg/kg bw/day from GD 15 to PND 16. The animals from the first two blocks were nonetheless grouped when data analysis was performed. In the third block, dams from the high-dose group received 100 mg/kg during the whole treatment period and were therefore included as part of the middle dose group in the data analysis. Because of this alteration in dosing scheme, combined with a low pregnancy rate from the animal breeders, final group sizes differed considerably (Table 1).

The day after delivery, the pups were counted, sexed, weighed, checked for anomalies, and anogenital distance (AGD). Bw were measured again on PND 6, 13, 24, 31, and 45. Offspring were examined for the presence of nipples/areolas on PND 13 and for motor activity levels on PND 14, 17, and 23 (all procedures are further described in Axelstad *et al*., 2008). On PND 16, three male and two female pups per litter were sacrificed, when possible. They were weighed and decapitated, and testes, epididymis, ventral prostate, ovaries, liver, adrenals, and thyroid glands were excised, weighed, and prepared for histopathological examinations. On PND 24, 2–4 pups per litter were weaned and kept for assessment of developmental neurotoxicity, whereas the dams and one male per litter were sacrificed. In dams, the thyroids were excised, weighed, and prepared for histopathology and the number of implantation scars in the uterus was registered. In offspring, thyroid gland and testes were excised, weighed, and prepared for histopathology. All organs intended for histopathological examinations were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histological evaluations were made if statistically significant changes in organ weights were seen. Pup thyroids, testes, and ovaries on PND 16 were evaluated histologically, irrespectively of organ weight changes.

Several blood samples were collected for hormone analysis. On GD 15, dams were anesthetized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum (midazolam) and blood was drawn from the tail vein. From offspring on PND 16 and PND 24 and from dams on PND 24, trunk blood was collected. Total T_4 content in plasma was measured in all samples using modified Delfia T_4 kits (as described in Axelstad *et al*., 2008). Testosterone was extracted from male PND 16 serum as previously described (Vinggaard *et al*., 2005) and was measured by time-resolved fluorescence using commercially available fluoroimmunoassay kits (PerkinElmer Life Sciences, Turku, Finland).

The adult offspring (3–7 months old) were tested in a battery of behavioral tests. Experimental setup for testing of spatial learning in a radial arm maze and motor activity in activity boxes is described in Axelstad *et al*. (2008). Testing for acoustic startle response was performed as described in Kjaer *et al*. (2010), with some modifications, by use of the SR-Lab TM SDI startle response system (SanDiego Instruments, Inc., Europe). Specifically, no background noise was delivered in the two chambers during testing. After 5 min acclimatization, the test session commenced with five 120-dB(A) white noise startle trials for habituation followed by 100 test trials delivered in semirandomized order: 10 startle trials of 120 dB(A) white noise; 10 each of 8 prepulses (25, 30, 35, 40, 45, 50, 55, and 60 dB(A) white noise, respectively), and 10 trials with no stimuli. Movement of the tube was registered for 100 ms after onset of the startle stimulus and amplified, and the average response over 100 ms (AVG) was calculated. For each level of prepulse, AVGs were averaged and used for calculation of prepulse inhibition (PPI). PPI was expressed as percent reduction in AVG compared with the average of the 10 middle 120-dB startle trials: % PPI = 100 − [(AVG at prepulse + startle trial)/(AVG at startle trial) 100%].

Animals were weighed before each behavioral test. At 8 months of age, all offspring were anesthetized by CO_2/O_2 and decapitated. Ovaries and thyroid glands were excised, weighed, and prepared for histopathology.

TABLE 1
Pregnancy and Litter Data of Dams and Offspring Exposed to 0, 50, 100, Or 150/100 mg Mancozeb/kg bw/day from GD 7 to PND 16. Data Represent Group Means Based on Litter Means ± SDs. Asterisks Indicate a Statistically Significant Difference Compared with Controls * $p < 0.05$, *** $p < 0.001$

	Control	50 mg Mz	100 mg Mz	150/100 mg Mz
Dams and litters				
No. of litters	$n = 15$	$n = 19$	$n = 21$	$n = 9$
Dam bw gain, GD 7 to GD 21	75.47 ± 12.1	60.30 ± 14.9*	55.36 ± 20.2***	29.89 ± 20.4***
Dam bw gain, GD 7 to PND 1	9.93 ± 8.8	2.84 ± 9.0*	−4.62 ± 11.2***	−22.44 ± 13.1***
Dam bw gain, PND 1–17	36.93 ± 14.8	35.16 ± 15.6	39.70 ± 19.3	57.56 ± 20.7**
Gestation length (days)	22.60 ± 0.5	22.95 ± 0.4	22.82 ± 0.5	22.67 ± 0.5
% Postimplantation loss	8.79 ± 8.4	17.38 ± 21.3	10.85 ± 21.5	14.83 ± 30.6
% Perinatal loss	10.68 ± 10.1	21.29 ± 26.5	16.47 ± 28.1	14.83 ± 30.6
Litter size	11.33 ± 2.6	9.50 ± 4.5	10.26 ± 4.3	10.10 ± 3.8
% Perinatal deaths	2.1 ± 6.0	7.4 ± 23.2	6.2 ± 21.6	0.0 ± 0.0
% Males	47.1 ± 15.4	44.0 ± 16.9	46.0 ± 17.5	47.8 ± 12.1
Offspring				
Mean birth weight	5.84 ± 0.32	5.92 ± 0.557	5.65 ± 0.34	5.22 ± 0.33***
AGD males (mm)	3.43 ± 0.17	3.39 ± 0.21	3.43 ± 0.25	3.19 ± 0.12
AGI males (mm/cubic root bw)	1.89 ± 0.08	1.86 ± 0.09	1.91 ± 0.12	1.83 ± 0.05
AGD females (mm)	1.76 ± 0.10	1.72 ± 0.16	1.74 ± 0.08	1.70 ± 0.05
AGI females (mm/cubic root bw)	0.99 ± 0.06	0.96 ± 0.07	0.98 ± 0.04	0.99 ± 0.04
Nipples (areolas) males	0.48 ± 0.6	0.70 ± 1.1	0.49 ± 0.5	031 ± 0.5
Nipples (areolas) females	12.4 ± 0.4	12.3 ± 0.3	12.3 ± 0.3	12.4 ± 0.2
Mean bw PND 6	12.63 ± 1.3	12.08 ± 2.1	11.65 ± 1.1	11.10 ± 0.8*
Mean bw PND 13	25.26 ± 3.3	24.64 ± 5.0	23.03 ± 2.7	22.22 ± 1.7
Mean bw PND 24	59.42 ± 4.8	55.69 ± 7.5	55.48 ± 4.3	54.07 ± 2.6
Mean female bw day 31	87.6 ± 5.7	85.5 ± 9.3	83.3 ± 7.2	82.6 ± 3.3
Mean male bw day 31	97.8 ± 6.8	94.7 ± 10.5	92.6 ± 6.9	88.8 ± 5.3
Mean female bw day 45	138 ± 7	136 ± 13	134 ± 10	134 ± 8
Mean male bw day 45	179 ± 13	176 ± 20	177 ± 11	167 ± 7

Note. Mz, mancozeb.

Statistical analysis of data with normal distribution and homogeneity of variance were analyzed using ANOVA followed by a Dunnett *post hoc* test as described in Axelstad *et al*. (2008). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random, and nested factor in ANOVA or analyses were done on litter means. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a nonparametric Kruskall-Wallis test was used. Trend analysis on dose-response relations for hormone levels, body, and organ weights were performed using Spearman's test. Statistical analyses of the effects on macroscopic lesions and histopathology were done using Fisher's exact test.

RESULTS

The results from the two studies showed that doses of 150 mg/kg bw/day and above caused toxic effects in the dosed dams, as severe weight loss and transient paralysis of the hind limbs were observed. In the range-finding study, most dams were sacrificed on GD 14. At the time of necropsy, almost no food content was found in the ventricles and intestines of the sacrificed animals. The severity of hind limb paralysis was independent of pregnancy status but was dose dependent, as four out of six animals in the highest dose group received the most severe paralysis score, whereas two out of six animals in the middle dose group and none in the low-dose group received this score (data not shown). In the dams surviving after GD 14, maternal and pregnancy data, organ weights, and T_4 concentrations were not analyzed because of the very small final group size in dams giving birth ($n = 2$–3).

In the main dose-response study, the two high-dose dams that were sacrificed on GD 16 received the mildest paralysis score. In the rest of the animals, maternal bw gain from GD 7 to GD 21 was significantly lowered in the 50, 100, and 150/100 mg mancozeb/kg bw/day groups compared with controls ($p = 0.0119$, $p = 0.0009$, and $p < 0.0001$, respectively) (Table 1). Maternal weight gain from GD 7 to PND 1, i.e., adjusted for uterine content, was also significantly reduced in all three dose groups compared with controls ($p = 0.050$, $p = 0.0001$, $p < 0.0001$), and both endpoints showed a significant dose-dependent downward trend ($p < 0.0001$). During the lactation period, exposed dams generally gained more weight than controls, but the difference was only statistically significant in the high-dose group ($p = 0.007$). The additional weight gain was, however, not enough to eliminate differences in dam bw, and at the time of weaning (PND 24), high-dose dams had significantly lower bw than controls ($p = 0.031$) (Table 3), and trend analysis showed a dose-dependent decrease in dam bw

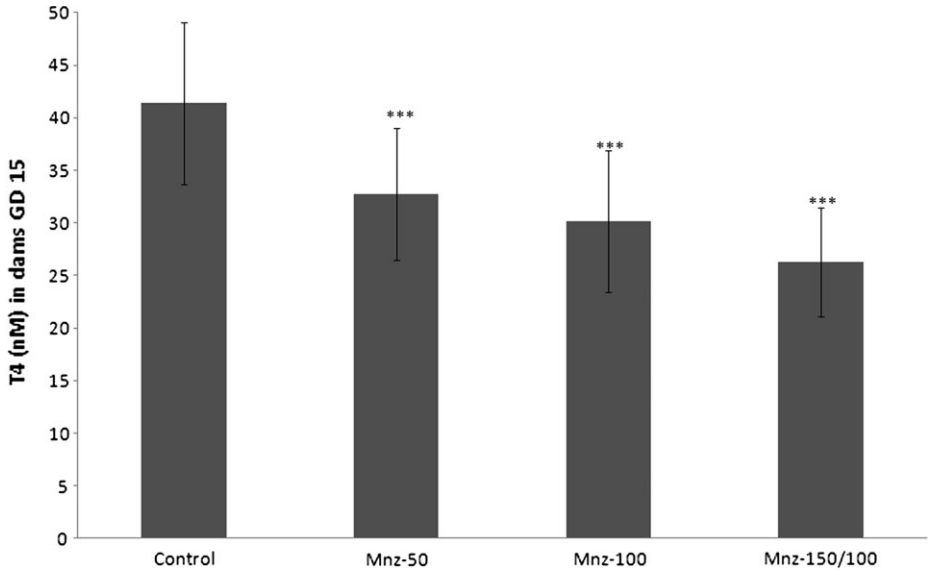

FIG. 1. T_4 levels (nanomolar) in dams on GD 15 after 1-week exposure to 0, 50, 100, or 150/100 mg mancozeb/kg/day. Data represent group means ± SDs, n = 9–21. Asterisks indicate a statistically significant difference compared with controls; ***$p < 0.0001$.

($p = 0.0134$). Gestation length, litter size, postimplantation loss, neonatal deaths, gender distribution, AGD, and nipple retention were similar in the four groups (Table 1). Offspring bw were significantly lowered in the high-dose group at birth ($p = 0.0004$) and on PND 6 ($p = 0.036$), with a significant dose-dependent downward trend on both days ($p = 0.0018$ and $p = 0.0077$, respectively). From PND 13 to 45, offspring bw in the highest dose group seemed lower than in controls, and the trend analysis showed a significant downward trend on days 13, 24, and 31 ($p = 0.0154$, $p = 0.0185$, and $p = 0.0059$, respectively); however, the difference between groups was not statistically significant (Table 1). From PND 45 and onward, no effect on bw was seen (data not shown).

On GD 15, a significant dose-dependent downward trend was seen for T_4 levels in dam serum ($p < 0.0001$) and the levels were decreased by 21, 27, and 37% in the three mancozeb groups, respectively, compared with controls ($p = 0.0004$, $p < 0.0001$, and $p < 0.0001$) (Fig. 1). This effect was no longer evident 1 week after dosing had stopped (PND 24) (Table 2), at which time also thyroid gland weights were unaffected (Table 3) and no dose-dependent trends were seen for any of the endpoints.

The offspring T_4 levels on PND 16 showed no dose-dependent trends, and group means did not differ significantly from controls in any dose group (Table 2). On PND 24, no significant differences between groups were seen on offspring T_4 levels (Table 2); however, a significant dose-dependent upward trend was seen ($p = 0.0242$), indicating a possible reactive overshoot because of recovery from previously compromised thyroid hormone status. Thyroid gland weights in the offspring were unaffected on PND 16 as no dose-dependent trends and no significant differences between groups were seen (Table 3). Also no histopathological effects on the offspring thyroids on PND 16 were seen (data not shown). On PND 24 (Table 3) and in adulthood (data not shown), offspring thyroid weights were also unaffected and therefore histopathology was not investigated.

TABLE 2

Effect of Mancozeb on T_4 and Testosterone Levels in All Dams and Offspring, after Exposure to 0, 50, 100, Or 150/100 mg Mancozeb/kg bw/day from GD 7 to PND 16. Data Represent Group Means ± SDs. Asterisks Indicate a Statistically Significant Difference Compared with Controls ***$p < 0.0001$

Hormone measurements (nM)	Control	50 mg Mz	100 mg Mz	150/100 mg Mz
No. of samples	14–15	15–20	17–22	8–10
T_4 in dams GD 15	41.4 ± 7.7	32.7 ± 6.3***	30.15 ± 6.7***	26.26 ± 5.2***
T_4 in dams PND 24	24.8 ± 12.9	21.2 ± 7.9	19.6 ± 7.8	23.24 ± 8.6
T_4 in offspring PND 16 (litter mean)	32.5 ± 7.5	35.8 ± 8.0	34.6 ± 5.4	33.1 ± 8.8
T_4 in offspring PND 24 (male)	18.5 ± 7.0	19.8 ± 7.6	22.1 ± 5.8	24.3 ± 6.2
Testosterone PND 16 (males)	1.05 ± 0.75	1.58 ± 1.70	1.54 ± 1.70	1.65 ± 1.40

Note. Mz, mancozeb.

TABLE 3

Effect of Mancozeb on Terminal Bw and Relative Organ Weights in Male and Female Rat Offspring Sacrificed at PND 16 and PND 24, after Exposure to 0, 50, 100, Or 150/100 mg Mancozeb/kg bw/day from GD 7 to PND 16. Data Represent Group Means Based on Litter Means ± SDs. Liver, Thyroid, and Adrenal Weights Are Litter Mean Values from Male and Female Offspring Combined. Asterisks Indicate a Statistically Significant Difference Compared with Controls *$p < 0.05$

	Control	50 mg Mz	100 mg Mz	150/100 mg Mz
Organ weights PND 16				
No. of litters	15	19	21	9
Bw (g)	30.60 ± 3.6	29.41 ± 6.4	28.06 ± 3.3	26.94 ± 2.0
Adrenal gland (mg/100 g bw)	33.59 ± 4.2	32.01 ± 5.1	32.14 ± 2.3	31.07 ± 2.5
Liver (mg/100 g bw)	2674 ± 116	2656 ± 63	2631 ± 117	2637 ± 130
Thyroid gland (mg/100 g bw)	16.36 ± 2.3	16.92 ± 2.4	16.83 ± 1.7	17.97 ± 1.7
Ovaries (mg/100 g bw)	43.39 ± 7.2	43.45 ± 7.3	44.85 ± 5.9	49.07 ± 5.2
Testes (mg/100 g bw)	353.3 ± 24.4	349.5 ± 24.5	350.5 ± 20.1	347.6 ± 11.8
Epididymis (mg/100 g bw)	97.94 ± 19.2	94.01 ± 22.6	88.06 ± 21.3	111.4 ± 26.2
Prostate (mg/100 g bw)	40.23 ± 11.6	44.83 ± 11.9	40.30 ± 6.5	46.91 ± 9.2
Organ weights PND 24				
Dam bw (g)	254 ± 17	250 ± 24	247 ± 14	234 ± 13*
Dam thyroid (mg/100 g bw)	6.52 ± 0.72	6.12 ± 0.85	6.57 ± 1.04	6.32 ± 0.99
Bw in male offspring (g)	61.43 ± 5.7	57.50 ± 12.7	55.20 ± 3.5	55.00 ± 4.3
Thyroid gland (mg/100 g bw)	13.04 ± 3.07	14.52 ± 3.31	13.18 ± 2.64	14.76 ± 5.74
Testes (mg/100 g bw)	569.9 ± 34.2	585.8 ± 71.96	573.5 ± 61.23	556.6 ± 54.64

Note. Mz, mancozeb.

On PND 16, weights of testes, epididymis, prostate, ovaries, and liver in the offspring were also unaffected by mancozeb exposure (Table 3). Statistical analysis of the relative organ weights and of absolute organ weights analyzed with bw as covariate indicated no differences between treatment groups, and no dose-dependent trends were seen. No histopathological effects were seen in the ovaries and testes of the offspring (data not shown), whereas histopathology of the other organs was not investigated. On PND 24, there were no effects on testes weights (Table 3), and in adult female offspring, there were no effects on ovary weights (data not shown).

None of the performed behavioral tests showed effects of mancozeb exposure, as neither activity levels in young or adult offspring, performance in the radial arm maze, or acoustic startle response were affected nor were any dose-dependent trends seen (data not shown).

DISCUSSION

The observed neurotoxic effects in the dams corresponded well with the toxicity effects generally seen after higher dithiocarbamate exposures (ataxia and paralysis of the hind limbs caused by demyelination and degeneration of peripheral nerve cells) (WHO, 1988); however, the dose levels were surprisingly low compared with doses causing adverse effects in other published studies. Dose-dependent weight loss and hind limb paralysis were observed in dams from all dose groups in the range-finding study. Furthermore, some of the animals dosed with 150 mg/kg bw/day in the dose-response study displayed clinical signs of intoxication and had to be sacrificed after 7 days of dosing.

In the published literature, similar toxic effects of mancozeb have been reported but at much higher dose levels (500–1500 mg/kg bw/day) and after longer dosing periods than seen in the present study (Kackar *et al*., 1997a,b; Trivedi *et al*., 1993). In these studies, mortality rates between 15 and 20% were seen in animals receiving 500 mg/kg bw/day for a year, whereas no signs of weight loss or any clinical signs of neurotoxicity were reported in female rats receiving between 500 and 800 mg mancozeb/kg bw/day for a month (Baligar and Kaliwal, 2001; Mahadevaswami *et al*., 2000). In contrast to these published findings, a number of unpublished industry studies of mancozeb exist. The results from these are used for risk assessment purposes and are referred in reports from the Joint Meeting on Pesticide Residues (JMPR, 1993). In these studies, the mancozeb doses used were generally much lower than seen in the published mancozeb literature, and the no adverse effect levels (NOAELs) for mancozeb were between 5 and 10 mg/kg bw/day, whereas lowest adverse effect level (LOAELs) were between 10 and 50 mg/kg bw/day. These were based on a number of short- and long-term toxicity, reproductive, and teratogenicity studies in rats, and the critical effects were most often bw reductions and decreased food consumption but also organ weight changes, histopathological findings (often in the thyroid), and altered hormone levels of T_4 and TSH (Gallo *et al*., 1980; Goldman *et al*., 1986; Hooks *et al*., 1992; Muller, 1992 in JMPR 1993; Stadler, 1990; Sundar, 1999; Tesh *et al*., 1988). Hind leg paralysis was reported in one study, where pregnant dams were dosed with 360 mg mancozeb/kg bw/day from GD 6 to 15 but was not seen in the 60 mg/kg bw/day dose (Tesh *et al*., 1988). Which other dose levels between 60 and 360 mg/kg/day would have caused this effect is speculative.

It is unclear why the dose levels inducing toxic effects in the published literature were so much higher than seen in the industry studies and in the present one. Many different rat strains have been used, and the animals have been dosed either by gavage or in the feed, but no consistency in sensitivity of specific strains or dosing method was evident from the data. A possible explanation could be the purity of the tested mancozeb. In all the unpublished studies, technical grade quality with purity between 82 and 86% was used. In the published literature, the mancozeb was often a commercial grade quality, with no stated purity, and it may therefore have differed from the technical grade quality either in the amount of mancozeb present or in the amount or types of impurities.

Mancozeb exposure reduced plasma T_4 levels in the dams in a dose-dependent manner, with the highest dose of 150/100 mg/kg bw/day causing a 37% decrease after 7 days of dosing. Because of the switch in dosing from 150 to 100 mg/kg bw/day, it was not possible to discern if effects seen in the highest dose group were caused by the short-term exposure to 150 mg mancozeb/kg bw/day or the overall dose produced by combined 150/100 mg/kg.

In some of the published literature, T_4 reductions around 50% were first seen after either 2 months or 1 year of dosing with 1500 mg mancozeb/kg bw/day (Kackar *et al*., 1997b; Trivedi *et al*., 1993). However, in a more recent study, mancozeb exposure induced reductions in T_4 levels comparable to those of the present study, as a 50% reduction in T_4 levels was seen after 4 days of dosing with 250 mg mancozeb/kg bw/day. Effects on body and other general toxicity effects were not seen in this study, even at quite high-dose levels (250, 500, and 1000 mg/kg/day), but this was probably because of the relatively short dosing period of 4 days (Flippin *et al*., 2009). In the mancozeb studies referred in the JMPR report, LOAELs for T_4 reductions in two long-term toxicity studies were between 450 and 750 ppm mancozeb in the diet (equal to approximately 20–30 mg/kg bw/day) (Hooks *et al*., 1992; Stadler, 1990), which supports our findings of effects in the dams at 50 mg/kg bw/day.

In contrast to the many studies reporting increased thyroid weights (Hurley *et al*., 1998; JMPR, 1993; Kackar *et al*., 1997b; Trivedi *et al*., 1993; WHO, 1988), no effect on thyroid weight was seen in dams from the present study when measured on PND 24. However, at this time, dosing had already been discontinued for 7 days, and an effect on the maternal thyroids might therefore have been reversed.

Interestingly, offspring levels of T_4 were not significantly lowered in any dose group compared with controls when measured on PND 16. It is, however, unclear whether the thyroid hormone system of the offspring was unaffected by mancozeb during the entire dosing period. The fact that a significant dose-dependent upward trend was seen in offspring T_4 levels on PND 24 may indicate a possible reactive overshoot because of recovery from previously compromised thyroid hormone status. Therefore, measurements of T_4 levels during fetal or early postnatal life might have revealed other results than seen on PND 16. Such a pattern in postnatal T_4 levels can be seen in a recent publication from Paul *et al*. (2010). Here the TDC triclosan was given to rat dams during gestation and lactation, and whereas offspring T_4 levels were reduced by 30% on PND 4, no significant effects were seen on PND 14 and PND 21. According to the literature, the thiocarbamates and their metabolite ETU can cross the placenta and are found in breast milk (Brucker-Davis, 1998). It is, however, possible that the mancozeb did not cross the placenta or pass to the milk in sufficient amounts to affect the thyroid status of the offspring. Possibly, toxicokinetic factors may have affected maternal transfer of mancozeb into milk and thereby limited lactation exposure to the pups. Alternatively, mancozeb may not have triggered the same toxicodynamic effects in the offspring during the lactation period as in exposed dams. Measurements of maternal and offspring T_4 levels during the early postnatal period would have been very helpful in discerning whether dams were more susceptible to the thyroid-disrupting effect of mancozeb than the offspring and for identifying whether the offspring's thyroid system was affected at any time point during the dosing period. In future studies, measurements of mancozeb and its metabolites in milk, urine, or blood samples from the offspring would be helpful in order to learn more about transfer of mancozeb from dams to the offspring and to compare the exposure to human levels.

Developmental hypothyroidism in rats is known to reduce growth from the age of approximately 2–3 weeks and onward (Akaike *et al*., 1991; Kobayashi *et al*., 2005; Noda *et al*., 2005). In the present study, high-dose offspring were only significantly smaller than controls at birth and on PND 6; however, significant dose-dependent trends were seen on days 13, 24, and 31, and it is therefore unclear whether the weight reduction in the offspring was a consequence of developmental hypothyroxinemia or a direct effect of mancozeb, possibly related to the lower weight gain seen in the high-dose dams.

AGD, nipple retention, testosterone levels, reproductive organ weights, and histology on PND 16 were not affected by mancozeb, indicating that the perinatal exposure did not affect reproductive development. Previously, adverse effects on the reproductive system have been reported in the published literature, as treatment of adult male rats with mancozeb has caused adverse effects on testes and epididymis weights and histology (Kackar *et al*., 1997a). However, the effects were only seen after long-term exposure (180–360 days) to very high doses of mancozeb (1000–1500 mg/kg bw/day). Treatment of adult female Wistar rats with 500, 600, 700, or 800 mg mancozeb/kg bw/day for 15 and 30 days, respectively, affected the female reproductive system in the two highest dose groups. Effects included decreased ovarian weight, number of healthy follicles and estrous cycles, prolonged diestrus phase, and increased number of atretic follicles (Baligar and Kaliwal, 2001; Mahadevaswami *et al*., 2000). However, in these studies, no general toxicity effects were seen, which makes it difficult to compare the dose levels directly to the present ones. In the reproductive and developmental studies

referred in the JMPR, NOEALs for reproduction were set much higher than for general toxicity effects, e.g., body changes and adverse effects on the thyroid hormone system. As described earlier, because of toxicokinetic and toxicodynamic effects, the mancozeb may not have passed to the offspring in sufficiently high amounts to affect their reproductive development. Furthermore, many of the endpoints affected in previous studies like estrous cyclicity, weight, and histology of reproductive organs in adult animals (that were still being dosed) were not investigated in the present study.

None of the investigated behaviors were affected by mancozeb exposure. Based on a large body of published literature on this subject, as well as on a developmental neurotoxicity study of the thyrotoxic compound propyl thiouracil from our own group, we had expected developmental hypothyroxinemia to cause elevated activity levels and impaired maze performances in the adult rat offspring (Akaike *et al*., 1991; Axelstad *et al*., 2008; Goldey *et al*., 1995; Kobayashi *et al*., 2005; Noda *et al*., 2005). Furthermore, we have previously hypothesized that the degree of pre- and postnatal hypothyroxinemia could be directly correlated to changes in these behaviors (Axelstad *et al*., 2008), in a similar way to what was proposed by Crofton (2004) for hearing ability. This hypothesis was, however, not corroborated in a later study from our group (Axelstad *et al*., 2011). Here exposure to the UV-filter octyl methoxycinnamate during gestation and lactation caused severe hypothyroxinemia in dosed dams, during both gestation and lactation, but only small effects on offspring T_4 levels on PND 16 and no hyperactivity or impaired maze learning (Axelstad *et al*., 2011). We therefore hypothesized that in rats, T_4 levels in the offspring needed to be severely reduced postnatally for effects on activity and learning abilities to appear. The results from the present study corroborate this hypothesis, as moderate maternal hypothyroxinemia during early gestation did not affect any of the investigated behaviors in the offspring.

There are, however, other studies which have shown effects on neural development in rat offspring after short-term maternal hypothyroxinemia (Auso *et al*., 2004; Opazo *et al*., 2008), so more research in this area is needed in order to elaborate if maternal hypothyroxinemia during gestation in rats is enough to adversely affect the behavior of the developing offspring.

It is, however, quite clear that in humans maternal T_4 levels during the first trimester are crucial for fetal brain development, and even slight maternal hypothyroxinemia can result in adverse effects on the child's cognitive and motor function (Henrichs *et al*., 2010; Kooistra *et al*., 2006; Li *et al*., 2010; Pop *et al*., 1999, 2003). This means that even though developmental mancozeb exposure in the present study did not affect the investigated behaviors in the rat offspring, this should not lead to the conclusion that in humans mancozeb exposure is without risk for pregnant women and their fetuses. In the present study, 8 days of mancozeb exposure was enough to reduce T_4 levels significantly in all dose groups. The LOAEL of 50 mg/kg bw/day for T_4 decreases was much lower than previously reported in the published literature but corroborated the effects seen in industry studies quite well. Because humans are exposed to a variety of thyroid disrupters, all probably acting in a dose-additive manner (Flippin *et al*., 2009), mancozeb residues in food should be carefully monitored in order to protect pregnant women and their children from excessive exposure to TDCs.

In summary, the present study investigated the effects of developmental mancozeb exposure on thyroid hormone levels, reproductive, and neurological development of the offspring. We have shown that mancozeb exposure induced neurotoxicity in dams at and above 150 mg/kg bw/day. The observed changes in maternal T_4 levels did not cause any behavioral effects in the offspring, and we therefore hypothesize that in rats, moderate prenatal T_4 decreases in dams are not determining for adverse development of learning and motor skills in the offspring. However, 8 days of mancozeb exposure was enough to cause between 20 and 40% decreases in T_4 levels in rat dams, which indicates that mancozeb exposure may be a potential contributor to thyroid disruption in humans.

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