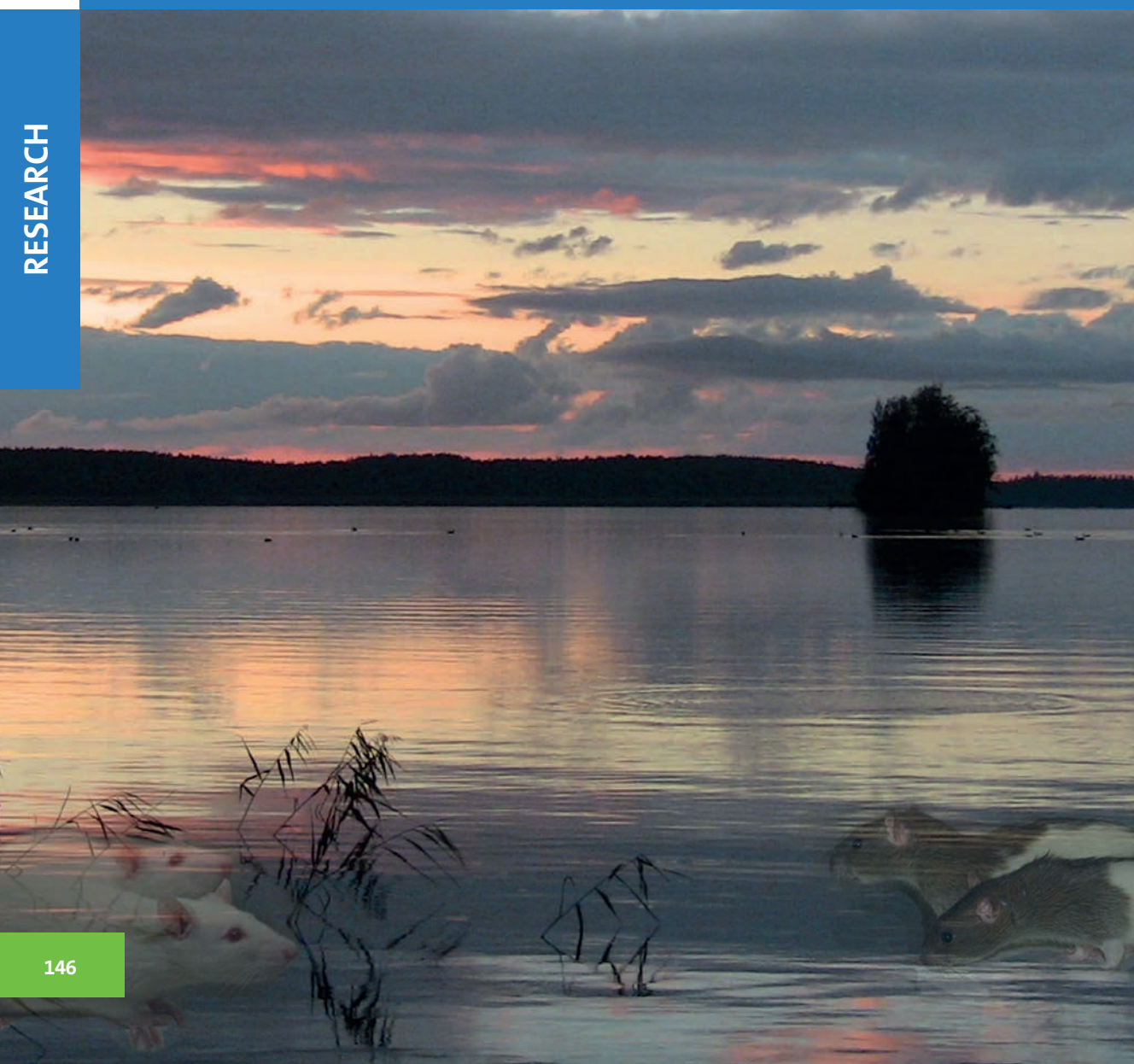
NATIONAL INSTITUTE FOR HEALTH AND WELFARE

Sanna Lensu

No Chocolate, Please!

Dioxin-Induced Responses in Feeding Related Behaviour and in Neuronal Activity

RESEARCH



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RESEARCH 146 • 2014

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ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Health Sciences of the University of Eastern Finland for public examination in Canthia Auditorium CA102 (L3), Yliopistonranta 1C, on December 19th 2014, at 12 at noon.

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Department of Environmental Health, Kuopio, Finland
and
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FOREWORD

”Department of Environmental Health at the National Public Health Institute (KTL), and its Laboratory of Toxicology started in Kuopio in the year 1983. In that laboratory, Raimo Pohjanvirta and Jouko Tuomisto made an interesting finding of a 1000-fold sensitivity difference in several forms of dioxin toxicity between outbred Han/Wistar and inbred Long-Evans rat in 1980s. Since then, this animal model has been utilised in many ways to enhance the knowledge of complicated mechanisms of dioxin toxicity in the highly complicated physiological organisms. Toxicity studies have made an enormous contribution for the sound and sophisticated health risk assessment of dioxins and of other agents that surround us in everyday life, such as many chemical compounds in food and water, cosmetics, drugs, and toys. In mechanistic toxicology and in many other fields of science animal experiments are esssential. While recognising the importance of “real-life” studies, we have to recognise that animals are living creatures, and their well-being and humane treatment are essential. This is even necessary to ensure good quality research results. This appreciation has been an important part of the working philosophy.

One circle closes with this thesis. It appears to be the last in the series of published dissertations in the THL (previously KTL), and the last using the differently dioxin-responsive rats in the now closed animal unit in the Laboratory of Toxicology. We are living in the world where the winds of change are continuously blowing. Despite any change in our society, in research organisations and funding agencies with their funding policies, I wish there will always remain wisdom to acknowledge the importance of toxicological research.”

Han/Wistar rats Long-Evans rats

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“What all agree upon is probably right; what no two agree in most probably is wrong.”

-Thomas Jefferson, 1817

Dedicated to my beloved ones

Abstract

Sanna Lensu. No Chocolate, Please! Dioxin-Induced Responses in Feeding Related Behaviour and in Neuronal Activity. National Institute for Health and Welfare (THL). Research 146. 185 pages. Kuopio, Finland 2014.
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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of a family of persistent organic pollutants called dioxins. These chemicals are highly toxic (at pico- and nanogram level), stable, and resistant to biological degradation in the environment. Human exposure to these compounds occurs mainly via ingestion of meat, fish and dairy products, as dioxins accumulate in the food chain. Since they have extremely long elimination halflives in humans their levels in adipose tissue increase throughout the lifespan. A key mechanism in mediating the effects of dioxin is binding to the intracellular aryl hydrocarbon receptor (AHR).

AHR is an ancient protein involved in many biological processes such as in several stages during development, but also in angiogenesis, in circadian clockwork, apoptosis, immune system function, and in metabolism – e.g. xenobiotic and fat metabolism. When activated by dioxins AHR participates in many cellular events. In the mice lacking AHR (AHR-knockout mice), dioxin does not exert any effects; in other living organisms the effects of dioxin are strongly dependent on dose, animal species and strain, gender and developmental stage, and observation time. In humans, chronic exposure to these endocrine disrupting chemicals has been postulated to play a role in the current obesity epidemic, and other metabolic disorders such as type II diabetes. In laboratory animals, one of the striking, dose-dependent effects of acute TCDD exposure is weight loss due to severely diminished food intake.

In this thesis, TCDD induced feeding related behavioural effects were studied in detail and the brain activated areas were screened. Experiments utilised rodent models with different AHR structures or expression levels and thus, with different sensitivities to TCDD lethality. At present, the most sensitive effects of dioxins are seen in the developing fetus, but here it was showed that in adult animals, the aversive response to a novel food item offered in close temporal proximity with TCDD, was an extremely sensitive, rapid, and longlasting response. This phenomenom appeared at equally low dose levels in all animals, independent of their sensitivity to dioxin lethality. The aversive response was missing in AHR-knockout mice. Thus, it seems that the housekeeping role of AHR is extended to behaviour: activation of AHR leads to rejection of such foods which appear as harmful by inducing metabolism, and thereby AHR activation enhances survival. Furthermore, by developing an automated system to follow ingestive patterns on a minute-to-minute basis, the diminished food intake was characterised in detail in sensitive Long-Evans and in resistant Han-Wistar rats. These studies revealed different feeding and drinking behavioural patterns over the day, with and without TCDD, evidence that there is interplay between the AHR and the circadian clockwork in the regulation of feeding. Regulatory pathways of these effects in brain and in periphery deserve further investigation.

The results implicate that TCDD induces two different feeding responses which both seem to be mediated by the AHR, but which occur at widely different doses. The one is related with the regulation of normal food intake and the other is related with the aversive response. The latter of these two is extremely sensitive occurring at the doses that do not affect normal food intake of animals. Physiological role of the AHR in mediating the aversive response is an interesting novel finding, and might be of importance for the development of medical treatment of anorectic diseases, such as cancer cachexia.

Keywords: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, TCDD; dioxin; feeding; drinking; behavioural patterns; aversive behaviour; neophobia; Aryl hydrocarbon receptor, AHR; rat; mouse

Tiivistelmä

Sanna Lensu. Ei suklaata, kiitos! Dioksiinialtistuksen vaikutuksia syömis- ja juomiskäyttäytymiseen sekä neuronaaliseen aktiivisuuteen. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 146. 185 sivua. Kuopio, Finland 2014.
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Dioksiinit ovat erittäin pysyviä ja laajalti levinneitä kemikaalisaasteita, joita ympäristössämme on käytännössä kaikkialla. Dioksiineja syntyy mm. epätäydellisen palamisen seurauksena polttoprosesseissa. Ihmiset altistuvat näille haitallisille klooriyhdisteille pääasiassa ruuan välityksellä, koska dioksiinit ovat rasvaliukoisia ja niin ollen kertyvät ravintoketjuissa. Ihmisen elimistöstä poistumisen pitkä, vuosia kestävä puoliintumisaika aiheuttaa sen, että dioksiinikertymä lisääntyy koko eliniän ajan. Haittavaikutukset riippuvat altistumistavasta, -annoksesta ja yksilöllisistä tekijöistä, mutta haitallisten vaikutusten syntymiseen riittävät erittäin pienet annokset dioksiinia (pg ja ng, milligramman miljoonasosat). Tyypillistä on, että eri lajien ja eläinkantojen välillä on suuria herkkyyseroja, ja nämä herkkyyserot ovat riippuvia myös toksisesta tai biologisesta vasteesta. Dioksiininkaltaisia kemikaaleja on useita, mutta kaikkein myrkyllisin on 2,3,7,8-tetraklooridibentso-*p*-dioksiini (TCDD).
Suurin osa dioksiinien biologisista vaikutuksista välittyy aryylihiilivetyreseptorin (AHR) välityksellä. Jos eläimillä ei ole toimivaa AH-reseptoria, ne eivät reagoi dioksiinialtistukselle juuri lainkaan. Lisäksi aiemmissa tutkimuksissa on havaittu, että koe-eläinten *Ahr* geenin ja toistaiseksi tuntemattoman *B*-geenin genotyypit vaikuttavat TCDD-herkkyyteen. AH-reseptorilla on myös fysiologisia tehtäviä elimistössä, kuten esimerkiksi vuorokausirytmien säätelyyn osallistuminen.
Yksi dioksiinialtistuksen vaikutuksista on näivettyminen: eläimet lakkaavat pikkuhiljaa syömästä, laihtuvat ja näivettyvät pois. Näivettyminen johtaa TCDD-annoksesta ja eläimen herkkyydestä riippuen kuolemaan, mutta yhä edelleen varsinainen akuutti kuolinsyy on tuntematon. Mikäli annos ei ole tappava, joidenkin päivien kuluttua eläimet ryhtyvät syömään, mutta niiden paino jää pysyvästi kontrollieläimiä matalammaksi. Nämä TCDD-altistuksen aiheuttamat syömisvaikutukset ovat tyypillisesti sellaisia, että niissä voidaan nähdä suuria herkkyyseroja riippuen eläimen lajista ja genotyypistä.
Tässä kokeellisessa työssä käytettiin malliaineena TCDD:tä tutkittaessa yhden kerta-altistuksen vaikutuksia koe-eläinten syömis- ja juomiskäyttäytymiseen ja solujen aktivaatioon aivoissa. Työssä käytettiin eri tavalla TCDD:lle herkkiä eläimiä: resistenttejä Han/Wistar (H/W) rottia, herkkiä Long-Evans (L-E) rottia ja niiden risteytyksiä sekä hiiriä. Hiiret olivat normaaleja villityypin C57BL/6-hiiriä tai C57BL/6-hiiriä, joilta *Ahr*-geeni oli poistettu. Tutkimuksessa kävi ilmi, että *Ahr*:n genotyypistä riippuen syömiskäyttäytyminen oli hyvin erilaista niin altistamattomilla kuin TCDD-altistetuilla eläimillä. Eroja oli mm. syömisaktiivisuuden vuorokausirytmissä ja aterioiden tiheydessä ja koossa. Työssä tutkittiin myös makuaversiota eli uuden ruoka-aineen välttämistä TCDD-altistuksen jälkeen. Tähän asti on oletettu, että TCDD-altistuksen aiheuttamat akuutit, nopeasti ilmenevät vaikutukset rajoittuvat solu- ja molekyylitasolle. Työssä selvisi, että

TCDD:n aiheuttama makuaversio on erittäin herkkä, nopea, ja kauan säilyvä biologinen vaste eikä se riipu eläimen herkkyydestä TCDD:n letaalisuudelle. Erityisen voimakkaan aversion tuotti suklaa, kun sitä tarjottiin eläimille samaan aikaan dioksiinialtistuksen kanssa uutena ruoka-aineena. Työssä selvitettiin myös mitkä aivoalueet aktivoituvat dioksiinialtistuksen jälkeen. Histologisen työn löydökset eivät kuitenkaan selitä käyttäytymisessä havaittuja ilmiöitä, ja näin ollen TCDD:n akuutit vaikutukset ruokahalun säätelyyn vaativat lisätutkimuksia.

Tämän väitöstyön merkittävä löydös oli se, että dioksiinialtistuksella havaittiin olevan kaksi erityyppistä vaikutusta syömiseen, ja nämä vasteet ilmenevät hyvin erilaisilla annostasoilla ja eri tavoin tutkituissa eläimissä. Toinen ilmiöistä liittyy ruokahalun säätelyyn: annos-vasteiseen syömisen vähenemiseen ja joko kuolemaan tai pysyvästi alentuneesen ruumiin painoon. Makuaversio oli hyvin herkkä vaste kaikissa tutkituissa eläimissä eivätkä tutkimuksessa käytetyt TCDD-annokset vaikuttaneet eläinten normaaliin ruokahaluun lainkaan. Makuaversion tiedetään liittyvän paitsi maistamiseen ja syömiseen, myös oppimiseen ja muistiin olennaisella tavalla. Keskushermosto on tärkeä tutkimuskohde sekä ruokahalun säätelyssä että muistissa ja oppimisessa, ja yksi tulevaisuuden haaste onkin selvittää ympäristömyrkky TCDD:n vaikutuksia aivoissa nykyistä tarkemmin.

Nämä tutkimukset antavat myös uusia viitteitä AH-reseptorin fysiologisista tehtävistä elimistössä. Tulosten perusteella vaikuttaa siltä, että AH-reseptori osallistuu syömisen säätelyyn, joko suoralla vaikutusmekanismilla tai esimerkiksi vuorokausirytmin säätelyn välityksellä. Toisaalta havaittiin, että hyvinkin pienillä TCDD-annoksilla tapahtuva AH-reseptorin aktivoituminen johtaa sellaisen ruuan välttämiseen, jota tarjottiin uutena ja yhtä aikaa altistuksen kanssa. Koska monet AHR:ää aktivoivat haitalliset aineet joutuvat elimistöön ruuan välityksellä, voidaan olettaa, että AHR:n aktivoituminen matalilla annostasoilla johtaa sellaisen ruuan välttämiseen, joka on aiheuttanut AHR:n aktivoitumisen. Toisin sanoen löydökset viittaavat siihen, että AHR:llä olisi myös suojaavia vaikutuksia: kun ruuan mukana tulleet aineet saavat aikaan AHR:n aktivoitumisen, kyseistä ruokaa ei enää syödä kertamaistamisen jälkeen. Tällä havainnolla voi olla merkitystä kehitettäessä lääkehoitoja, joilla voidaan parantaa monien sairauksien (esim. syöpä) yhteydessä ilmenevää heikentynyttä ruokahalua.

Avainsanat: 2,3,7,8-Tetraklooridibentso-*p*-dioksiini, TCDD; dioksiini; syöminen; juominen; aversiokäyttäytyminen, välttämiskäyttäytyminen; neofobia, uuden ruoka-aineen välttäminen; aryylihiilivetyreseptori, AHR; rotta; hiiri

Contents

List of original papers

I Lensu S., Tuomisto J.T., Tuomisto J., Viluksela M., Niittynen M., Pohjanvirta R. Immediate and highly sensitive aversion response to a novel food item linked to AH receptor stimulation. Toxicology Letters. 2011, 203 (3): 252-257.

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III Lensu S., Tiittanen P., Pohjanvirta R. Circadian differences between two strains in their feeding and drinking micro- and macrostructures. Biological Rhythm Research. 2011, 42 (5): 385 - 405.

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Abbreviations

AHR	Aryl hydrocarbon receptor (protein), AH receptor
Ahr	The gene encoding AHR
$Ahr^{-/-}$	AHR knock out (AHRKO); both alleles of *Ahr* are inactivated
$Ahr^{b/b}$	Allele of *Ahr* locus; high affinity allele; found in TCDD-responsive C57BL/6JKuo mice
$Ahr^{hw/hw}$	Han-Wistar –type allele of *Ahr* locus; related with resistance to acute toxicity and found in TCDD-resistant H/W rats and in line A rats
$Ahr^{wt/wt}$	Wild-type allele of *Ahr* locus; found in most rat strains sensitive to toxicity of TCDD, e.g. present in L-E rats
AHRR	AHR repressor; inhibits AHR function by insufficiently elucidated mehanisms
Arc	Nucleus arcuatus of hypothalamus in the brain
ARNT	AHR nuclear translocator; dimerizes with AHR and this heterodimer binds to DNA
B	An unknown gene B that affects TCDD-sensitivity
$B^{hw/hw}$	Han-Wistar –type allele of unknown B gene that affects TCDD-resistance; expressed in H/W rats (resistant) and in line B rats (moderately resistant to toxicity of TCDD)
BBB	Blood-brain barrier
bHLH/PAS	Basic helix-loop-helix /PAS [homologous region found in proteins period (PER), ARNT and single-minded (SIM)]: AHR belongs to this family of transcriptional regulators
BMAL-1	Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein 1, positive circadian rhythm regulator
CD36	Cluster of Differentiation 36, fatty acid translocase

CEA	Central nucleus of amygdala
c-Fos	Protein product of immediate early gene *c-Fos*; used as an early marker of cellular activity
clock, clockwork	General term for circadian regulation
CLOCK	Circadian locomotor cycles kaput, positive circadian rhythm regulator
Cry	Cryptochrome, repressive protein in circadian regulation
CS	Conditioned stimulus
CTA	Conditioned taste aversion
CYP	Cytochrome P450: xenobiotic metabolising enzymes – members of a CYP superfamily
C57BL/6JKuo	Mouse strain (bred in Kuopio); sensitive to the effects of dioxin
DBA/2	Mouse strain resistant to the dioxin effects
DRE	Dioxin (xenobiotic) responsive element in DNA: a specific sequence of DNA that binds the AHR-ARNT complex
ED_{50}	Effective dose 50%: the dose that is estimated to give 50% of the maximal response or the dose that achieves a response in 50% of dosed animals
efficacy	Intrinsic activity; ability to activate the receptor or magnitude of an effect; capacity of a compound to produce an effect
EROD	Ethoxyresorufin-*O*-deethylase; EROD activity is almost exclusively due to the enzyme CYP1A and therefore can be used as a tool to measure CYP1A-enzyme activity
FFA	Free fatty acid
GI	Gastrointestinal
GLP-1	Glucagon-like-peptide

H/W	Han/Wistar (*Kuopio*) rat strain; originally inbred but currently random bred strain; resistant to most effects of TCDD
IC	Insular cortex
i.g.	Intragastric administration
i.p.	Intraperitoneal administration
LD_{50}	Lethal dose 50%: the dose that is estimated to kill 50% of animals in an acute toxicity test
L-E	Long Evans (*Turku /AB*) rat strain; sensitive to most effects of TCDD
Line A rat	A cross-bred rat line (H/W x L-E); assumed genotype $Ahr^{hw/hw}$ $B^{wt/wt}$; very resistant to most effects of TCDD
Line B rat	A cross-bred rat line (H/W x L-E); assumed genotype $Ahr^{wt/wt}$ $B^{hw/hw}$; moderately resistant to most effects of TCDD
Line C rat	A cross-bred rat line (H/W x L-E); assumed genotype $Ahr^{wt/wt}$ $B^{wt/wt}$; sensitive to most effects of TCDD
NAcc	Nucleus accumbems
NBM	Nucleus basalis magnocellularis in the brain
NPY	Neuropeptide Y
NTS	Nucleus tractus solitarius
PAH	Polycyclic aromatic hydrocarbon
PaVN	Paraventricular nucleus of hypothalamus
PBN	Parabrachial nucleus in the brain
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran

Per	Period, repressive protein involved in circadian regulation
potency	A measure of the concentration or the dose eliciting an effect; affinity to a receptor – the higher potency the lower concentrations of a compound needed to occupy the receptor
SCN	Suprachiasmatic nucleus of the brain
SIRT1	Silent mating type information regulation 2 homolog 1; member of the sirtuin family of proteins that function as NAD^+-dependent protein deacetylases or ADP-ribosyltransferases
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin: the most potent congener of dioxins and therefore often used as a reference compound of dioxins
TEF	Toxic equivalence factor; factor used to compare dioxin-like compounds according to their potency to induce effects in relation to TCDD (whose TEF-value = 1)
US	Unconditioned stimulus, stimulus provoking e.g. GI-illness
VMH	Ventromedial hypothalamic nucleus of the brain

1 Introduction

Each of us must eat and breathe to stay alive. In addition to compounds essential for life, foodstuffs and the inhaled air contain unwanted products such as environmental contaminants. In many respects, archetypes of these agents are dioxins, which are widely distributed and stable in our environment.

Dioxins and other dioxin-like halogenated hydrocarbons are chemicals that strike fear into the general public. They are believed to cause harmful effects, even cancer: The International Association for Research on Cancer has classified the most potent compound of the dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as a human carcinogen (group 1; [1]) at extremely low amounts. Widespread public concerns about the presence of DDT and other environmental persistent contaminants were raised by Rachel Carson who published the book 'Silent Sping' at 1962. Since then, efforts to reduce environmental emissions of harmful agents including dioxin-like compounds have resulted in a lowering of concentrations in humans. The amounts of these chemicals to which humans can be exposed is regulated by the authorities; in the United States by the Environmental Protection Agency since 1985 [2]. Nevertheless, despite of all the efforts to limit the intake of dioxins, these compounds have attracted extensive public attention regularly and tragic cases of food contaminations or even deliberate poisonings have been news headlines throughout the world. The latest case emerged in Europe during January 2011, when animal feed in Germany was found to have been contaminated with dioxins.

The risk assessment of dioxins is challenging due to the strong dependence of the biological responses on many factors such as species, strain, age or developmental stage, sex, or tissue [3, 4]. The knowledge of these compounds has emerged from detailed mechanistical and epidemiological studies. For the latter, an important source of information has been studies conducted after the explosion at a chemical factory in Seveso 1976. One crucial initial finding was an appreciation of the key mechanism of action: practically all of the biological effects of these compounds: involve their binding to the intracellular aryl hydrocarbon receptor (AHR) [5, 6]. This is currently one cornerstone of dioxin risk assessment. AHR is phylogenetically a highly conserved protein and an important mediator of xenobiotic responses. However, a variety of pysiological functions has been found to involve AHR mediation highlighting its importance also in normal physiological systems. In deed, those include modulation of cell function and participation in the development of the brain and vasculature [7-9]. Further physiological roles of AHR, together with the physicochemical and structural characteristics of AHR ligands have been extensively studied, because an awareness of AHR-mediated pathways enhances our understanding of many toxic and biological events.

Despite numerous studies of the impacts of dioxin, there are also some responses that have remained rather elusive. One of these is the wasting syndrome: a dose-dependent reduction of body weight, permanently retarded growth and hypophagia in dioxin-exposed laboratory animals following even a single dose [10, 11]. Although it is rare that chemicals evoke these kinds of effects, another surprising feeding related behavioural observation has been made: exposure to TCDD caused avoidance of highly palatable food items such as chocolate [12].

Food intake and feeding behaviour have attracted increasing research interest during the past 10 – 20 years. This is not attributable to dioxins but instead it is due to current obesity epidemic spreading throughout the world. There are many unknown reasons, why the current western lifestyle – i.e. lack of physical exercise in automated environment combined with excessive availability of energy-rich food – has overwhelmed the physiological control of appetite and body-weight regulation. Huge research efforts have been spent in clarifying the key mechanisms in the control of energy homeostasis; however, the issue is complicated by the fact that feeding is much more than the intake of adequate amount of calories to meet metabolic needs. Learning and memory, and other non-metabolic factors such as food reward and its hedonic value are thought to play an important role in food intake, and both neural and peripheral systems participate in the complex control of energy balance regulation (reviewed e.g. [13-17]).

Food intake and the regulation of energy balance – even without dioxin exposure - are very seldom linked to a single gene but instead they involve a variety of genes and their interactive interplay throughout the day. Therefore experimental *in vivo* – models provide essential tools to study the energy balance, and behaviour in this context is an important parameter. In this thesis, several animal models, with known differences in the AHR structure or expression levels, were used to study feeding related responses after TCDD exposure, especially after low sublethal TCDD doses. These experiments intended to evaluate whether the AHR could be involved in mediating responses related to feeding behaviour. Moreover, although TCDD has dramatic effects on altered energy homeostasis and behaviour, many aspects of its properties on feeding related behaviour have remained poorly studied. In that respect, the studies in this thesis represent steps forward in understanding the dose-responsive effects of dioxin and providing clues to the AHR as one piece in the totality of factors involved in energy balance.

2 Review of the literature

2.1 Dioxins

The term ‘dioxin’ is rather inaccurate and it includes several chemicals: polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated bibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls. These compounds share not only structural similarities (Fig. 1), but also physical and biological characteristics, namely binding to an intracellular protein, the aryl hydrocarbon receptor (AHR, Chapter 2.2). These chemicals contain numerous congeners and isomers, but only those PCDDs and PCDFs having chlorine in lateral substitutions at positions 2,3,7,8 and coplanar PCBs (unsubstituted or monosubstituted in the ortho-positions, Fig. 1) are able to adopt a planar position and to induce dioxin-like toxicity. Among the dioxins, the potencies to induce (toxic) effects differ, and therefore the most potent congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is often used as a reference compound of dioxins [18-20].

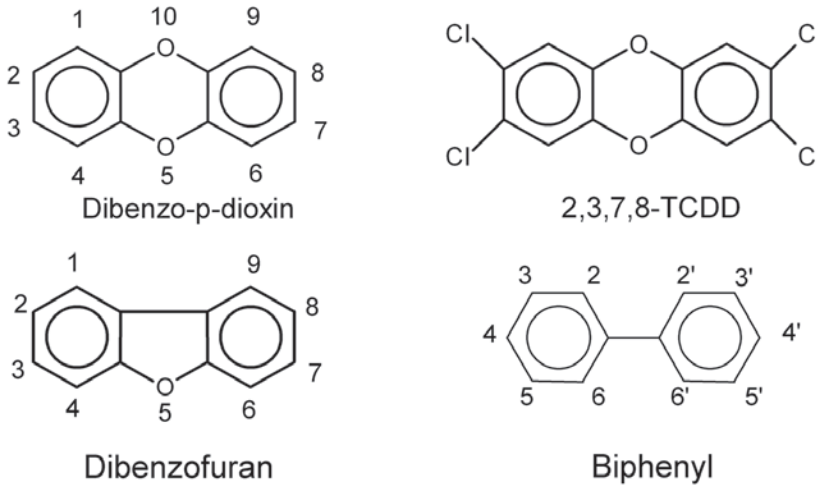

Figure 1. Main structures of different dioxin congeners, and structure of the most potent congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

All dioxin-like congeners need to be taken into account in the dioxin health risk assessment because usually they appear in mixtures, and exposure to a single dioxin-type pollutant rarely happens. For risk assessment purposes, if one wishes to characterise the toxicity of each dioxin congener and their mixtures, then it is

customary to estimate toxicity equivalence factors (TEFs). The TEF is a relative potency factor which has been assigned to each congener possessing dioxin-like toxicity and it is calculated relative to TCDD, which has the value of 1. Hence, TEF-values of dioxin-like compounds vary from 0.00003 to 1. TEFs are also commonly used in the dose-metrics in the dioxin literature, although originally the values were defined for risk management by an expert committee, which took account the existing scientific data and assumptions [21, 22]. The total sum of all partial equivalents in the mixture (toxic equivalent, TEQ) can be calculated by summing up the amount or concentration of an individual compound multiplied by its TEF. It is important to appreciate that the TEF methodology is valid only if the chemicals have been structurally well defined, exhibit additivity, and they produce the same toxicity through the same mode of action [21, 23].

All dioxins are effective at extremely low concentrations (at pg and ng levels). Responses vary (*in vivo* and *in vitro*), and there are several factors determining sensitivity differences (described later in Chapter 2.1.3.1) which complicate the mechanistic studies of dioxins. However, mechanistic studies represent the foundation for the sound but challenging risk assessment of dioxins [21, 24].

2.1.1 Sources and properties of dioxins

Dioxins are a ubiquitous and feared group of persistent organic pollutants (abbreviated to POPs). In practice, dioxins can be found everywhere. PCB-compounds have been utilized as technical oils e.g. in hydraulic and electronical equipment and as plasticizers in the plastic industry until the 1980s. Although PCDDs and PCDFs have never been intentionally made (except for scientific use), they are easily formed in the burning processes in the presence of chlorine and oxygen. Hence, they are able to end up in the environment as unwanted and harmful by-products e.g. in the production of PCBs and chlorophenols, waste incineration, metal industry, and chlorine-based bleaching-processes. While industrial dioxin pollution is nowadays strictly controlled, uncontrolled burning at low temperature is still a present-day source of dioxins. An example of this is a dumpsite fire or burning of wastes in an uncontrolled manner (Fig.2) [25, 26].

Once these chemicals are produced, they are extremely stable, resistant to biological degradation, and they remain in the environment since they become bound to particulate matter (usually first in the air and then in the sediments) where they remain for ages [27, 28]. They are poorly soluble in water and even in organic solvents [29, 30]. Dioxins accumulate in the food chain. In the environment, they are very slowly broken down by ultra-violet radiation and biodegradation [31].

Figure 2. Increasing recycling of e-wastes and their illegal or accidental incineration are potential sources of dioxins, constituting a global environmental hazard. [1]

2.1.2 Absorption, distribution, metabolism, and excretion (ADME) of dioxins

Dioxins are fairly well absorbed from the gastrointestinal (GI) and pulmonary tracts [32, 33], and penetration via skin is also possible, although relatively slow [34, 35]. Generally, the extent of absorption is critically dependent on the solubility of dioxins, whereas the administration route has only a minor role [36, 37]. Once inside the body, they are transported in blood and lymph associated with chylomicrons and lipoproteins, and rapidly deposited in the liver and fat. In these tissues, they accumulate in lipid droplets, lipoproteins, and in mitochondria. In liver, dioxins bind avidly to cytochrome-P450 protein 1A2 (CYP1A2). Their metabolism is slow and the elimination follows first-order kinetics. In humans and in most other mammals, dioxins are mainly excreted in the faeces [38].

There is only some data about congener specific half-lives, and it appears that they differ. Elimination rate depends on species and developmental stage, i.e. elimination is faster in the young than in grown-up individuals. Furthermore, high doses (or repeated exposures) seem to induce elimination and accelerate the rate of dioxin elimination [39]. In general, these compounds have long elimination half-lives,

[1] Figure from: http://nagesh-biradar.blogspot.com/ (Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License)

ranging from 12 to 31 days in the rat and from 5 to 11 years in humans [38, 40, 41]. In human beings the body burden increases every year; by the time an individual is about 60 years old, there has been a 5- to 10-fold increase in their concentrations in the body [42, 43].

Because of all these issues, the bioavailability of dioxins is important with the exposure: although these compounds can be found even at high levels in the environment, only the amount gaining access to the body (body burden) is able to exert effects. Nevertheless, although the body burden would be the most optimal surrogate for the dose metrics of dioxins, this information is very rarely available [44].

2.1.3 Toxic responses in experimental animals

After a dioxin exposure, the induction of metabolic enzymes as well as up- or down-regulation of other AHR mediated genes occurs rapidly [45-48], but lethality even after a high dose takes at least a week, and there is a wide variety between animals (Chapter 2.1.3.1) [49, 50]. In most vertebrates, dioxin exerts many responses i.e. both hypoplastic and atrophic as well as hyperplastic and proliferative effects. In general, some responses can be considered as adaptive (such as induction of metabolic enzymes), whereas some of them are clearly harmful and lead to death in a dose-dependent manner (e.g. reviewed [3, 51-53]). Despite numerous studies examining the toxic effects of TCDD, the critical factor(s) responsible for death have remained a mystery. A peculiar wasting syndrome precedes death: dose-dependent diminished feeding, severely disturbed lipid metabolism and profound weight loss. In addition, rather high doses of TCDD have been shown to induce an aversion towards novel food items and to alter food preferences. These feeding related outcomes will be described in more detail in Chapter 2.4.

Dioxins exert toxicity by acting on many cellular signaling systems, such as protein kinases, cytokines, and growth factors, as well as altering the function and concentration of receptors. They also alter the phosphorylation patterns of proteins regulating cell cycle and apoptosis [54]. The effects on the endocrine system include accelerated elimination and impaired synthesis of hormones. However, the effects are dependent on the dose, the studied organism and its sensitivity to dioxins, combined with the time of observation. Moreover, the effects are somewhat tissue specific, as different tissues concentrate TCDD differently. Many of the effects are secondary to the wasting syndrome encountered with high doses [3].

Dioxins are non-genotoxic carcinogens (as demonstrated in animals) but this effect is seen only at relatively high doses when compared to the general toxicity [55]. Although dioxin exposure is linked to a higher risk of many cancers [56], initiation of carcinogenesis seems to be secondary to tissue damage. It has been shown that dioxin induced oxidative stress, and the production of reactive oxygen species contribute to the DNA damage in many tissues [57-59].

The most sensitive effects of dioxins seem to be developmental defects. These compounds can pass through the placenta and are present in the mother's milk [60, 61]. Although there are still uncertainties of exact timing and mechanisms through which TCDD exerts these adverse effects, non-toxic (< 1 µg/kg) maternal dioxin exposure has been shown to cause developmental delays and to harm male reproductive system [62-64]. There are also other low dose (< 1 µg/kg) prenatal exposure effects e.g. disturbances in neuroendocrine functions and brain development [65, 66], interfering with learning and memory [67-69]. Nevertheless, these behavioural effects in animals are restricted to *in utero* and lactational exposure; in adults much less research has been conducted, and it seems that other behavioural responses than those related to feeding are missing [70].

2.1.3.1 Factors affecting sensitivity differences between animals

Sensitivity to the toxic effects of dioxin differs not only between species but even in substrains, as well as during development. According to the literature, sensitivity to lethality ranges from the most sensitive lake trout sack fry [LD_{50} value (lethal dose killing 50% of exposed animals) of 0.074 µg/kg, injected into egg [71]] to the most resistant animal, adult male Han/Wistar rat subsrain (H/W; *Kuopio*, LD_{50} value well above 9600 µg/kg [72, 73]). The LD_{50} value of the most sensitive rat substrain, an originally inbred (later random-bred) Long-Evans rat (L-E, *Turku/AB*) is about 7 µg/kg for females and about 18 µg/kg for males [50, 73]. In other words, this rat model showed about a 1000-fold difference in the sensitivity within the same species. *Ahr*$^{-/-}$ mice, originating from the C57BL/6-mice strain, are largely unresponsive to TCDD effects [74, 75], but in most mice strains LD_{50} values vary from 150 to 300 µg/kg [3]. For the resistant DBA/2 strain lethal dose is 2600 µg/kg while the corresponding value for the C57BL/6J mice is about 180 µg/kg [49]. Gender also affects sensitivity: in rats, females are more sensitive than males [50, 73, 76] whereas in mice, the situation appears to be reversed (at least in some substrains) [77]. Furthermore, age and developmental stage are important determinants: Not only are the differences in embryo/fetal toxicity or in early postnatal toxicity among species and strains much smaller [78], but the developing individuals are also much more sensitive than the adults (e.g. [64, 79]).

There are convincing data indicating that the wide variety in responses cannot be solely explained by kinetic differences in the metabolism and excretion of TCDD. Intra- and inter-species differences mostly involve the AHR, but there do seem to be some additional, still unknown, factors determining the toxic outcome (reviewed in [10]). In many animals, the ligand binding affinity to AHR accounts for the response sensitivity: this applies to mice and birds and explains within-species sensitivity differences [49, 80, 81]. The 10-fold difference between C57BL/6J and DBA/2 mice in TCDD toxicity as well as in sensitivity to the biochemical effects results from the polymorphism in the **ligand binding domain of AHR** of the resistant DBA/2 mouse (Fig. 3A) causing diminished affinity in ligand binding [82]. In the resistant H/W rats, the mutated AHR is smaller protein than that of the sensitive L-E rats [83], but the

binding affinity of TCDD to AHR does not differ between the strains [84]. Generally, in mice all responses to TCDD show similar sensitivity differences whereas in resistant H/W rats, some responses occur equally (**type I dioxin effect**, e.g. induction of metabolic enzymes) in sensitive L-E rats while some others (**type II dioxin effect,** e.g. lethality, wasting and diminished feeding) differ [3, 85]. The mechanism behind these different efficacies among responses is not fully understood, but alternative splicing in the **transactivation domain at the C-terminus** (Fig. 3A) of the H/W-type AHR apparently makes the ligand-bound receptor ineffective at acting as a transcription factor. In support of this proposal, there are the numerous differences noted in responses and in AHR-mediated regulation of gene transcription between the two strains [45, 46, 86].

In an attempt to clarify the unknown factors and the roles of resistance genes in TCDD toxicity H/W and L-E rats were crossbred in our laboratory. The outcome of crossbreeding was three rat lines with different types of resistance related genes, *Ahr* and an unknown gene *B*. Line A rat has a mutated H/W type allele of *Ahr* and wild-type *B* allele (genotype $Ahr^{hw/hw}$, $B^{wt/wt}$). The genotype of line B rat turned out to be $Ahr^{wt/wt}$, $B^{hw/hw}$, whereas line C rat possesses neither of the resistance alleles ($Ahr^{wt/wt}$, $B^{wt/wt}$). The unknown gene B is associated with resistance since the rats carrying the B^{hw} allele are intermediately resistant to TCDD [73, 87].

2.2 Aryl Hydrocarbon Receptor (AHR)

The aryl hydrocardon receptor is an intracellular, ligand-dependent transcription factor having a modular structure (Fig. 3A). It belongs to basic Helix-Loop-Helix/Periodic, AHR nuclear translocator, Single-minded (bHLH/PAS) proteins. Several transcriptional activators, and importantly, repressors are associated with regulating transactivation of these proteins, and they are involved in developmental and adaptive processes [88]. PAS domain proteins [e.g. *period's* (PER) homologs, single-minded's (SIM) homologs, and hypoxia inducible factors (HIFs)] have multiple roles in neurogenesis and in environmental adaptation: hypoxia, circadian rhythms, and xenobiotic metabolism to mention but a few [89, 90].

The responses to AHR activation are mediated via canonical (also known as classical) and non-canonical signaling pathways (see Fig. 3B), although these may occur simultaneously and they both affect the final outcome. The toxic effects arise from abnormal activation or dysregulation of target genes of the AHR. The best-known pathway for the AHR is the activation via classical, canonical pathway by ligand binding (Fig. 3), typically leading to induction of metabolic phase I enzymes [enzymes in cytochrome P450 family: CYP1 family and also CYP2A1)] and phase II enzymes (e.g. [91, 92]). The involvement of AHR in xenobiotic responses and in biology will be briefly described in chapters 2.2.1 and 2.2.2, a more comprehensive information of multiple roles of AHR in biology and toxicology can be found in the recently published book edited by Prof. Pohjanvirta [93].

2.2.1 Activation of AHR – mediator of xenobiotic responses

The AHR is the only vertebrate member of the bHLH/PAS family that is known to bind and to be activated by small chemical ligands. The binding of a ligand into a receptor protein (Fig. 3B) is like a key opening secondary effects, up- or downregulation of gene transcription. In short, ligand-binding is followed by AHR entry in the cell nucleus, chaperone proteins are dissociated, and AHR heterodimerises with another bHLH/PAS protein, Ah receptor nuclear translocator (ARNT). The heterodimerised complex binds further to xenobiotic response elements (XREs, known also as dioxin response element, DRE or AHR element). DNA binding is followed by interactions of the AHR/ARNT dimer with transcriptional coregulators [94, 95]. There are many AHR regulated genes [46, 91] and some of them modulate dioxin kinetics - e.g. induction of CYP1A2 causes dioxin accumulation in the liver [96, 97] - whereas some regulate AHR function: induction of AHR nuclear repressor (AHRR) inhibits the transcriptional activity of AHR [98]. This inhibitory mechanism of AHRR seems to be more complicated than originally thought [99] and the interactions between AHR and AHRR have also appeared to be tissue-, cell-, and context-specific (reviewed in [100]). The nuclear export and ubiquitin-mediated degradation are of importance in the termination of dioxin signaling [101, 102].

The ligand molecule's planar shape facilitates its binding affinity to the AHR, and the ligand's potency depends to a large degree not only on how well it fits into the receptor but also on its persistence and metabolic inactivation. According to a structure-activity modelling, it now seems that two different electrical binding mechanisms exist for different AHR ligands [103]. Furthermore, with the creation of a homology model of the ligand binding pocket of AHR and molecular docking simulations, it has been demonstrated that certain physicochemical characteristics of the internal ligand binding cavity are needed for optimal binding of a high affinity ligand, such as TCDD [104, 105].

AHR activation is also able to induce effects via a non-canonical signaling pathway (Fig. 3B), without DRE binding or heterodimerization with ARNT. The contribution of the alternative signaling pathways to dioxin toxicity is still far from clear, but the interference of AHR with estrogen or androgen receptor or β-catenin signaling pathways are examples of this kind of signalling [106].

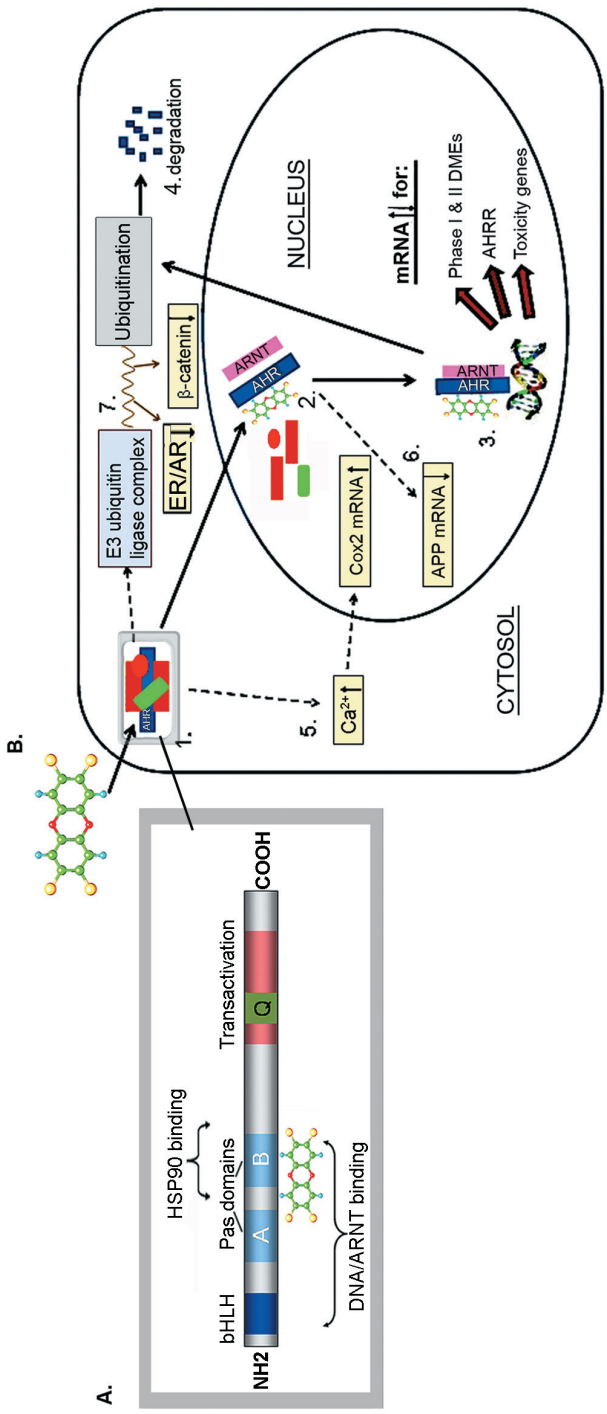

Figure 3. Structure of AHR[2] (**A**.) with the subregions involved in AHR action (**B**).

[2] Figure 3 is slightly modified from the original source 10. Lindén J, Lensu S, Tuomisto J, et al. Dioxins, the aryl hydrocarbon receptor and the central regulation of energy balance. *Frontiers in neuroendocrinology*. 2010;31(4):452-478., adapted with permission from Elsevier.

Figure 3. (*Continued*). **A.** The ligand binding domain is indicated with TCDD molecule at the N-terminal end of AHR. N-terminal end contains also bHLH and PAS domains, structures for heat shock protein binding, and for nuclear transport and export. The basic region is responsible for DNA binding, the HLH motif is involved in heterodimerization and the PAS domain determines the specificity. The transactivation domain at the C-terminus is involved in determining the responses following AHR activation. **B.** The schematic figure illustrates the signaling pathways following AHR activation. If not activated, AHR with its stabilizing chaperone proteins is present in the cytosol in the protein complex. The complex is unable to enter nucleus but is optimal for ligand (such as TCDD) binding (1.). In the classical pathway (solid black arrows) ligand binding leads to translocation into the cell nucleus, dissociation of chaperone proteins (2.), heterodimerization with ARNT, and DNA binding at dioxin response elements (DREs) (3.) affecting the expression of many target genes. Activity is terminated by the export of AHR into cytosol, and the ubiquitin-proteasome mediated degradation (4.). In addition, AHR activation seems to induce alternative pathways, to modulate functions of interactive proteins (dashed black arrows; non-canonical pathways i.e. effects occurring without DRE binding or heterodimerization with ARNT). These include an increase in intracellular Ca^{2+} levels (5.) that in turn may lead to an increase in cyclooxygenase 2, suppression of acute-phase proteins (6.), or (7.) AHR can act as an E3 ubiquitin ligase targeting β-catenin, or estrogen or androgen receptor for proteasomal degradation.

Other compounds can also bind to AHR but the ligand binding does not induce dioxin-like toxicities. In many cases this is due to their efficient metabolism [107-109]. Additionally, the previously mentioned structural and electronic differences in the binding mechanisms seem to be important [103, 105]. Other ligands of AHR include many synthetic and naturally occurring planar molecules resembling TCDD in size and shape. Examples of these planar compounds and high-affinity ligands are polycyclic aromatic hydrocarbons (PAH compounds) or indolo(3,2-b)carbazole. Furthermore, there are both endogenous and exogenous ligands, such as flavonoids, tryptophan and its metabolites, bilirubin, indirubin, indigo, and arachidonic acid metabolites which can act as agonists, partial agonists or antagonists of the AHR (reviewed in [110]). Interestingly, it has been demonstrated that the very same ligand can act (as shown *in vitro*) as an agonist or an antagonist, depending on the species and the origin of the cell line [111].

It is also possible that ligand binding is not always needed for the activation of AHR. An example is the regulation of CYP1 family enzymes, well-known to be dependent on AHR, but apparently their induction does not always involve ligand binding. That seems to be the case (at least in some species) for omeprazole [112, 113] or nicotine [114, 115]. In addition, high glucose concentrations [116], cyclic adenosine monophosphate (cAMP) [117], or low-density lipoprotein (LDL) [118] have been claimed to be capable of activating AHR (*in vitro*).

2.2.2 Physiological roles of AHR

The generation of AHR knockout mice in three different laboratories [75, 119, 120] and studies with these animals have provided enlightening information of the

physiological roles of AHR. The regulation of AHR in the absence of exogenous ligand(s) can be seen as its constitutive activity. The conserved structures of both the AHR and its negative repressor, AHRR are thought to reflect their importance in the evolution. Although the expression of AHRR is more tissue-specific than that of AHR, it may also have physiological roles (at least in regulating AHR and hypoxia inducible factor) [100].

AHR has been shown to be involved in the normal fetal development of many tissues [121-123], being especially important in angiogenesis [124, 125]. Its ability to regulate apoptosis [126] likely contributes to the regulatory role of AHR in many biological events, one good example being reproduction [127, 128]. In malignant and non-malignant cell migration [129-131] there are several additional proteins participating in the actions of AHR. For example, AHR and estrogen receptor interact in many ways, and it has been shown that by increasing degradation of estrogen receptor or β-catenin, in some cases AHR may even act as a tumour suppressor [132, 133]. AHR is involved also in the regulation of autoimmunity and in the maintenance of normal immune functions [134-137]. The possible role of AHR in the circadian regulation will be discussed in Chapter 2.4.2.

AHR-deficient mice are viable but have reduced fertility [128] and a 40 – 50% neonatal lethality. They also seem to be susceptible to premature degeneration [121] along with shortened life expectancy [138]. Studies show them having depleted splenic lymphocytes, a 25 – 50 % reduction in liver size and a 100% frequency of persistent *ductus venosus,* a fetal portocaval shunt from the portal vein to the inferior vena cava that normally closes at birth [125]. For their first weeks of life, their growth rate is retarded compared to wild-type controls [75, 119]. At adulthood and during aging, these animals exhibit cardiohypertrophy, hyperplasias of glandular mucosa and the forestomach stratified squamous epithelia, and hyperkeratosis and marked dermal fibrosis [121]. *AHR*$^{-/-}$ mice are susceptible to bacterial-related inflammation since they exhibit defects in T cell differentiation [139-141].

There are rather recent studies supporting the role of AHR in intermediary metabolism: Wang and coworkers reported that AHR-deficient mice tended to have lower blood glucose (throughout the day) than wild-type mice, and AHR deficiency improved glucose tolerance and insulin sensitivity as compared with normal C57BL/6J mice [142]. Furthermore, there are reports that AHR also may have a role in fatty acid metabolism and synthesis, as well as in lipid transport [143-145].

2.3 Overview of some issues affecting energy homeostasis and food intake behaviour

During evolution it has been beneficial for the survival to synchronise energy intake and consumption i.e. balancing anabolic and catabolic processes throughout the day (circadian rhythm): adjust physiology and behaviour efficiently in such a way that a function is optimal for the occurrence (linking external cues from the environment to

the individual needs, such as food availability and energy intake in conjunction with the activation of metabolism and induction of xenobiotic enzymes). Sufficient time is needed for both metabolic activities and resting (internal processes). Highly conserved and complex adaptive mechanisms defend the lower limits of adiposity and maintain appropriate energy and water balance. In the optimal situation, this regulation guarantees adequate intake for the basic physiological and biochemical processes involved in metabolism, in thermogenesis, in skeletal muscle action, as well as in growth and reproduction. Impaired regulation leads to diseases, for example obesity, metabolic disorders, or anorexia. Furthermore, because both human beings and animals consume food within meals, the factors affecting individual meal size, duration, and their appearance are important determinants of total daily food intake [13, 146].

This is an area of intense research activity: a literature search for 'food intake' identifies each year hundreds of published review articles, a trend which has continued over the past years. Since this is such a broad field of science, Chapter 2.3 provides an overview of some issues contributing to energy homeostasis, especially to feeding behaviour, the issues most relevant to this thesis.

2.3.1 Meal regulation and the control of energy balance

The primary peripheral players of energy balance are the gustatory system and GI-tract, pancreas, liver, muscle and adipose tissue. GI-tract offers space for digestion and absorption processes which are optimised by the direct actions of gut hormones. Since the sensations of hunger and satiety are orchestrated in the brain the control of energy homeostasis requires bidirectional, integrated signaling between brain and periphery either through direct neural connections or via blood [15, 16]. Already in the 1950's Gordon Kennedy proposed that the brain monitors body weight and adiposity which are defended against challenges to maintain a constant level, 'set-point' – also called the lipostatic hypothesis [147, 148].

Control of meal taking consists of within-meal related signals (satiation) as well as meal-to-meal, between meal related signals (satiety). These feeding-related terms are often collectively called 'satiety signals', although this term neglects the important difference between the terms satiation and satiety. Hunger drives meal initiation, but there many other contributing factors affecting satiety and the time between meals. These include factors like hedonic value of food, learning, appearance of food, and social circumstances – factors which are more dependent on other cues rather than the energy status of the body. Although they are known to be important contributors to energy balance (especially in humans) their regulatory mechanisms are not as well known as those controlling satiation, individual meal size and its termination.

Meal taking is under the direct, short-term control of various sensory signals and hormones related to the satiation process during ingestion of food. An important

location for these signals in the bidirectional pathway between the brain and periphery is the nucleus tractus solitarius (NTS) in the hindbrain (Figure 5). The hindbrain is also responsible for the motor coordination of ingestion. However, meals are also affected by other factors such as levels of circulating nutrients, correlates of stored energy (adiposity signals leptin and insulin), rhythmic factors (e.g. estrous cycle), and cues from the ambient environment (such as temperature or seasonal changes). These factors may be considered as indirect factors regulating energy balance [149]. Figure 4 represents the primary regulatory sites and pathways of the regulation of energy balance and feeding.

2.3.1.1 Taste pathways and role of tasting system in food intake

The understanding of taste mechanisms has increased enormously following the identification and cloning of the taste receptor genes (for a review, see [155]). Flavour and somatosensory sensations have their own pathways differing from the tasting system, including the gustatory signals that originate from the epithelial cells of the taste buds (triggered by water-soluble compounds), the olfactory signals that originate from the epithelial cells in the nasal cavity (triggered by volatile compounds), and the somatosensory signals that activate ion channels in the somatosensory nerve fibers (such as capsaicin) [156]. In mammals, nutrients are sensed in the oral cavity and in the GI-tract (Figure 4), with the mechanisms being currently most well-known for the sweet, amino acid (umami) and bitter. They are detected by the family of G-protein coupled taste receptor families (T1Rs for sweet and amino acid, T2Rs for bitter). In short, gustatory stimuli – sweet, bitter or umami – excite taste buds leading to adenosine triphosphate (ATP) release from the receptor cells that downstream stimulate afferent nerves and presynaptic cells leading to neurotransmitter (serotonin and/or noradrenaline) release. For sour and salty compounds, the tasting mechanism involves ion channels the former being sensitive to the H^+ ion (acids) and the latter to Na^+ ion (reviewed in [156-158]). The status of the fat taste receptor is still a topic of debate but apparently fatty acid detection involves many different mechanisms, including inhibition of K^+ channels in taste cells, activation of fatty-acid transporters (e.g. CD36), and specific G-protein coupled receptors. Fatty acids act via these receptors and they have been shown to trigger an increase in intracellular calcium concentrations that in turn stimulates the release of numerous neurotransmitters and gut hormones [159].

Taste pathway from the tongue and mouth (Figures 4 & 5) reaches **rostral** part of the nucleus tractus solitarius (NTS) through cranial nerves from where the pathway projects to the parabrachial nucleus (PBN). In addition, taste receptors in taste receptor cells are also found in the intestine and from there an important route for visceral sensory signals to the brain is the vagus nerve, another route being via bloodstream through area postrema (AP) in the caudal brainstem. These visceral pathways converge at the **caudal** part of NTS to project to PBN. Gustatory neurons of PBN project to the forebrain either via thalamocortical pathway [thalamus –

insular cortex (IC)] or via lateral hypothalamus (LH, or lateral hypothalamic area, LHA) and bed nucleus of stria terminalis to specific regions of amygdala, where there are partly reciprocal connections to the responsive sites of the brain [16, 150, 154].

2.3.1.2 Some integrators participating in the regulation of energy balance

Vagal stretch and tension sensors that work in harmony with mechanosensitive transducers and neurotrophic factors mediate information about the stomach fullness and nutrients within the GI-tract. Thus, they play a role at least in meal regulation and presumably they also contribute to long-term energy balance. It seems that vagal sensory activity and its modulation by regulatory peptides (see below) participates also in the control of meal frequency and in the initiation of a new meal [150].

Enteroendocrine cells in the gastrointestinal epithelium release hormones in response to nutrient stimuli. For a schematic drawing of the regulatory pathways, see Figure 4. In addition to communicating with each other, feeding regulatory peptides have modulatory effects in the brain [160]. Cholecystokinin (CCK) suppresses feeding by acting on receptors of vagal nerve terminals in the mucosal lamina propria [151]. Peptide YY (PYY) and glucagon-like-peptide (GLP-1) are intestinal peptides mediating their anorectic effects on brain via vagus-brainstem pathway, although also other mechanisms are at play. PYY acts as a direct anorexigen via neuropeptide Y2 (NPY2) receptors in the hypothalamus, whereas GLP-1 acts there on its own receptors. All the previous peptides inhibit also gastric emptying [161]. Furthermore, GLP-1 acts on pancreas to stimulate insulin and inhibit glucagon release i.e. it is an effective regulator of glycemic balance. GLP-1 is shown to have an acute diminishing effect on the ongoing meal, leaving the intermeal interval and subsequent meal sizes intact [162]. GLP-1 is one of the site-specific cleavage products of *proglucagon*, the others being intestinal oxyntomodulin (OXM) and pancreatic glucagon. In addition to diminishing meal size, OXM may participate in the control of energy expenditure, along with glucagon. Glucagon regulates blood glucose level affecting the mobilisation of glucose and stimulating hepatic gluconeogenesis; it also reduces meal size [163, 164].

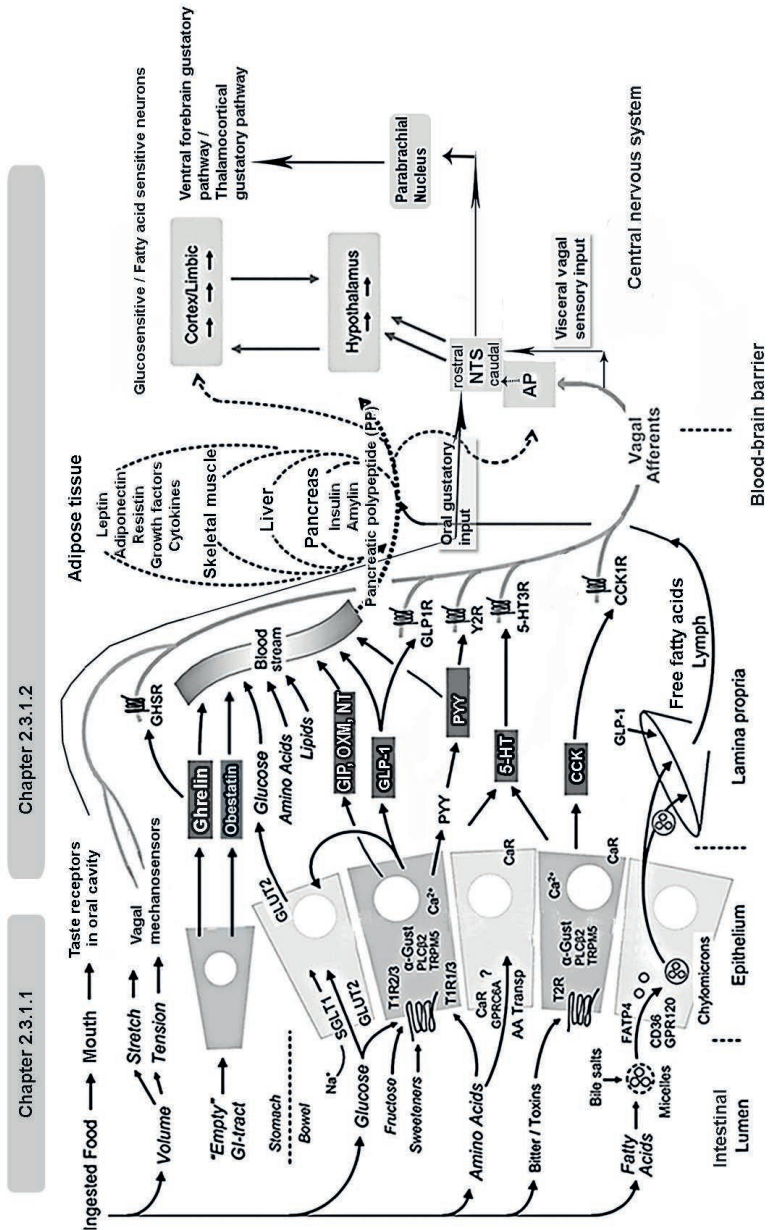

Figure 4. The gut-brain communication in the regulation of food intake and energy balance.

Figure 4 (*Continued*). Schematic drawing shows the complicity of the regulatory pathways. In the figure there are shown primary regulatory sites and mechanistic pathways for the detection of eaten food and nutrients. Figure is modified from [150], according to [14, 16, 151-154]. Abbreviations: α-Gust=α-gustducin; AA transp.=amino acid transporter; AP=area postrema; BBB=blood-brain barrier; CaR=calcium receptor; CCK=cholecystokinin; CCK1R=receptor for cholecystokinin; 5-HT=serotonin; 5HT3R=serotonin receptor; CD36=fatty acid binding-protein; FATP4=fatty acid transporter protein; GHSR=G-protein coupled growth-hormone-secretagog receptor (ghrelin receptor); GLP-1=glucagon-like peptide-1; GLP-1R=receptor for glucagon-like peptide-1; GLUT2=glucose transporter 2; GPCR6A=G-protein coupled receptor-6, amino acid sensing receptor; GPR120=fatty acid-binding G-protein coupled receptor; NT=neurotensin; NTS=nucleus tractus solitarius; OXM=oxyntomodulin; PBN=parabrachial nucleus; PLCβ2=phospholipase β2; PP=pancreatic polypeptide; PYY=peptide YY; SCN=suprachiasmatic nucleus; SGLT1=sodium glucose co-transporter; T1Rs=taste receptor 1 family members; T2Rs=taste receptor 2 family members; TRPM5=taste selective cation channel; Y2R= receptor for PYY, neuropeptide Y2 receptor.

The only known hunger signal is ghrelin, a hormone excreted by the oxyntic X/A-like cells in the gastric mucosa of the empty stomach (differing from other peptides which are released postprandially) [165, 166] and by specific neurons in the hypothalamus [167]. Its effects are mediated via vagal afferents and bloodstream [168]. Other intestinal peptides involved in meal control include obestatin, neurotensin (NT), glucose-dependent insulinotropic polypeptide (GIP), and vasoactive instestinal peptide (VIP) together with pancreatic peptides amylin and pancreatic polypeptide [161, 169]. For example, pancreatic amylin and pancreatic polypeptide are anorectic by promoting meal termination, diminishing meal size [170].

The presence of glucose in the intestine can be signalled to the brain via many routes. The blood glucose level is an indicator of the nutritional state, and a transient decline is related to meal initiation [171, 172]. In the periphery, the hepatic portal vein and pancreatic β-cells are innervated by vagal afferent fibers which have the ability to sense blood glucose levels [150]. Furthermore, the blood glucose concentration is sensed in the intestine through gluco- and osmoreceptors, and in the CNS via many neural pathways. In addition, nutrients are known to be able to modulate energy and glucose balance also by acting directly on the brain. Sweet taste receptors are expressed within the brain [173] and some hypothalamic neurons appear to be glucosensitive, being able to adjust their firing rate according to the extracellular glucose concentration. In addition, fatty acids can act as cellular messengers within brain, informing fatty acid sensitive neurons about the energy status. It seems that glucose and fatty acid sensing neurons show inverse responsiveness depending on metabolic signals, and thereby these neurons contribute to energy balance by adjusting feeding behaviour, hepatic glucose production or insulin secretion accordingly (reviewed in [174, 175]). For example, increase in FFA level induce anorectic signalling within CNS [174].

Currently, leptin and insulin are considered to serve as key circulating signals of body energy stores and adiposity. They exert short- and long-term effects on metabolic balance in the brain and periphery including direct effects on intermediary metabolism, feeding, and thermogenesis. Insulin regulates blood glucose levels (e.g. by facilitating glucose transport into muscle and adipose cells, and by promoting protein and lipid synthesis) and it sends information of body adiposity to the CNS along with leptin, a peptide secreted by white adipose tissue [176, 177]. Adipose tissue is an important and active metabolic organ, releasing many regulatory messengers: leptin, adiponectin, resistin, and many growth factors and cytokines. Furthermore, adipocytes produce angiogenic factors which are involved in neovascularisation of adipose tissue, thus body adiposity is controlled also by that means [178, 179]. While insulin and leptin are synthetised in the brain only in small amounts (if at all), they are transported into the brain by a saturable transport system via the blood-brain barrier (BBB), thus the transport system modulates leptin and insulin action [180]. When inside the brain, they are both able to induce direct neural effects via receptors in neural cells [181, 182]. In experimental models, if either leptin or insulin effects are blocked in the brain the outcome is increased food intake and adiposity [177, 183]. In obesity, hypothalamic and peripheral resistance develops to both of these hormones [184, 185]. NTS appears to be a very important relay station for the actions of leptin. There are leptin responsive neurons within NTS, and ascending pathways from the periphery to the brain and descending projections from the leptin-activated hypothalamic neurons pass through the NTS [149].

The hypothalamus in concert with endocrine organs (pituitary, thyroid and adrenal glands) has an important role in the maintenance and control of basal metabolism and thermogenesis. The pituitary gland which is under the control of hypothalamic neurons synthetises and releases various endocrine hormones (e.g. somatotropin or growth hormone, thyroid- and melanocyte-stimulating hormones, gonadotropins, corticotropins, oxytocin and antidiuretic hormone) that affect directly energy and water balance and regulate gene transcription throughout the body. Together with the hypothalamus and the thyroid, the pituitary gland controls hunger, thirst, temperature, and behaviour according to the environmental and metabolic cues (Figure 5). One needs a balance between the hypothalamus, pituitary and adrenal system in order to keep endocrine system functional and this involves many regulatory feedback loops, including direct and indirect neural pathways (e.g. reviewed in [186, 187]).

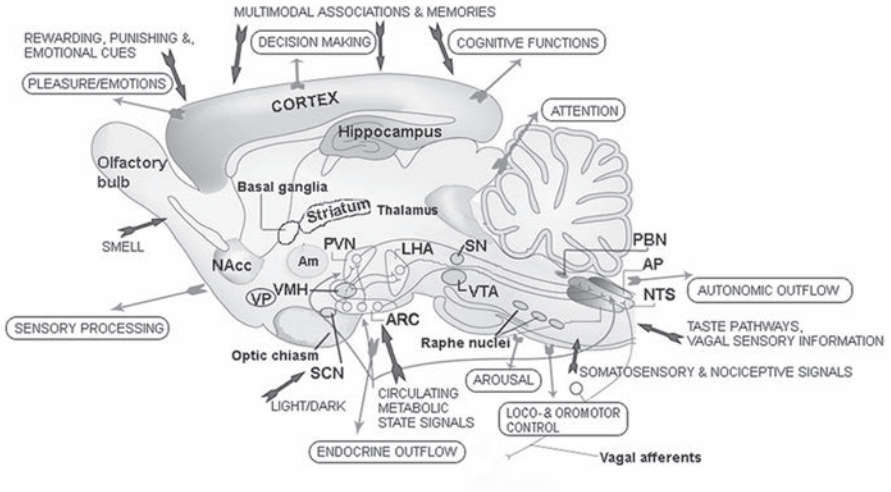

Figure 5. Schematic drawing of neural areas and numerous factors that affect regulation of eating, taste processes, and energy balance. Various inputs and outputs (outputs encircled) are written with capitalised letters, and arrows indicate an approximate site that is responsible for the control. Modified from [188], according to [14, 160, 177, 189, 190]. Abbreviations: Am, Amygdala; AP, Area postrema; ARC, Nucleus Arcuatus; LHA, Lateral hypothalamic area; NAcc, Nucleus accumbens; NTS, Nucleus tractus solitarius; PBN, Parabrachial nucleus; PVN, Paraventricular nucleus; SCN, Suprachiasmatic nucleus; SN, Substantia nigra; VMH, Ventromedial hypothalamic nucleus; VTA, Ventral tegmental area.

2.3.2 Behavioural aspects of feeding and drinking: conditioned taste aversion and neophobia with their neural substrates

Gustatory neophobia and taste memory in mammals play a role in survival: when a novel food is offered, neophobia prevents an animal from eating until the food appears to be safe [191]. Rodents are opportunistic omnivores and unable to vomit. Therefore they display especially strong neophobic responses when a novel, and potentially harmful, food item is offered. There are two behavioural phenomena that prevent an animal eating potentially harmful, toxic food item: the first is the neophobia, an innate organised and adaptive behaviour, and the second is conditioned taste aversion (CTA), a learned behaviour that prevents consumption of a food previously provoking gastrointestinal discomfort [192]. In experimental settings, the consistency of the aversive effect depends on novel food item properties (olfaction, taste, nutritive value) and on the social facilitation between individuals (if possible). In addition, the gender and age, as well as the experimental conditions may affect the outcome. Food restriction, and additional odours, flavours, visual or

auditory cues coupled with a conditioned stimulus (CS) may potentiate the aversion [193-198]. In addition, taste-recognition memory has a key role: the animal needs to remember the consequences of comsumption and be able to associate either the familiarity or the spatio-temporal relation between illness and food/drink intake during the second encounter [154].

Increasing diet variability enhances fitness and therefore it is evolutionarily beneficial to taste new food. New foods are sampled and if a postingestive malaise does not follow within a few hours then neophobia dissipates and food intake increases rapidly [199]. That novel stimulus becomes familiar and neophobia is attenuated demands associative learning i.e. learning that there is no malaise linked with the taste. This is termed as appetitive memory [154, 200, 201].

If malaise does occur, then the taste becomes an aversive signal, resulting in aversive avoidance behaviour. CTA can be recognised in various animal species from reptiles to mammals, including humans. For example, in humans, CTA is one of the adverse side effects encountered in cancer therapy (reviewed in [191]). Experimentally, taste can be considered as a CS and the resulting digestive illness as an unconditioned stimulus (US). Most typically, lithium chloride is used as an US to produce gastrointestinal illness. Differing from the other types of condititioning (a phenomenom that was first characterised by the Russian physiologist Ivan Pavlov around the turn of the 20th century), taste aversion in rats may occur even if there is a long time lag (up to 12 hours) between CS and US while a very short (minutes or seconds) interval may not result in CTA [202, 203]. That evidences the need for certain post-ingestive period, the time for the stimulus to be detected and the memory to be formed (from minutes up to several hours). In this process, the orosensory properties of food (CS) are involved, affecting also gastrointestinal chemoreceptors which potentiate CTA response [196, 204]. A positive stimulus can also be used in conditioning, for example to test flavour-nutrient learning [205]. The learned CTA response diminishes if CS is presented without US: a phenomenom called extinction in Pavlovian terms.

Many experimental manipulations during US presentation (such as anaesthesia or sleep deprivation) have proved ineffective in preventing the formation of CTA, whereas they do disrupt or modify attenuation of neophobia [206-209]. These findings indicate that there are two different neural mechanisms both for appetitive and aversive memory acquisition and consolidation. At an early stage, taste recognition is similar for both phenomena, using the pathways described in Chapter 2.3.1.1. Then, depending on the gastrointestinal consequences, the different pathways and memory processes come into play [210]. Finally, in both aversive and appetitive taste learning processes, long-term memories need to be formed and long-term potentiation together with plastic changes in neural functions are needed. Thus, activation of genes, protein synthesis, and synaptic arrangements are required for memory formation, consolidation and extinction [211, 212]. The entire process from

novel food item presentation leading finally to its consumption involves many complex pathways, of which the main regulatory networks will be described below.

NTS and PBN are important relay locations for both the taste and for the visceral sensory signals. Both areas display region specific activity depending on the novel taste stimulus [213-216]. In addition, neurons in the nucleus accumbens (NAcc) detect novel taste by decreasing their spontaneous activity, and subsequent changes in dopaminergic, glutamatergic and cholinergic activity follow (reviewed in [200, 201]). Cholinergic system of the basal forebrain including nucleus basalis magnocellularis (NBM) has innervations to the cortical mantle, hippocampus and amygdala, which are important areas in safe memory processes. NBM modulates acetylcholine (ACh) release that increases in response to novelty, to gustatory stimuli and during appetitive conditioning. It has been shown that increased Ach release in NAcc shell occurs if the novel food produces malaise, but not if the food appears to be safe [200, 217]. Thus, cholinergic system participates in the recognition and in the categorisation of novel stimuli [154].

PBN is an important location for the aversive taste memory (for the location in the brain, see Figure 5), since it is the site where signals of visceral malaise and taste signals interact. PBN receives glutamatergic projections from the NTS, and glutamatergic activity via metabotropic receptors in the PBN seems necessary for the association between aversive taste and gastric malaise [218, 219]. Hence, cholinergic and glutamatergic systems act synergistically since cholinergic activity is needed for the novel taste recognition and glutamate participates in transforming the safe taste into an aversive one. An aversive stimulus increases cholinergic activity in IC, but also glutamatergic N-methyl-D-aspartate (NMDA) receptors in the NAcc are believed to be involved in aversive memory processing, in the interplay with IC and basolateral amygdala. In addition, dopaminergic activity (modulated by the taste and visceral inputs innervating the PBN) in the NAcc participates in taste encoding and taste memory formation (reviewed in [200, 201, 220]).

The areas of importance in consolidation of aversive memories and in the formation of long-term taste memories are IC and amygdala, especially the central nucleus of amygdala (CeA), and the basolateral amygdala, that receive visceral signals from PBN. In IC, CeA, and basolateral amygdala glutamatergic system is intimately involved in CTA memory processing [221-224]. IC receives also ascending taste information from thalamus. Other cortical areas, e.g. perirhinal, entorhinal and medial prefrontal cortices participate in the formation of taste memories, and are interconnected with many taste processing areas [201]. Moreover, these systems are modulated by neurotransmitters and neuropeptides [211, 225]. Previously taste memory formation was not believed to involve any major interplay of hippocampus with cortical structures, but more recent studies argue that hippocampus may have a role in the specific modulation of taste memory [226, 227]. In addition, De la Cruz and coworkers showed that protein synthesis in the hippocampal and cortical (perirhinal

and insular) areas is required for the safe taste memory consolidation as well as for the attenuation of neophobia [228].

Although behavioural reponses have been reported to occur even after several months, the offering of CS without US leads to extinction, a process which requires dynamic activity changes in distinct brain areas [229, 230]. There are several studies demonstrating that extinction is not associated with forgetting – unlearning the original association with malaise (US) and taste stimulus (CS) – but instead it involves learning of a new association that competes with the original one (reviewed in [191]). If the malaise-inducing agent remains absent, it seems that the appetitive memory trace strengthens, and it has been shown that IC has also an important role in extinction processes. However, it has been claimed that CTA consolidation and consolidation of CTA extinction appear to be two different memory processes, and both require newly synthetised proteins [228, 231, 232].

2.3.3 Circadian rhythmicity

Circadian rhythms constitute an important regulatory network for optimal physiological and behavioural adaptation into an ambient environment. At the molecular level structurally similar circadian oscillators can be found in the brain and in peripheral organs, and they actively synchronise behavioural activities and rhythms of hormone secretion, temperature, blood pressure, metabolism, and sleep-wakefullness with environmental cycles [233, 234].

The master clock was found already in the early 1970's, the suprachiasmatic nucleus (SCN) in the hypothalamus (Figure 5) [235, 236], where heterogenous circadian clock cells generate circadian rhythms involving clock genes and their protein products. Most important are the positive regulators brain and muscle ARNT-like protein 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK), and repressors Period1 and 2 (Per1 and 2), Cryptochrome1 and 2 (Cry1 and Cry2). In short, this evolutionarily conserved regulation functions as follows: BMAL1 and CLOCK proteins dimerize in the cytoplasm, translocate in the nucleus, and bind to *cis*-regulatory element (E-box) sequences of repressor genes to activate their transcription. The protein products of the repressors inhibit the activity of *Bmal1* and *CLOCK*, thus generating a negative feedback loop for their own transcription. However, it should be borne in mind that the control of circadian rhythmicity is controlled by the transcription and translation of numerous other genes; altogether approximately 600 genes are expressed in a rhythmic manner within SCN [237-240].

Information from the retinal ganglion cells uses direct retinohypothalamic track to entrain suprachiasmatic neurons to light-dark cycles. Additonal afferent systems to SCN include geniculohypothalamic track and the brainstem serotonergic system. These pathways use many classical neurotransmitters in their signalling [e.g. excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA), neuropeptide-Y (NPY), acetylcholine, and histamine]. Via its many efferent

pathways, the SCN entrains the rhythmicity on neural systems, on peripheral organs (which similarly to SCN, express clock genes and have a high number of rhythmically regulated genes), and on endocrine and immune functions [240-243]. Although SCN is the master clock, many other systems are able to maintain molecular clockwork even without the control of the SCN [240, 244, 245]. One example of that is the food-entrainable system [246].

A food-entrainable circadian pacemaker is located (still at an unknown location) outside of the SCN, as SCN-lesioned animals or animals with disturbances in SCN-generated rhythms are able to generate a feeding related circadian rhythm [247, 248]. Feeding and fasting entrain rhythms in peripheral organs and in extra-SCN areas in the brain (food-anticipatory rhythms), whereas the SCN clock has been considered impervious to feeding cues, especially if the photic stimulus is present. Nevertheless, it has been shown that timed availability of food and macronutrients, especially if associated with hypocaloric conditions, lead to phase-changes in SCN-controlled rhythms and also in the SCN clock [249-251], possibly due to a hypometabolic state [252]. Furthermore, increasing evidence exists nowadays that various metabolic cues are linked to circadian rhythmicity, constituting a bidirectional loop between peripheral metabolism and SCN [253, 254]. In other words, nutritional inputs and peripheral clock signals can alter signaling pathways and rhythmicity in the brain, while the circadian rhythmicity of circulating hormones (e.g. insulin) is modulated by the SCN.

Currently there is ample evidence showing how the metabolic state can affect the circadian clockwork. A typical example is the interplay between sirtuin1 (SIRT1), clock genes (*BMAL1* and *Per2*) and nicotinamide adenine dinucleotide (NAD^+). SIRT1 is a NAD^+ dependent mitochondrial deacetylating enzyme involved in transcriptional silencing, genome stability, and longevity, whereas NAD^+ functions as an indicator of the cellular metabolic state. SIRT1 is known to deacetylate BMAL1 [255] and Per2 [256], thus it participates in the circadian clockwork by regulating clock gene expression. As the enzymatic activity of SIRT1 is regulated by its coenzyme NAD^+ [257], and there is a report that adenosine monophosphate -activated protein kinase (AMPK) is involved in enhancing SIRT1 activity by increasing NAD^+ [258], the cellular metabolic state has a crucial modulatory potential for regulating rhythms. There are many other players involved in the coupling of circadian clockwork to metabolic cycles, e.g. glucose sensors and many nuclear receptors (reviewed in [234, 254, 259]).

In addition to light and feeding rhythm, sleep deprivation, aging, high-fat diet, pharmacologic agents in rodents, even availability of a running wheel can alter the circadian oscillation of molecular clockwork and vice versa. These changes can be detected as alterations both in behaviour and in gene and protein expression. The motivational value of food has also many entraining properties on the regulatory pathways, even though these effects are modified by species and gender-related factors, as well as the composition of offered meal [244, 260-263]. In this context the use of pair-fed control animals (feed-restricted in TCDD experiments: given food

relative to that consumed by exposed animals) should be mentioned. Pair-feeding is a useful way to compare the effects of altered feeding *per se* to those of the compound being studied [17]. Nevertheless, similar daily energy consumption does not necessarily determine factors like adiposity or body weight level (e.g. [264-266]). It was shown by Pohjanvirta [267] that the signalling pathways activated by feeding cues do not remain similar during a period of restricted feeding. Similar weight-loss but different durations of feed restriction caused entrainment of peripheral oscillator genes and disturbances in circadian rhythms. Since the pair-feeding usually occurs during the daytime – at the time of lowest feeding in the case of many rat strains – it apparently modifies many feeding related outcomes and metabolic cycles in different ways, if one compares pair-fed animals to their *ad libitum* fed counterparts and study subjects. It has been shown that a timed offering of a palatable food item in a remarkable amount (relative to daily energy intake), even without food restriction, can entrain various aspects of circadian rhythmicity. Not only the behaviour [268, 269] can be modulated but also gene expression in brain and in peripheral tissues [245, 269-271]. Provision of a hypercaloric, high-fat diet appears to dampen diurnal rhythmicity of feeding and metabolism [264], conversely, the contrasting situation: severe feed restriction has been shown to lead to phase-advances, as already mentioned. However, phase-advances have been found in many endpoints such as in body temperature, hormone levels, molecular pathways and in many kinds of behaviours. After extended periods on a hypocaloric state nocturnal animals have become somewhat diurnal (reviewed in [245]).

2.4 Effects of dioxins on energy homeostasis and feeding behaviour

2.4.1 Wasting syndrome

The feeding related effects of dioxins are rather atypical manifestations of chemicals. The wasting syndrome as an outcome of dioxin exposure is typified by a dramatic reduction in feeding and the corresponding decline in body weight in exposed animals [272]. Lethality is delayed, but if the dose is high enough (bearing in mind that the lethal dose varies tremendously between animals) animals refuse to eat and they lose as much as 50 % of their body weight before they succumb within 1 – 8 weeks [3, 273]. At doses higher than the lethal dose, the time to the death is not markedly affected by the dose, but usually exposed animals do not show total feeding refusal - feeding declines progressively. At sublethal doses permanently stunted growth, decreased feeding, and alterations in feeding behaviour occur (reviewed in [3, 4, 10]). Furthermore, at sublethal doses after 1 – 2 weeks (depending on the dose and the animal) feeding is enhanced and there is subsequent weight gain. Nevertheless, exposed animals seem to maintain body weight at a lower level in comparison to their controls. The weights of the treated animals stabilise

permanently to a lower level, which has been termed the 'lowered body weight set-point' due to exposure. There are several results supporting the lowered body weight set-point, e.g. TCDD-exposed rats are able to defend their lowered body weight level against various feeding challenges, and they are able to gain weight or to increase feed intake after fasting [273-277]. The body weight set-point hypothesis was tested with streptozotocin-induced diabetic rats (animals which are hyperphagic and underweight), even after TCDD treatment diabetic H/W rats remained hyperphagic relative to their non-diabetic counterparts. Feed intake and weight gain were only mildly affected by TCDD as compared to the situation in diabetic rats. Although insulin abolished the body weight loss in both groups, the TCDD-group stabilised weight to the level of TCDD-treated non-diabetic rats [278]. These findings reveal that hypophagia as such is a tool for adjusting the body weight level in response to TCDD.

There are studies revealing that animals do not suffer from nausea [279], and neither gross malabsorption nor increased energy expenditure offer explanatory roles in wasting [273, 277, 280]. Hypophagia and thereby depleted energy stores could be regarded as the cause of death following dioxin exposure: approximately at that time also the pair-fed controls died because of the reduced energy intake [281, 282]. Nevertheless, these findings do not provide a full explanation for the lethality, and other mechanisms are also at play. This is supported by the findings that force feeding cannot prevent the death of TCDD-treated animals; this has been demonstrated in rats [283, 284] and in guinea pigs [285]. Furthermore, dietary modulation of TCDD-induced wasting proved ineffective, as neither obesity nor high-energy diet could prevent weight loss in the resistant H/W rats. Provision of a balanced liquid feed prevented the weight loss in L-E males while a high-fat diet seemed to be related to shortened life-span in TCDD-treated L-E female rats [284]. In general, fat has appeared to be the only macronutrient that is an unfavourable energy source after exposure to TCDD in rats [12, 286]. In contrast, in one study in female mice (harbouring a high-affinity AHR for TCDD, LD_{50} value was not given) the consumption of a high-fat diet combined with TCDD treatment resulted in a significantly accelerated body weight gain at the highest dose (100 µg/kg *i.p.*) tested, while the lower doses (1 or 10 µg/kg) were ineffective, with or without the high-fat diet supplement [287].

The group of Rozman has examined whether TCDD can affect brown adipose tissue and induce thermogenesis (see below). Brown adipose tissue cells contain several small droplets of fats, and these cells are loaded with mitochondria capable of generating ATP. Additionally, there is also proton conductance that causes uncoupling of oxidative phosphorylation from the generation of ATP, leading to the generation of heat, known as nonshivering thermogenesis. Fat cells and blood vessels within brown adipose tissue have a sympathetic innervation, thus enhanced sympathetic activity may induce lipolysis and heat production, and loss of energy [288]. At sublethal and lethal doses, TCDD has been reported to evoke morphological alterations in the interscapular brown adipose tissue [289, 290]: the number of lipid

droplets in brown adipose tissue diminished and their size was enlarged, followed by progressive lipid depletion. However, no activation of thermogenesis as a consequence of exposure was reported; on the contrary, exposed rats appeared to be hyposensitive to noradrenaline-induced thermogenesis in brown adipose tissue [291].

Factors related to stress responses or direct stomach effects vary, and do not explain wasting. TCDD has been shown to **decrease** gastric emptying and gastric acid secretion, but there was no evidence of hypoplasia of the acid-secreting parietal cells in the stomach [292]. Gastrointestinal hemorrhages following exposure appeared to be related to problems in blood clotting due to impaired hepatic production of coagulation factors [293, 294]. Stress factors from the hypothalamus-pituitary-adrenal axis exhibit varying responses to TCDD depending on the dose, animal, and other experimental settings (reviewed in [3, 10]). For example, in H/W rats and L-E rats plasma corticosterone levels did not change at all, or there was an insignificant increase in response to TCDD, respectively [295].

The liver is the target organ of TCDD and low doses induce many hepatic enzymes, a reversible response relative to tissue concentration of TCDD. At relatively high doses, liver hyperplasia and necrosis occur in many species. Liver toxicity is associated with impaired biliary clearance, degenerative changes and oxidative stress (reviewed e.g. in [3, 4]). During the wasting process, body fat stores are mobilised, fat synthesis as well as several lipogenic enzyme activities decline and fat infiltrates into the liver [286, 296, 297]. In rats, the serum free fatty acid (FFA) levels increase, and the disturbed lipid metabolism causes also enhanced levels of serum triglycerides and cholesterol. According to the results from many animal studies, TCDD can induce specific time- and dose-responsive effects on glucose and fat metabolism, e.g. on cholesterol biosynthesis, and fatty acid synthesis and oxidation. These effects have been most extensively studied in liver tissue, and can be observed in many species also at low doses (e.g. [45, 91, 298, 299]).

The effects of dioxin on adipose tissue include insulin-resistance like symptoms: downregulation of insulin receptors and glucose transporters, and a subsequent suppression of insulin-stimulated glucose uptake *in vivo* and *in vitro* [300-302]. Serum insulin levels decrease, apparently due to hypophagia and the body weight loss at high dose that causes also a reduction in blood glucose levels [303, 304]. However, blood insulin [305] and glucose levels decrease also at sublethal levels, and the inhibition of gluconeogenetic enzymes may contribute to lowering of the blood glucose concentration, namely the inhibition of the key enzyme, phosphoenolpyruvate carboxykinase (PEPCK) [306, 307]. TCDD was shown to relieve hyperglycemia encountered in high-fat fed and streptozotocin induced diabetic rats [308]. In the glucose tolerance test, blood glucose levels were similar in TCDD-treated mice and their controls although controls had higher insulin levels [309]. After TCDD exposure, rats became hypersensitive to insulin-induced hypoglycemia [274, 304, 310]. Thus, although exposure has been shown to impair pancreatic insulin secretion *in vivo*, *in vitro* and *ex vivo* [309, 311], administration of TCDD seems to enhance insulin

function and sensitivity in animals. In this context it should be noted that continuous exposure and thus the elevated body burden of dioxin-like compounds in humans has been linked to induction of type II diabetes [312]. In contrast to the situation in rodents, in humans exposure seems to increase blood glucose and insulin, and to enhance insulin resistance [313-315]. Apparently this issue warrants more research as there are also studies which have detected no association between human diabetes and TCDD [316, 317].

2.4.2 Alterations in circadian rhythms - possible role of AHR in the regulation?

A wide variety of physiological functions display circadian rhythms (Chapter 2.3.3), e.g. most aspects of xenobiotic metabolism are under the control of circadian regulation. Although the metabolism has its own regulatory component being activated by xenobiotics, detoxifying enzymes do show expression patterns in a circadian fashion. The oscillation of proteins and their encoding mRNAs may have different characteristics, but in recent years emphasis is given for the fact that the fluctuations can affect drug efficacy and/or toxicity throughout the day [233, 234]. On the other hand, many xenobiotic compounds can perturb the regulation of circadian rhythms by affecting circadian genes or via interactions with metabolic proteins. Furthermore, bearing in mind that food is one of the important peripheral circadian timekeepers (Chapter 2.3.3), exogenous compounds capable of altering feeding behaviour may also entrain circadian rhythmicity by that means.

TCDD exposure has been shown to alter circadian feeding rhythms in various rat strains. After H/W rats have reached their lowered body weight (about 2 weeks following high TCDD dose) they shift their feeding to the daytime, i.e. their food and water consumption is mainly unaffected during daytime in comparison to darkness. This effect remained for months in resistant H/W rats [274, 275], and similar kinds of responses have been found in L-E [318] and Sprague-Dawley rats [281]. Studies with mice also revealed altered circadian rhythmicity, with a high dose level of TCDD causing phase advances in locomotor activity ([319, 320], congress abstracts). Circadian expression of various hormones is affected by TCDD, exemplified by corticosterone which was rendered without any rhythm [321], or melatonin whose peak levels were suppressed after TCDD [322, 323]. In addition to TCDD, other activators of the AHR are capable of altering feeding behaviour. For example nicotine, an inducer of CYP1A enzymes [114], has been shown to flatten circadian peaks in feeding and to diminish eating by decreasing both meal size and meal numbers in rats [324].

While the role of AHR in the circadian rhythm regulation without a bound exogenous ligand is still a target for intensive studies, several other proteins belonging to the same PAS family, such as BMAL1 (referred also ARNT3 due to structural similarity to ARNT) and CLOCK, are key regulators of the circadian rhythms [325-327]. The expressions of PAS proteins (including AHR) and their

encoding mRNAs fluctuate during the day in several mouse and rat tissues [90, 328]. On a normal light/dark rhythm, the peak in *Ahr* transcripts occurs during the time of lights off in SCN, and in liver 4 hrs earlier [329]. PAS proteins have revealed the tight interplay in AHR-mediated responses in the presence of an agonist [330-332], for example in mice TCDD disrupted rhythms or light-induced phase shifts in the behaviour and in immune responses, and they appeared to be linked with alterations in molecular clockwork [320, 329, 333].

Clock time has been shown to modulate AHR activity. Furukawa and coworkers found that the induction of CYP-enzymes via AHR displayed circadian rhythmicity with the peak occurring in the middle of the night [334], thus making the inductive effect of an AHR agonist to be dependent on the exposure time, as confirmed later [261]. The interplay between clockwork regulation and the AHR is further supported by the findings obtained in *Per1* knockdown mice: in these animlas the effect of TCDD on CYP induction was markedly enhanced in liver and in mammary gland [335, 336]. Furthermore, TCDD repressed the magnitude of *Per1* and *Per2* expression [333], while disruption of these circadian clock genes abolished the diurnal fluctuation of the typical AHR mediated response, induction of *CYP1A1* [261]. In 2013 Xu and coworkers showed that administration of another AHR-agonist, β-naphthoflavone, could block hepatic and SCN induction of *Per1* expression in normal mice and *in vitro* [337]. Hence, in the presence of an agonist there are several reports supporting the interplay between the circadian timekeeping and the AHR.

2.4.3 Novelty avoidance and CTA after TCDD exposure

TCDD induces specific alterations in feeding behaviour. Exposure of adult rats has been shown to cause not only a reduction in the amounts of feed consumed, but also alterations in feed preferences and strong aversive responses. Usually rodents prefer sweet and fatty tastes, but exposure of TCDD has caused a persistent avoidance of these types of foodstuffs as shown by Tuomisto and coworkers [12]. In their experiments, the selective changes in chocolate consumption emerged rapidly, during the first dark period (chocolate was offered at the beginning of darkness) after the exposure. In that study, differently TCDD-sensitive rats were exposed to sublethal (but rather high, 10 – 100 µg/kg) doses of TCDD at different times of day during the light period. The diminished chocolate intake occurred independently of the time of exposure and of the sensitivity of an animal to overt TCDD-toxicity. Furthermore, the results of various experiments were concluded to favour the TCDD-induced neophobia: the novel food item aversion was most evident following simultaneous offer of the novel food item with TCDD exposure [12].

The main characteristics of novel food item aversion were identified in the same study. A habituation period of 16 days to the selected novel food items before the exposure was a long enough period to familiarize the rats with these items and alleviated the immediate avoidance for chocolate or cheese. Nevertheless, selection

of chocolate as a food diminished and remained low for five weeks postexposure. These studies also indicated that the chocolate aversion endpoint - at the doses used - was not critically depending on the gender. It is noteworthy that in this experiment all of the novel food items (cheese, chocolate and sucrose) were freely available increasing the total energy intake and body weights of the rats on these special diets.

Studies investigating the role of CTA in the feeding responses following TCDD are complicated by the persistent properties of TCDD and by its feeding and weight reducing effects. Nevertheless, in the studies of Pohjanvirta and coworkers, TCDD exposure induced a positive CTA response for saccharin at a dose 1000 µg/kg in H/W rats while the lower, 50 µg/kg dose proved ineffective in both H/W and L-E rats [279]. Further support of CTA was the finding that differently sensitive rats avoided chocolate at the repeated testing after 19 days' postexposure while in the same experiment there was no difference in novel food item (cheese) comsumption between the exposed and control rats at day 13 postexposure (i.e. cheese was offered as a novel food item on that day). This study revealed that the avoidance of chocolate on day 19 postexposure could not be related to the food energy because there was no neophobic response associated with cheese in the exposed rats. The emergence of the chocolate avoidance appeared to depend on its previous offering during the night immediately after the TCDD exposure [12].

3 Aims of the study

Specific alterations in feeding and drinking behaviours following dioxin exposure have been observed in adult laboratory animals, but the sensitivity and the detailed characteristics of these effects among different laboratory animals have remained unknown. The aim of this work was to characterise acute feeding related responses in behaviour and in brain after a single TCDD dosing in adult rodents with different sensitivities to TCDD due to their recognized differences in their AH-receptor structures or expressions.

The specific aims of this thesis were:

1. To characterise the effects of TCDD on ingestive behaviours and determine their time- and/or dose-responses to provide a more dynamic and comprehensive perspective of the effects of TCDD (I-II, IV).

2. To elucidate the possible role of the AHR in consummatory and aversive behaviours by comparing the feeding responses of studied animals (I-IV).

   Aims 1 and 2 will include following steps:

   Time- and dose-responses of novel food item avoidance will be assessed in adult rats, after a single low dose exposure of TCDD (I-II).

   The novel food item avoidance behaviour will be characterised and it will be determined if the avoidance is dependent on: animal strain and genotype, properties of foodstuff and the factors associated with the exposure of TCDD (I-II).

   Time-responses in feeding behaviour will be assessed, after two different TCDD doses. Studies will include characterisation of diurnal micro- and macrostructures of feeding and drinking behaviours in two differently TCDD-sensitive rat strains. These experiments will be done with and without TCDD (III-IV).

3. To screen which areas of the brain are activated at 24 hours after a single TCDD exposure in two differently TCDD-sensitive rat strains (V).

4 Materials and Methods

4.1 Animals and animal husbandry

The rats and mice used in the experiments of this thesis are shown in tables 1 and 2. The sensitivities of these animals to TCDD lethality as well as their ED_{50} values (effective dose; causing a 50% response in exposed animals) to some toxic endpoints of TCDD are shown in Table 1. Table 2 displays a summary of experiments and their results.

Table 1. Lethality of TCDD (LD_{50} values) along with ED_{50} values for EROD activity (measure of CYP1A1 induction) and body weight change for the animals used in the experiments (I – V). nd = not determined; ND = not detectable.

Animal species; strain; sex	Genotype	Lethality, LD_{50} (male/ female; µg/kg)	EROD activity, ED_{50} (male/female; µg/kg)	Body weight change, ED_{50} (male/female;µg/kg)
rat; H/W	$Ahr^{hw/hw}$, $B^{hw/hw}$	> 10000 [72] / > 9600 [72]	nd / 0.77 [85]	nd / 19 [85]
rat; L-E	$Ahr^{wt/wt}$, $B^{wt/wt}$	18 [73, 50] / 7 – 10 [73, 50]	nd / 0.39 [85]	nd / 6.3 [85]
rat; line A	$Ahr^{hw/hw}$, $B^{wt/wt}$	> 10000 [73]/ > 2000 [73]	nd / 0.15 [87]	nd / 20 [87]
rat; line B	$Ahr^{wt/wt}$, $B^{hw/hw}$	830 [73]/ 410 [73]	nd / 0.28 [87]	nd / 21 [87]
rat; line C	$Ahr^{wt/wt}$, $B^{wt/wt}$	40 [73]/ 19 [73]	nd / 0.14 [87]	nd / 5.5 [87]
mouse; C57BL/6Kuo	$Ahr^{b/b}$, $B^{?/?}$	~350 [77]/ >5000 [77]	0.79[338]/1.01[338*] (or nd / 1.47 [339])[#]	nd
mouse; AHR-knockout C57BL/6	$Ahr^{-/-}$, $B^{?/?}$	ND	ND	ND

* CYP1A1 mRNA induction, data from [338]; [#] For ovariectomized C57BL/6 mice, data from [339]

All rats (H/W, L-E, line A, B and C) were obtained from breeding colonies of the National Public Health Institute (Kuopio, Finland) maintained in a specific pathogen free barrier unit. In addition, mice (two genotypes of C57BL/6 mice, C57BL/6Kuo and AHR knockout mice, *Ahr-/-*) were bred at the same location, although the *Ahr-/-* mice originated from The Jackson Laboratories, Bar Harbor, ME, USA. Line A, B and C rats have been developed by cross-breeding H/W and L-E rats in this laboratory [73]. FELASA recommendations were followed in regular health surveillances of animals and in the training of employees working with laboratory animals [340, 341]. Health surveillances confirmed that the animals were free of typical rodent pathogens.

In the experiments, healthy adult animals were used (Table 2). Before the start of the experiments, all animals were habituated for at least a week to ambient conditions and handling by the experimenter. In experiments I-II and V, the rats were individually housed in a stainless steel wire mesh cages (38 x 20.5 x 19.5 cm). Mice were kept individually in Macrolon® polycarbonate cages (36 x 19.5 x 15 cm) covered with wire mesh lids. Autoclaved aspen chips (Tapvei Co., Kaavi, Finland) were used as mouse bedding and nesting material. In experiments III – IV, Coulbourn Habitest® cages were used (see Chapter 4.3.2 and Figure 6). The air-conditioned animal rooms were regulated for light (12/12 h light/dark cycle, lights on at 7 a.m.), temperature (22 ± 2 ^{o}C) and relative humidity (50 ± 20%). Temperature and humidity were monitored by a computer based system (Honeywell XBSI, Honeywell, Morristown, USA).

In experiments I – II and V, the animals were fed with regular feed which was available together with tap water ad libitum, unless otherwise stated in the experimental design (Chapter 4.5 and Table 2). The regular feed for mice and L-E rats was Altromin 1314F feed (Altromin GmbH, Lage-Im Seelenkamp, Germany; energy content 12.5 kJ/g). H/W rats, line A, B and C rats and also L-E rats in the experiment (V) were fed with R36 feed (previously Ewos, Södertälje, Sweden and at the time of experiments Labfor/Lactamin, Kimstad, Sweden; energy content 12.6 kJ/g). In experiments III – IV, the rats were fed with 45-mg dust-free precision pellets (Bio-Serv®, Frenchtown, NJ, US). These pellets and tap water were available ad libitum. The energy content of the precision pellets was 15.1 kJ/g (or 3.6 kcal/g).

During and after the experiments, all materials possibly contaminated with TCDD were collected and sent to a hazardous waste treatment plant (Ekokem Oy Ab, Riihimäki, Finland) for professional incineration.

All the animal experiments were reviewed and approved by the Animal Experiment Committee of the University of Kuopio and Kuopio Provincial Government, and they were conducted in accordance with the Guidelines of the European Community Council directives 86/609/EEC.

4.2 Chemicals

TCDD was purchased from the UFA-Oil Institute, Ufa, Russia. It was over 99% pure as confirmed by gas chromatography -mass spectrometry (GC-MC) [342, 343]. A weighed amount of TCDD was dissolved in diethyl ether (BDH Laboratory Supplies, Poole, UK) for storage (in the dark, at room temperature). To prepare the dosing solution, an appropriate volume of TCDD-diethyl ether solution was mixed with corn oil (Sigma-Aldrich, C8267, St. Louis, MO, US) and the ether was allowed to evaporate thoroughly. Before each administration dosing solutions were mixed with magnetic stirrer and sonicated in warm water bath for 20 minutes. The administration volume for both TCDD and vehicle in all experiments was 4 ml/kg which was administered by intragastric gavage (*i.g.*) unless otherwise stated. From all dosing solutions at least one quality control sample was taken and stored in a freezer (-20 ^{o}C) for the confirmatory analysis of TCDD by gas chromatography-mass spectrometry. Chemicals other than TCDD and all laboratory equipment used in the experiments are detailed in the original publications.

4.3 Methods

4.3.1 Indices of weight and food intake

Two different variables were used for the body weight and food intake (I – IV). Since these rat lines grow at different rates and because the exposure affects both weight gain and energy intake, in addition to normal weight (g), the metabolic weights of rats were calculated. In this approach, the difference in the body weight and its possible effect on feeding were taken into account by relating the total daily feed consumption (g) to the metabolic mass of each rat [BW(kg)$^{0.67}$]. Hence, this estimate provides a measure of energy intake according the metabolic needs within the same species (discussed in [344, 345]).

4.3.2 Behavioural responses (I - IV)

4.3.2.1 Characterisation of aversive responses (I-II)

In experiments characterising details of novel food item avoidance and aversive behaviour after TCDD exposure, differently TCDD-sensitive rats and mice were exposed to TCDD. Various doses of the chemical were used to enable dose-response analysis and data modeling [85, 87]. Regular chow in a novel form (in powdered form), chocolate (milk chocolate, Panda, Vaajakoski, Finland; energy content 23 kJ/g), 0.25 % saccharin (S-6047, Lot. 075K00071 Sigma; contains no energy) and 10 % sucrose (ICN Biomedicals, 821713, Lot. 8681C, OH, USA; 10 % solution contains 1.7 kJ/g energy) were used as a food stimulus. Saccharin and sucrose were dissolved in tap water. The experimental designs are described in Chapter 4.5 and in Table 2.

Unless otherwise stated in the experimental design, regular feed and tap water were available *ad libitum*.

4.3.2.2 Drinking bouts and meals (III-IV)

Before L-E or H/W male rats were moved into test cages (Fig. 6), they were habituated to eating 45-mg pellets for about one week. They were further accustomed to the Habitest® cages for at least one week, and the experiments were started only when their feeding and drinking had stabilized to a normal level (number of licks ca. 7000 and number of pellets ca. 450 per day). Only those rats meeting these criteria were included in the study. The data were gathered from pairs (one control and TCDD-treated rat at the same time).

Figure 6. Automated device for monitoring of feed and water intake.

Patterns of feeding and drinking behaviours were compared either across rat strains and subsections of time (III) or across treatments, measuring days and subsections of time (IV).

A single meal or a drinking episode was defined as an event where any two consecutive pellets or licks had a lag shorter than five minutes between them. From the episodic data, various variables could be analysed reflecting feeding and drinking behaviors separately, both describing individual meals and drinking bouts but also the total consumptions within a day. In the analysis of circadian variation in behaviours, episodic data were pooled separately for each rat in chronological order. In all analyses, missing events (if a rat was not drinking or eating during a subsection of time) were encoded as zero values.

Feed and water spillage in the experiments were controlled. In the total feed consumption, the spilled amount was taken into account, but not in feeding microstructural analysis because it was impossible to define afterwards the exact time and the specific meal during which the spillage had occurred.

4.3.3 Automated monitoring system (III-IV)

The continuous feeding and drinking monitoring was performed with the Coulbourn Habitest® (Coulbourn Instruments, Whitehall, PA, US) system, controlled by Graphic State 3.02 software (Coulbourn Instruments, Whitehall, PA, US). This system is run by a standard computer and consists of a special cage equipped with a feeder and a lickometer described in original publications III – IV. Each time the feeder or the lickometer was activated, the time of the event was recorded in ms with reference to the start of the measurement. Stored binary data were further processed into feeding or drinking episodes with SPSS 15.0 (SPSS Inc., Chicago, IL, US). The measuring device is shown in figure 6. To prevent any data loss e.g. due to power failure, all measuring devices were connected to an uninterruptible power source.

4.3.4 c-Fos Immunohistochemistry (V)

C-Fos-immunostaining was used as an indicator of activated cells [346-348] in the brains of L-E and H/W rats. In the immunohistochemistry (V), the brains were cut into 30 µm coronal, serial sections with vibratome (Leica VT1000S, Leica Instruments, Wetzlar, Germany). All sections were cryoprotected and frozen (-20 ^{o}C) until used for immunohistochemistry. Serial brain sections were stained free-floating using immunoperoxidase methods to visualize c-Fos protein. As a primary antibody, polyclonal rabbit primary c-Fos antiserum (1:2000, sc-52, Santa Cruz Inc. U.S., Lot. K089) was used. The exact protocol of immunostaining is described in original publication (V).

C-Fos positive cells were mapped using an integrated hardware-software application: the set-up contains a PC-computer connected to an ECLIPSE E600 microscope (Nikon, Tokyo, Japan) via CCD colour video camera (HV-C20, Hitachi, Tokyo, Japan). Data were collected with the aid of the software programs Stereo-Investigator and NeuroExplorer (MicroBrightField, Colchester, VT). To determine the total number of activated, c-Fos positive cells, all darkly, intensively stained neuron nuclei of relevant size and shape were counted in the target area (µm^2, achieved during the plotting).

4.3.5 Statistical analyses and assessment of ED_{50}

In the statistical analyses, different versions of SPSS software (SPSS Inc., Chicago, IL, US) were used, and p-values of less than, or equal to 0.05 were considered significant. In the behavioural analyses (I-IV), the data consisted of repeated measures from the same animal that had been measured for time periods of varying lengths. However, the repeatability and the dependency of an observation from a previous one and for each animal was taken into account in the statistical analysis of the data [349]. For continuous responses, a linear mixed model and for count responses a generalized linear Poisson model with generalized estimating equations (GEE) were used. Autoregressive and exchangeable correlation structures were used

accordingly. Type III tests of fixed effects with Sidak's adjustment for multiple comparisons were used.

In experiment V, immunohistochemical data were assessed by two-way analysis of variance after verifying the homogeneity of variances by Levene's test. A full-factorial model with contrast coefficient was calculated to assign the statistical differences among the groups. If the interaction of strain and TCDD-treatment was not statistically significant, the data were analysed by the analysis of variance followed by post-hoc testing (Dunnett's 2-sided t-test and Dunnett's T3-test).

ED_{50} values and their confidence limits for TCDD induced chocolate avoidance (III) were estimated by benchmark dose analysis [Benchmark Dose Software 2.1.2. (BMDS), by US EPA (http://www.epa.gov/ncea/bmds/index.html)]. The continuous Hill model was used for the estimation. The form of the response function in Hill model is:

$$Y[dose] = \gamma + [\nu * dose^n/(k^n + dose^n),$$

where Y[dose] is the observed effect at [dose]; γ = Intercept (control effect, Y_{min}); k = Slope, (ED_{50}); n = Power factor, Hill coefficient; ν = Sign, ($Y_{max} - Y_{min}$).

In the model, variance was assumed to change as a power function of mean value. The non-homogenous variance for the *ith* dose group was estimated as follows:

$$\delta_i^2 = \alpha[\mu(dose_i)]^{\rho},$$

where α = Power parameter, $\mu(dose_i)$ = observed mean (from the model) for the *ith* dose group and ρ = Coefficient of variation.

In the Hill model, ρ was set to 1 because in the present data, variances were proportional to the mean. Furthermore, in the model, α was restricted to be ≥ 1 and $n > 1$. If $\alpha < 1$, then the slope of the dose-response curve becomes infinite at the control dose.

Table 2. Summary of animal experiments (I-V refers to original publication) together with their main findings.

Number of original publication; experiment question(s) asked	**Rat strain(s); sex**	**Group size; age (at exposure); weight (at exposure, mean ± SD)**	**Dose(s) of TCDD[a] (µg/kg); adm. route; adm. volume**	**Studied outcome; measuring period (after TCDD)**	**Main result(s)**
(I) Dose-response of novel food item avoidance?	Line A, B, C rats; male	3 – 10; 7 – 12 wks; 251 ± 43, 276 ±45, 280 ±21g	0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10; *i.g.*; 4 ml/kg	Chocolate consumption; daily for three days	ED_{50} values (for mean chocolate consumption during 3 days): line A: 0.36 µg/kg, line B: 1.07 µg/kg, line C: 0.34 µg/kg.
(II) Time-course of (chocolate) aversion?	Line A; male	8; 12 wks; 258.8 ± 20 g	0, 3; *i.g.;* 4 ml/kg	Chocolate consumption; 0.5 – 12 h periods for 48 h	Chocolate was rejected after TCDD, the difference from controls reached significance by 5.5 h.
(I) Is foodstuff aversion detectable in mice; role of the AHR?	C57BL/6Kuo & AHR knockout mice; male	5; 14 – 26 wks; 28.2 ± 2 & 27.8 ± 2 g	0, 5 and 100; *i.g.*; 10 ml/kg	Chocolate consumption; daily for three days	Aversion of novel chocolate emerged in mice but the effect was more transient in mice compared to rats. TCDD-treated *Ahr*$^{-/-}$ mice liked chocolate.

Table 2 *(Continued)*

(II) Effect of time lag (between exposure and access to chocolate: 0, 1, 7 or 14 days) on aversion? Duration of aversion?	H/W; male	5 - 6; 9 wks; 259.5 ± 16 g	0, 10; *i.g.;* 4 ml/kg	Chocolate consumption; daily for 1 week (time lag 14 days) or 2 weeks (time lag 0, 1, 7), thereafter 24 h on days 27, 34, 41, 48, 55, 62, 69 and 76 after TCDD	Chocolate avoided if the lag 1 day or less. Most persistent aversion if chocolate presentation coincided with TCDD exposure: attenuation of the effect started in 6 weeks.
(II) Is feed texture critical? Is feed avoided if the texture is changed at the exposure?	Line C, male	5 – 6; 15 – 18 wks; 348.6 ± 34 g	0, 3; *i.g.*; 4 ml/kg	Consumption of regular chow, pelleted or powdered; daily for 4 days	Novel texture of familiar feed avoided after TCDD exposure.
(II) The role of conditioned taste aversion?	Line A, male	6; 11 wks; 257.5 ± 26 g	0, 3; *i.p.*; 4 ml/kg	Consumption of powdered feed and chocolate; 24 h on days 0 and 7	At subsequent challenge, the choice coupled to TCDD exposure avoided.
(II) Effect of administration route on the avoidance?	Line A, male	7; 8 wks; 202.2 ± 20 g	0, 3; *i.g.*or *i.p.*; 0, 100 *i.p.;* 4 ml/kg	Consumption of chocolate; daily for 3 days	Administration route affects the response: 3 µg/kg orally has the same effect as 100 µg/kg peripherally.

Table 2 *(Continued)*

(II) Is liquid food equivalent to solid? Does habituation interfere?	H/W, male	4 – 5; 9 wks; 235.9 ± 26 g	0, 3; *i.g.*; 4 ml/kg	Consumption of sucrose (10%) or saccharin (0.25%) solutions; habituation > a month; follow-up daily for 16 days	Also liquid food avoided. Habituation prevented saccharin aversion.
(II) Effect of habituation on chocolate aversion?	L-E, female	4; 10 wks; 172.1 ± 10 g	0, 3; *i.g.*; 4 ml/kg	Consumption of chocolate; habituation > a month; follow-up daily for 8 days	Habituation diminished chocolate aversion, but only for a day.
(III) Differences in feeding and drinking behaviours between H/W and L-E rats?	H/W & L-E; male	13; 17 – 20 wks; 404.4 ± 48 & 316.9 ± 39 g	-	Micro- and macrostructures of feeding and drinking behaviours; 8 days	H/W rats had highest consummatory activity during darkness, L-E rats had two peaks occurring around light transition phases.
(IV) Effect(s) of single exposure of TCDD on feeding and drinking behaviours?	H/W & L-E; male	13 – 14; 18 ± 4 wks; 414.8 ± 41 & 324.3 ± 33 g	0, 10, 100, 1000; *i.g.*; 4 ml/kg	Micro- and macrostructures of feeding and drinking behaviours; 5 – 14 days after TCDD	H/W feed intake diminished due to fewer meals while L-E rats had smaller meals after TCDD. Exposure affected circadian patterns of feeding and drinking: intakes were most decreased during mornings in L-E rats, in H/W rats during constant light or darkness.
(V) Which brain areas are activated by TCDD?	H/W, L-E; male	4 – 5; 14 – 15 wks; 338.0 ± 19 & 297.3 ± 21 g	0, 50; *i.g.*; 4 ml/kg	c-Fos immunohistochemistry after 24 h of TCDD and after 2 h of leptin (1.3 mg/kg, *i.p.*) or NaCl injection	TCDD alone did not alter hypothalamic c-Fos expression after 24 h, during daytime.

[a] 0 is the control: controls were treated by equal route and volume with corn oil, the vehicle for TCDD.

5 Results

5.1 Characteristics of TCDD-induced novel food item avoidance after low dose exposure

The avoidance of novel food items appeared to be an extremely sensitive, rapid and persistent response to TCDD at doses far below those affecting energy intake or body weight gain (**I, II**). Intake of a palatable food item, chocolate, was rapidly diminished in response to TCDD. The difference in the cumulative intake achieved statistical significance at 5.5 hours after the exposure (Fig. 7). In this preference test, control male rats of line A shifted their feeding to chocolate while the TCDD treated counterparts (3 µg/kg, *i.g.*) preferred normal feed.

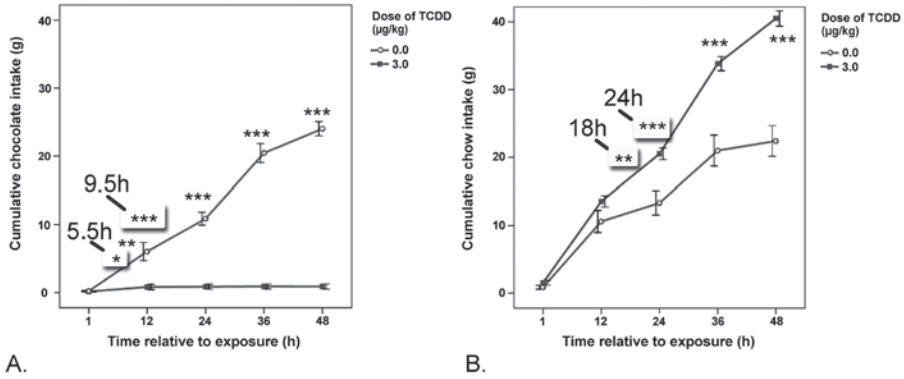

Figure 7. Male line A rats (n = 8 per group) avoided novel chocolate (**A**) offered in conjunction with the exposure of TCDD (3 µg/kg) at the beginning of the darkness (time 0) while controls preferred chocolate to chow (**B**). Lines represent means (± SE) of cumulative intakes within groups. The time points when the differences between groups reached statistical significance are shown in panels (Linear mixed model for repeated measures, ***$p \leq 0.001$, **$0.001 < p \leq 0.01$, *$0.01 < p \leq 0.05$).

The avoidance of novel food item was not restricted solely to chocolate: rats avoided palatable liquids (H/W rats, **II**: Fig. 6) and a feed that had a novel texture (line C rats, **II**: Fig. 3). The total avoidance was most persistent (for several weeks in H/W rats, **II**: Fig. 2) when there was a lag of one day or less with the exposure and the offering of chocolate. TCDD treated H/W male rats ate less chocolate than controls up to 76 days (end of observation) in case the chocolate presentation coincided with the exposure of TCDD. If the lag between exposure and chocolate offer was one week or more, or if the rats had been habituated to a novel food item, aversive behaviour either disappeared in male H/W or line C rats (**II**: Figs. 2 and 6) or was

relieved in L-E females (**II**: Fig. 7). However, the relief of avoidance of chocolate was transient, it lasted only for a day. It is noteworthy that the one month habituation to a liquid saccharin solution in H/W males (**II**: Fig. 6) and for chocolate in L-E females (**II:** Fig. 7) differed between the groups the former being *ad libitum* while the latter was provided in restricted amounts daily. While saccharin contained no energy and did not affect weight gain, by restricting the amount of energy-rich chocolate (0.2 – 0.5 g per day) it was possible to avoid enhanced body weight gain during the habituation period.

The administration route of TCDD appeared to interfere with the aversive outcome (**II**: Fig. 5). In line A male rats, the dose of 3 µg/kg by gavage appeared to have the same effect as 100 µg/kg administered intraperitoneally: the 24 hours' chocolate consumption following exposure was significantly less than that of their corn-oil treated counterparts. At that time, intraperitoneal administration of 3 µg/kg of TCDD did not have any effect on chocolate consumption. Thus, the total energy intake (per metabolic masses of rats, $kJ/kg^{0.67}$) was enhanced due to increased chocolate consumption equally to controls. Nevertheless, within 48 hours of exposure, chocolate consumption was diminished also in the group treated with 3 µg/kg dose intraperitoneally.

5.1.1 Role of the conditioned taste aversion?

Small, 3µg/kg (*i.p.*) dose of TCDD to dioxin-resistant line A rats induced conditioned taste aversion to the novel food they had received at the exposure. One week after the exposure, the rats were challenged with one novel food item and also with the food item they had been offered at the exposure. If the exposed rats were offered powdered feed, they chose chocolate; if they were offered chocolate, they chose powdered feed in the selection test (**II**: Fig. 4).

5.2 Animal species and strain (AHR genotype) determining differences in the feeding and drinking responses; role of the AHR in the regulation of feeding and aversive behaviour

5.2.1 Novel food item avoidance

Within and between species differences in the TCDD-induced novelty avoidance were determined using C57BL/6Kuo male mice and in differently dioxin-sensitive rat lines. To assess the dose-response and ED_{50} value of TCDD-induced novel food item aversion, the mean data of three days' chocolate consumption for the rat lines A, B, and C was used. During the first days at doses below 1 µg/kg (*i.g.*), all rats displayed some variations in their chocolate consumption. Therefore the mean value of three days' consumption was used in the ED_{50} estimation. However, chocolate consumption diminished significantly already on the day of exposure in line A and C rats at doses equal or higher than 3.0 µg/kg and 1.0 µg/kg, respectively. In line B

rats, the reduction reached significance during the second day after the exposure at doses equal, or higher than 3.0 µg/kg. In all (male) rats, exposure caused a total aversion at dose 3.0 µg/kg (Figure 8). The ED_{50} values (µg/kg) appeared to be 0.36 for line A, 1.07 for line B, and 0.34 for line C. For the confidence intervals, see Figure 8 and **I**: Table 1. Neither body weights nor normal feed consumptions (**I:** Fig. 1) were altered in the experiment.

The appearance of TCDD-induced novelty avoidance for chocolate in species other than rats was established by using wildtype C57BL/6Kuo mice. TCDD-induced avoidance for chocolate appeared at both tested doses, 5 and 100 µg/kg (*i.g.*), although the neophobia-like avoidance was weaker and more transient than that encountered in rats, lasting only for two days (**I:** Fig. 2). In control mice, chocolate was palatable despite its novelty: although the difference in chocolate consumption during the exposure day did not quite reach statistical significance between individual TCDD-treated groups and controls (5 µg/kg vs. ctrl: $p = 0.159$ and 100 µg/kg vs.ctrl: $p = 0.078$, linear mixed model), the diminishing effect of TCDD did achieve statistical significance ($p = 0.027$, linear mixed model).

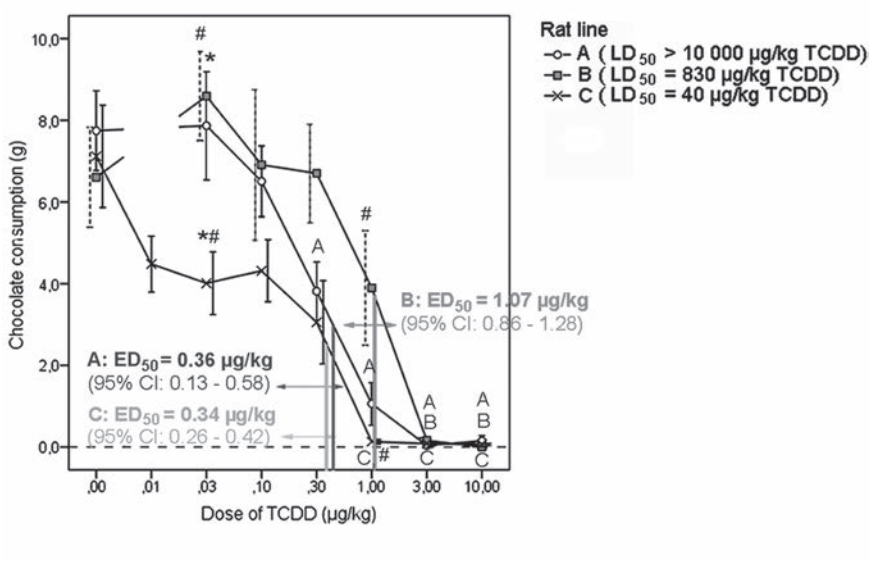

Figure 8. TCDD exposure induced dose-responsive chocolate aversion in differently dioxin-sensitive male rats of lines A, B and C. Average chocolate consumption (mean ± SE) for three consecutive days following TCDD is shown for each group (n = 7, range 3 – 10), but in the statistical evaluation, the repetitive nature of observations was taken into account. Significant differences ($p \leq 0.05$, linear mixed model) from the control level within each strain are shown as capitalised letters A, B, C, respectively. Differences among lines at identical TCDD doses are indicated as follows: *between lines A and C and # between lines B and C. ED_{50} values with 95% confidence intervals were estimated by benchmark dose analysis.

5.2.2 Diurnal micro- and macrostructures of feeding and drinking behaviours in differently TCDD-sensitive rat strains – with and without TCDD

In the automated monitoring of diurnal feeding and drinking activities, TCDD-sensitive L-E and TCDD-resistant H/W rats displayed distinct differences. It is noteworthy that although the figures and tables of original publication (**III**) show the data combined for the whole observation period (seven days), the repeatability of measures was accounted in each rat and measuring day in the analysis of data. Statistical significances of results can be found in the original publications (**III, IV**) with supplementary data included (**IV;** Suppl. Figs. 1 – 3 & Suppl. Tables 1 – 2).

The main difference between rat strains without TCDD-treatment was found in H/W rats which exhibited no peak in their feeding activity during the morning light shift (**III:** Figs. 1 and 2) whereas in L-E rats, the highest activities were recorded during the dawn and dusk (**III:** Figs. 1 – 4). Daytime was the time of least feeding activity in L-E rats whereas H/W rats diminished their feeding and drinking before the dawn and started to increase consumption already during daytime. Thereafter H/W rats increased feeding and drinking steadily towards the night, the time of highest activity in these rats (**III**: Figs. 1 – 4). Meal sizes varied according to the general circadian feeding activity patterns. Furthermore, at the times of lights-on and lights-off L-E rats had more meals that were larger than those of H/W rats (**III:** Fig. I). L-E rats had longer lasting meals and ate at a lower rate compared with H/W rats. Although H/W rats had more drinking episodes than L-E rats, the bouts were larger and lasted longer in L-E throughout the day when compared with H/W rats (**III:** Fig. 3).

TCDD exposure affected intakes at different times of day in these rats: in the sensitive L-E strain, feeding and drinking were suppressed mainly at the dawn, around the light transition phase (at supralethal dose 100 µg/kg, **IV**: Fig. 3A,C & 6C,E) whereas in H/W rats both doses, 100 or 1000 µg/kg reduced food intake (in grams, **IV**: Fig. 3B) during all phases other than the morning. This tended to shift diurnal feeding of H/W rats from daytime to night (after 100 µg/kg) or to evening (after 1000 µg/kg) while the proportion of morning feeding relative to total diurnal food intake remained mainly intact (**IV**: Fig 3D). Drinking was most severely suppressed in H/W rats during night at both doses (**IV**: Fig. 6D). Except for the 10 µg/kg for L-E rats, all TCDD doses induced increased feed spillage (**IV**: Figs. 2C,D).

Following a sublethal dose of 10 µg/kg to L-E rats suppression of daily food intake achieved statistical significance from the fifth day onwards (**IV**: Fig. 2). This diminished intake of food was a result of slightly fewer meals during daytime (**IV**: Fig. 4C). In addition, the total feed intake (g) was suppressed slightly below the controls during morning, the time of active food consumption in this strain (**IV**, Fig. 3A). Total daily drinking tended to remain below the level of the controls

throughout the observation period (**IV**: Fig. 6A), mainly due to the fact that there were fewer drinking bouts in the morning (**IV:** Fig. 7A).

In L-E rats, the high 100 µg/kg dose suppressed meal size (except during daytime, **IV:** Fig. 4C). The phase with the most severe suppression occurred in the morning when the reduction in meal size (from the 1st day after the exposure) was accompanied later by fewer meals (from the 4th day postexposure, **IV:** Figs. 4A,C). In this group, the proportion of daytime drinking enhanced as a result of increased numbers of daytime drinking episodes. Furthermore, the size of a drinking bout showed a downward tendency at all other phases except during the daytime (**IV**: Figs. 7A,E).

In the resistant H/W rats, both TCDD doses caused rather similar alterations in feeding and drinking patterns despite the 10-fold difference in the dose (both sublethal). In the H/W rats, the response in total food intake was bidirectional (**IV:** Figs. 2A,B): meal frequency diminished almost throughout the day supplemented with a slight but significant decrease in meal size during the first few days postexposure (**IV**: Figs. 4B,D). After about one week, meal sizes and their durations started to increase throughout the day although the frequency remained below that exhibited by the controls (**IV:** Figs. 4B,D,F). The drinking was most severely affected during the night, when the decreased size of drinking bouts significantly diminished total amount of drinking (**IV:** Figs. 6D, 7F).

5.2.3 Role of the AHR in the novelty avoidance behaviour

Rats with different AHR structures but fully functional AHR had only minor differences in their sensitivity to TCDD-induced novelty avoidance (**I**) and the characteristics of the effect did not seem to depend on the sensitivity of an animal to overt dioxin toxicity (**II**). In order to seek further support of the possible role of the AHR mediating the TCDD-induced chocolate avoidance, it was studied in *Ahr*$^{-/-}$ mice. In these animals, no signs of exposure related aversion were detectable (**I**: Fig. 2). Corn oil treated control *Ahr*$^{-/-}$ mice were neophobic and avoided novel chocolate on the day of exposure, whereas their TCDD-treated counterparts (100 µg/kg) ate chocolate avidly, in similar amounts to the wildtype control mice. Thus, mouse genotype and TCDD treatment had a significant interactive effect on chocolate consumption [$F(1, 20) = 5.269$, $p < 0.05$, linear mixed model].

5.3 Involvement of the CNS in the early phase of TCDD intoxication

During the lights-on hours of the day, the effects of 50 µg/kg (*i.p.*) exposure to TCDD on the induction of immediate early gene activation were minor in the brains of H/W and L-E rats. Twenty-four hours after the exposure, TCDD induced an increase in the total number of c-Fos positive cells in the dorsal nucleus of premammillary area (**V:** Table 1). Treatment with the positive control, leptin,

increased the total number of c-Fos positive cells and the density of these cells in ventromedial hypothalamic nucleus (VMH) and arcuate nucleus (Arc). Combined treatment with TCDD and leptin increased the total number of c-Fos positive cells in VMH as compared with combined corn oil and saline treatment (**V:** Table 1).

As was the case in H/W rats, TCDD exposure on its own in L-E rats did not have any significant effect on c-Fos-ir in the studied areas of the brain as compared with the control treatment. Treatment with leptin - with or without TCDD exposure - increased the total number of c-Fos positive cells in VMH ($p = 0.003$) and in Arc ($p = 0.013$). In PaVN ($p = 0.088$) and in ventral premammillary nucleus ($p = 0.07$), leptin treatment exhibited a subtle tendency to decrease the activation. Combined treatment with leptin and TCDD in L-E rats did not cause a similar upward trend in c-Fos-ir to that found in H/W rats.

6 Discussion

6.1 Studies of energy homeostasis and body weight regulation – why use TCDD and animal models?

Research on food intake and metabolism is an extensive field of science, but there are only a few compounds capable of inducing persistent anorexia and which could thereby be utilised in the research into diminished feeding and starvation mechanisms. Therefore the exposure of dioxins, especially the 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin - a potent anorexigen, represents a good model to induce a wide variety of effects on metabolism and on comsumption of various foodstuffs. Although a vast amount of data regarding the effects of TCDD does exist, there were no details of time- and dose-responsive definitions of the feeding related outcomes in behaviour. Hence, the focus of this work was characterising *in vivo* effects of TCDD on neural activation as well as on diurnal feeding, drinking, and avoidance behaviours at mainly sublethal TCDD dose levels.

Different animal models have been used to screen the possible factors affecting the variation in responses of TCDD, as the animal model, exposure time and the dose are critical determinants of the outcomes. Animal models with different sensitivities to dioxin toxicity can be exploited to find the explanations for the wasting phenomenom: outcomes occurring equally at rather similar, low dose level in differently dioxin-sensitive animals are of interest or vice versa, those that differ between substrains. Evidently, high (lethal) and low dose effects involve very different phenomena within the studied organisms. The low dose effects are especially interesting with respect to the feeding regulatory pathways involved in the lowered food intake and maintenance of the weight at a lowered level after TCDD administration [276]. In many instances, it is impossible to differentiate the high dose effects from secondary phenomena to diminished feeding and body weight loss, and therefore it is important to undertake time- and dose-response studies with TCDD. One property of interest in TCDD-toxicity is the cytosolic aryl hydrocarbon receptor, AHR. In addition to mediating the effects of xenobiotics, it participates in the regulation of many biological events, as described in a recent book edited by Prof. Pohjanvirta [93]. Hence, by using TCDD, a well-known and potent agonist of the AHR, it is possible to broaden also the understanding of the AHR's role in many physiological and biological processes.

Feeding and its neural regulation have been the targets of scientific studies for decades. Ethologists published consummatory studies already at the beginning of the 20th century [350] and the first publications of neophobia appeared in the 1960's [351]. As early as the 1940's, the importance of hypothalamic nuclei in the feeding regulation was acknowledged [352, 353]. Furthermore, the concept of an idea of neural correlate of

the defended body weight settling point was proposed by the British scientist Gordon Kennedy at that time [147, 148], supported later by Hervey [354]. In the 1960's, Hoebel published his studies regarding self-stimulation of hypothalamic nuclei in the control of feeding and the role of gastric load in this effect [355]. These preliminary works used rather robust techniques in rats, and at that time, little was known about the neural connections involved. Tracing studies and targeted neural stimulation have thereafter provided more information of the whole hypothalamic network, and following exploitation of various molecular and visualisation methods, and identification of neuropeptides – namely leptin [356] – the field has taken huge steps forward.

Behaviour constitutes an essential part of feeding and drinking, and is a good example of an experiment in which *in vivo* models are essential. On the other hand, food intake is one of the most common variables measured in experimental studies. Nevertheless, even in a strictly controlled experiment results can be biased by many factors, exemplified by study settings, sampling frequency, animal model, feeding protocol or gender or species [17, 357-360]. Simply choosing the controls is sometimes a difficult task: the common use of *ad libitum* fed controls can be criticised due to their unnatural excess food consumption and the lack of natural physical activity. These "controls" have appeared to be susceptible to suffer metabolic disorders and age-related deficits in neural functions, leading to shortened life-span in comparison with their restrictively fed counterparts [361, 362]. On the other hand, although a sedentary lifestyle with excess calories may be considered as rather atypical among wild animals, it does resemble current lifestyle among humans rather well.

All experimental *in vivo* studies are limited by the fact that necropsies can be performed only once, and the necropsy in conjunction with its timing represents a significant determinant for the results of the experiment. In many instances, *ex vivo* or *in vitro* studies provide time- and cost-efficient ways to study biological events and their mechanisms. Nevertheless, metabolic and cellular processes that are related in a spatio-temporal manner to the experimental treatment, as is the case with TCDD, result from chain(s) of event(s) that are difficult to model thoroughly in *in vitro*. For many years in several fields of science mechanistic studies will need to rely on animal testing, despite all the progress in *in vitro* methodologies and the techniques emerging from computerised data modelling. The maximal utilisation of experimental data includes all of these parameters, thereby favouring also the reduction and refinement of the animal experiments. It is a fact that without animal experiments, a vast amount of the current knowledge in medical science and in drug therapies would never have been discovered, as has been concluded by many scientists [161, 363, 364].

6.2 Characteristics of novel food item avoidance as clues of TCDD-targeted regulatory pathways - neophobia or CTA?

Several memory processes are involved in feeding (e.g. review [201]). Taste learning is a necessary and adaptive response appearing during the evolution to ensure the proper identification of nutritive foods and to promote the dietary variability, which can enhance the fitness of the individual. Bearing in mind that rodents are unable to vomit, when confronted with a new food item they may use two different strategies to avoid intoxication as discussed in Chapter 2.3.2. It is known that while neophobia dissipates and appetitive memory is formed if no malaise occurs after the tasting of a new food [154], CTA is routinely acquired after a single trial with CS and US, and may persist for extended periods, although a short time lag (from minutes to hours) is needed for CTA to develop [203]. US intensity (e.g. dose of LiCl, a typical malaise inducing compound) affects CTA, i.e. a low intensity US may not lead to altered gene expression and protein synthesis, and thus is not able to evoke permanent modifications in neural circuits.

Previously it had been shown that TCDD-exposure evoked an intense novel food aversion in the rats of the same strain or line [12] that were used in this thesis. Previous results were in favour of TCDD-induced neophobia although the interpretation of the studies was complicated by the fact that in most cases there were several palatable food items available simultaneously, and the rat was expected to make a choice between them. Furthermore, when Tuomisto and coworkers studied the role of habituation on TCDD-induced food aversion, the availability of palatable food items (*ad. lib.*) increased the total energy intake, and thus the weight gain in rats [12]. Since the doses were also rather high, one could speculate that the rats had attained the lowered body weight level as a response to TCDD by diminishing their consumption of high energy foodstuffs. Here it was confirmed that chocolate avoidance was not related to the body weight change: neither the habituation nor the TCDD exposure affected weight gain. In further support of specific avoidance behaviour is the fact that in these experiments, exposed rats had not lost their normal appetite since their consumption of standard feed remained unaffected. The reason for the strong avoidance of chocolate remains unknown, but may be related to its properties: it is a mixture of many nutrients and tastes, and has high energy content. Although fat appeared to be unfavourable after TCDD [284, 286], in the previous study the cheese aversion was not as intense as that encountered with chocolate [12]. Furthermore, aversive behaviour can be affected by the stimulus itself [191, 365]. It is also possible that cacao-derived compounds of chocolate (such as flavonoids)[366] are able to activate AHR and potentiate the aversive effect of TCDD.

The discrimination between the effects, whether TCDD caused novelty avoidance or CTA, is clearly dependent on the study settings. Many findings here are in favour of novelty avoidance, i.e neophobia induced by TCDD while some parts of the data support a role for CTA. Intense novelty avoidance was closely

linked to exposure, occurring at extremely low dose levels, and emerging very rapidly in adult rats - within hours (**I**, **II**). Avoidance was not restricted to chocolate: very low doses of TCDD caused aversion of liquid food items or of familiar feed presented in a novel form. When chocolate was offered for the first time one week postexposure, neophobic response was similar to controls. Two weeks postexposure there were no signs of neophobic response in both TCDD-treated and control animals, raising the possibility that rats had accustomed to the cholate smell in the animal room by that time. Preceeding habituation even for a month (with an item not affecting daily energy intake) did not prevent chocolate aversion. The effect of habituation might be stimulus dependent as the preceeding habituation with liquid saccharin (*ad. lib*.) was able to alleviate avoidance. This difference in observations might also derive from different genders, as it has been shown that prenatal exposure to TCDD induced saccharin avoidance in female pups but not in males [367]. The gender differences in responses to feeding challenges are found also in the H/W and L-E rats [279, 284]. One possibility to explain the difference in the outcomes may arise from different habituation. The energy-deficient saccharin was offered freely throughout the day to the male H/W rats whereas the chocolate was only a tiny piece offered during daytime to the female L-E rats. Thus, further studies are still needed to clarify the possible gender differences and the effect of preceeding habituation protocol in TCDD induced aversion.

CTA was supported by the finding that only that food item offered at the exposure was avoided one week postexposure (**II**). However, studies to reveal the role of CTA in the feeding responses following TCDD are complicated by the persistence of TCDD in the body. In general, offering of the CS without US should ultimately lead to extinction [212], but the kinetics and long elimination half-life of TCDD challenge the testing of CTA-response without US. With repeated offers of CS, the US should not be present any longer. In experiments with TCDD this means that in a (full-grown) rat the body-burden of TCDD-exposure is still almost the same one week later as it was on the day of exposure (the elimination following first-order kinetics and the elimination half-life of a single sublethal dose of TCDD being about 16 - 31 days [32, 368, 369]). In that sense the time of one week used here to test the CTA response was definitely too short. In the previous studies conducted by Pohjanvirta and coworkers only a high dose of TCDD (1000 µg/kg) was effective in evoking a CTA response in H/W males [279]. However, since a low dose of TCDD (3 µg/kg) to resistant rats caused a long-lasting chocolate aversion, with attenuation of this response starting only after 6 wks (**II**), this is interpreted that a low dose of TCDD is able to form and consolidate persistent, long-term aversive memory in rats. Although after a week, there was a positive CTA response, the question of whether the TCDD-induced avoidance is merely defective alleviation of neophobia or related to conditioned taste aversion cannot be unambiguously answered. These results would need confirmation with testing of the CTA response after a sufficiently long enough time following a low dose exposure to TCDD. In practice, this would

require at least 4 – 5 elinimation half-lives (when there is 1/16 or 1/32 of the dose remaining in rats) representing about 64 – 155 days, depending on the interindividual and interstrain variation [40, 369]. Although that is a substantial part of a full-grown rat's lifespan, these kinds of behavioural experiments can be performed. Houpt and coworkers have detected the CTA response and c-Fos activation in the NTS for as long as 6 months, after only a few pairings with US and CS which had been conducted 6 months previously [230].

Although one cannot make any precise definition of avoidance behaviour, it was an extremely sensitive response and there were hardly any sensitivity differences in the dose-responses between the strains (representing the type I dioxin response [85]) (**I**). TCDD efficacy values for the novelty aversion were not calculated, neither the relative changes in efficacy ratios. Therefore, the classification of the aversion as an endpoint of TCDD toxicity in the rats with different *AHR* genoypes is based on the similarities in the ED_{50} values for the aversion and for the EROD-activity (Figure 8 and [87], and **I,** Table 1). The calculations of exact estimates for efficacy [85, 87] would provide more detailed information of the aversion.

Whether the avoidance can be extended to other behaviours in addition to feeding remains to be clarified, but surprisingly, even their regular feed when dispensed in a novel form was recognised as novel and avoided by the exposed rats. This excludes the possibility of novel tastes or flavours being the sole inducers of avoidance behaviour. It should be mentioned that it has been shown previously that TCDD-treated rats do not suffer deficits in their tasting abilities [275].

Based on this data, it seems that after about two elimination half-lives (after 6 weeks) the learning process of new association with chocolate started in H/W rats (**II**), but it remains to be tested whether the malaise-inducing taste, chocolate, had become palatable again when the TCDD had been elimated, requiring about 110 days in H/W rats [369]. Possibly an even longer time period would be needed, as the effects of (rather high, 1000 µg/kg) TCDD dosing on gastric preloads were tested and positive responses observed even after 100 days [370]. Studies of this phenomenom and its regulatory pathways could shed some light on this behavioural phenomenon, and in that respect it might be possible to identify which taste memory process is responsible for the effect. Interesting target areas would be NTS and PBN, since the taste stimuli communicates first of all with NTS and PBN [200, 201]. Furthermore, to ensure the consolidation of long-term memory, i.e. persistent CTA, permanent modifications need to occur in the regulatory sites – e.g. IC, CeA and basolateral amygdala [201, 212]. This applies also to the strengthening of appetitive memory trace during the CTA extinction, with responses occurring in IC, perirhinal cortex and amygdala [232]. Hence, with regard to the possibility that activation of AHR by TCDD causes CTA that becomes extinguished when the dose is eliminated means that the new association with the taste should be learned, along with the corresponding changes in protein synthesis should occur in the target areas of the brain [191, 371, 372]. A commonly used method to study the involvement of selected

brain areas is protein synthesis inhibition (e.g. [232]). Furthermore, use of the novel imaging methods of the brain (such as functional magnetic resonance imaging or positron emission tomography) could provide enlightening insights into the processes involved in the actions of TCDD. These could reveal the temporal responses in very same animals - provided that the repeated anaesthesia and other treatments would not harm the studied outcome.

6.3 Circadian ingestive patterns in TCDD-sensitive and –resistant rats providing indications of physiological status with and without TCDD

Food consumption in TCDD induced wasting is a critical variable, and the extensive difference in dioxin-sensitivity among the two rat strains makes this animal model a good tool with which to study the effects of TCDD exposure. However, the circadian consummatory patterns of these strains were unknown even without TCDD exposure, and therefore an automated monitoring system was devised to allow monitoring of food and water intakes throughout the day.

The results of this thesis showed that circadian activities of feeding and drinking were very different between the rat strains (**III**). Especially during the daytime, the hunger state differed between the H/W and L-E rats. Although in both strains most feeding (~2/3 or more of total eaten amount) and drinking (~3/4 of total number of licks) occurred during darkness (lights were off from 7 p.m. till 7 a.m.), L-E rats exhibited two feeding peaks around the time of light changes. The daytime, fully illuminated phase was the time of lowest feeding activity, with fewest and smallest meals of shortest duration in L-E. In contrast, H/W rats had their lowest intake around the morning light shift. They started to feed before noon and consumed increasing amounts of feed towards the night, with a similar mode of activity being detectable in drinking.

This observation raises many issues which need to be considered. First of all hunger and satiety signalling in these animals would be predicted to be in opposite phases in the morning or daytime, during normal working hours and thus, at the most typical time for collecting tissue samples. Furthermore, these differences in circadian activity may reflect differences in the circadian fluctuation of regulatory genes and proteins. Although it is not known in these two rat strains, there is a report that in two other rat strains the different behavioural rhythms did reflect fluctuations in many physiologic parameters (such as temperature or blood glucose) as well as hippocampal serotonin secretion [357]. It is known that in mice, *Ahr* transcripts express tissue-specific fluctuation patterns during the day [329]. Thus, depending on the time of observation, there can be between two to three-fold differences in AHR protein [328] or mRNA expression [329]. H/W and L-E rats are known to display about two-to three-fold differences in *Ahr* expression with and without TCDD [84, 373], however: the impact of circadian fluctuation was not evaluated

in these experiments. Collectively, these findings highlight the importance of circadian clockwork to be taken into account when this rat model is utilised in the experiments. In these rats further studies to clarify the amount of circadian fluctuation in the gene and protein expression would be essential.

As H/W rats with a mutated AHR grow at a faster rate, being heavier than L-E rats at adulthood, it would be interesting to study whether there is some overlapping regulatory pathway between metabolism, the circadian clockwork circuitry, and the AHR which could account for the difference in phenotype. In order to pinpoint the differences in feeding that may reflect the difference in the body weight between H/W and L-E, the variables with largest differences in behaviour should be evaluated. It is noteworthy that the total daily food consumption (grams) on two selected days (**III**, Table 1) did not differ between the strains, especially if consumption was related to body weight. The differences appeared when one considered the circadian timing of feeding and drinking behaviours, and the rates of consumption. Throughout the day, the feeding rate was higher in H/W rats than in L-E, but meals lasted longer in L-E rats. The drinking bouts of L-E rats were also longer almost throughout the day compared with those of H/W rats. Meal and drinking bout sizes varied during the day but in general, the fluctuation patterns followed the circadian feeding activity patterns in both strains. In general, H/W rats ate more and had more numerous meals during daytime and night in comparison with L-E rats. It has been shown that caloric intake does not necessarily determine the differences in weight or adiposity [264-266, 374], but meal patterns during a day and. microstructures of meals are important determinants for the weight gain [265, 374]. Furthermore, there exist data indicating that correct circadian rhythmicity is essential for the control of weight and metabolic health [264, 266, 375]. Thus, descriptive behavioural data of circadian feeding and drinking patterns can reflect the general activity rhythm and may provide some indications of body weight control in living organisms. In that sense the higher daytime activity of H/W rats may make them more vulnerable to gain weight in comparison with L-E rats.

In the presence of a potent AHR-agonist, TCDD, feeding and drinking patterns were differently altered in rats (**IV**). Both strains were treated with two different doses as a single exposure in the middle of the day: in the L-E rats the 10 µg/kg dose was sublethal (but high) and 100 µg/kg lethal [50]. In H/W rats both doses were clearly non-lethal [72]. In all groups, feed intake and body weight followed a downhill course following exposure. These responses have been classified as type II dioxin responses [107]. Sublethal TCDD dose induced a similar reduction in feeding in both H/W groups, this being larger (about 50% reduction) and occurring more quickly (within two days) than that induced by 10 µg/kg in L-E rats (about 30% reduction after five days postexposure). In general, the declines in feeding and drinking occurred mainly at the times of the highest consummatory activities: in L-E rats during the morning hours and in H/W in the middle of the day or night. TCDD induced phase-shifts in circadian rhythmicity of feeding and drinking, the exception being the dose 10 µg/kg

(for L-E rats). In L-E rats (following 100µg/kg dose) severe hypophagia flattened the diurnal peaks in the feeding, but as the daytime food intake was not affected (daytime food intake remained at its low level throughout the 5-day study period), the relative proportion of daytime feeding increased above the controls during the last two days. A similar phase-shift to enhanced daytime activity was detectable in drinking; in fact, the number of daytime drinking episodes displayed an increasing tendency. In contrast, the previously observed shift towards increased daytime feeding in H/W rats [274, 275] was now not observable during the first two weeks after TCDD. However, the phase-shift noted by Pohjanvirta and Tuomisto appeared at two weeks postexposure, following a 1000 µg/kg dose or higher. The present data indicates that during the first two weeks, the consummatory activities shift towards evening (1000 µg/kg) or night (100 µg/kg) in H/W rat. Feed and water intake remained close to zero during the mornings and in the daytime, but by the end of study, during the last four days rats dosed with 1000 µg/kg slightly appeared to increase their daytime feed consumption. Thus, the observation period for two weeks appeared to be too short to detect alterations in feeding and drinking patterns in resistant rats during the whole recovery phase and thereafter. It remained unclear whether circadian patterns in H/W rats were still about to change, and if this were to occur, how and when it happens.

The changes in the eating rate as a response to TCDD appeared to depend on meal size (**IV**, supplementary data). If the smallest meals (below 0.54 g) were excluded, in all of the TCDD-dosed groups the eating rate became slower as compared with their controls, the effect being least pronounced in 10 µg/kg dosed L-E group. On the other hand, if all meals were included, eating rates appeared to display an uphill curve. The consumption of very small meals has a clear elevating effect on the eating rate (**III**), thus the increased rate of all meals including the smallest ones reflects the increased numbers of very small meals after the TCDD. On the other hand, this bidirectional response is a good example of how the definition of the term "meal" affects the result(s). It is common that this kind of data is sensitive to the methodology used in the analysis, and in the definitions of meal or drinking episode. Therefore comparisons between studies can be difficult and special attention needs to be paid to the methodological issues [376, 377].

It is of interest that the treatment with sublethal dose of TCDD appeared in some way to balance the feeding rhythm of H/W rats: from the first day postexposure, a decline to almost zero feed intake occurred during daytime, and as a result the relative proportion of total food consumption during evening and night was enhanced. In addition, TCDD diminished the meal frequency of H/W rats, most consistently during the daytime throughout the study. At the sublethal 10 µg/kg dose in L-E rats, the total feed intake (g) was suppressed slightly below the controls during morning, the time of active food consumption in this strain. However, the effect did not change their diurnal distribution of feed intake. Furthermore, a slight but on some days a statistically significant reduction in meal number occurred

during the daytime in these rats. These feeding effects might be considered as type I responses to dioxin, because both phenomena occurred at the sublethal doses in both rat strains. However, additional experiments are needed to verify this hypothesis and to study the dose-responses of these feeding effects, at equally low doses in both strains. In this experiment the initiation of weight loss occurred in both strains, but it remained unknown whether the timing of diminished intake at the usual activity phase and/or the diminished motivation to start eating (diminished meal frequency, especially during daytime) is the critical factor(s) for weight loss. However, recently the meal frequency is suggested to be an important determinant for the weight gain in humans [378].

In the sensitive L-E rats, a lethal dose seemed to permanently override any factors related to within-meal satiation, to the physiological control of meal size, while the factors related to meal initiation i.e. satiety remained almost unchanged (in mornings also the satiety was increased). In other words, physiological control of feeding and body weight maintenance had been overwhelmed by enhanced satiation of meals after the lethal dose, and no correction in feeding occurred after the exposure. There was no attempt to increase food intake and thus prevent death. On the other hand, lethal and close to lethal doses are known to induce drastic effects in various tissues [3, 53], at the same time as there is a collapse in nutritional balance by disturbances in fat and glucose metabolism [46, 86, 91], and these changes also contribute to wasting and death. In the progress towards severe wasting, food intake appears to play a minor role. This hypothesis is supported by the facts that provision of additional energy or other dietary manipulations (such as force-feeding) were not able to prevent lethality, although these actions were able to modify the dioxin-induced weight loss and time to death [283, 284].

It would be important to clarify the changes in circadian patterns also in spontaneous activity and/or in indirect or direct measures of intermediary metabolism or thermogenesis in these rats. For example, activity data would have provided more information of the increased spilling of feed among TCDD-treated rats, a behaviour that was first reported some time ago [273] although its etiology is unknown. It certainly represents a motivated behaviour to start a meal as a rat takes a pellet from feeder but for some reason the pellet is not consumed, instead it is dropped. The reason might relate to increased satiety (as was observed following TCDD exposure) or it is possible that dioxin exposure causes a stereotypic stress response detectable as feed spillage, or the TCDD-treated rats may simply have increased need for gnawing [379]. Although spillage was detected in both strains, blood chemistry results of H/W and L-E rats (with the dose of 50 or 100 µg/kg) did not show a similar stress response between strains: in L-E rats serum corticosterone levels were increased or at least displayed an upward tendency in comparison with controls whereas in H/W rats this did not happen [295, 380]. Thus, one hypothesis to explain the increased spillage is that TCDD induces a hoarding behaviour: instead of accumulating extra energy as fat, the animal is trying to store extra food by hoarding

[381]. Hoarding could be viewed as an attempt to maintain body weight set-point at a target level despite fluctuations in energy sources [382]. The hypothesis of increased hoarding supports the original proposal that TCDD induced wasting was a secondary response to TCDD with the main target being the lowered body weight set-point [276]. Further studies of food spillage and its regulation could provide important information of TCDD toxicity.

6.4 Role of the AHR in the regulation of aversive behaviour or in the control of feeding?

Novelty avoidance is induced in rats at extremely low doses of TCDD, independently of their sensitivity to dioxin toxicity. The lack of any avoidance behaviour in *Ahr*$^{-/-}$ mice strongly supports the need for a functional AHR in this response. The dose-response of novelty avoidance following TCDD exposure in differently dioxin-sensitive rats is well correlated to the dose-response of CYP-enzyme induction in liver [87]. It can be postulated that avoidance behaviour represents a way to restrict consumption of potentially harmful foods whose consumption is stringently correlated to metabolic activation. It is not known how the activated AHR mediates its effects to prevent novel food consumption, but it seems that consumption is prevented for a considerable time (**II**), reflecting possibly the long elimination half-life of TCDD.

In phylogenetic terms, AHR is an ancient protein with a considerable degree of structural and functional homology between different vertebrate and even invertebrate classes, although the primitive AHR forms in some invertebrates are incapable of binding dioxin [383, 384]. In many freshwater or marine invertebrate species, there seems to be a lack of AHR homologues, or the issue is unknown. However, in these animals, induction of CYP-enzymes does occur in response to environmental PAH exposure [385, 386]. Interestingly, freshwater invertebrates are known to display extremely sensitive behaviour to avoid PAH-polluted sediments in their habitats and they strive to minimise their own exposure to these pollutants [387]. Thus, by some unknown mechanism they are able to sense PAH-compounds and actively avoid them by adjusting their behaviour accordingly; a strikingly similar phenomenom appears in these invertebrates as found in rodents. Whether avoidance is a more general and equally sensitive response across other species throughout the animal kingdom will require further experiments.

The role of AHR in mediating neophobia or in the regulation of conditioned taste aversion and taste learning will only be clarified by conducting further mechanistic experiments. Future mechanistic studies are important, as there are human individuals who are highly predisposed to food neophobia and in addition, neophobia is a common observation in some sicknesses. For neophobic individuals or those suffering from illness-induced neophobia or cancer cachexia, it is challenging to devise new diets or even to provide a normal and nutritionally

balanced energy intake. The impact of hedonic and reward value of food and psychological distress is likely to have an important role in loss of appetite e.g. in cancer patients, and therefore no single treatment is available to increase their appetites [388]. Some strategies have been applied to combat food neophobia (e.g. [389, 390]). Food neophobia seems to have inherited, genetic components in human populations although the genes involved are not known [391-393]. Interestingly, in studies of neophobia, the list of avoided foodstuffs contains many compounds that are able to activate AHR: not only fruits and vegetables (containing indolo(3,2-b)carbazole and flavonoids [394, 395]), but also herring was disliked by Finnish young adults who were neophobic [392]. Baltic herring is known to contain dioxin-like chemicals and therefore its consumption is restricted in Finland by EU regulations (latest update on year 2011: EU act 1259/2011). Thus, it could be hypothetised that metabolic induction and activation of AHR (at least in vertebrates) could be one of the factors linking the signals from the food and the environment to the behavioural responses. At present nothing is known about the AHR's role in human neophobia. Nevertheless, the behavioural findings of TCDD-induced novelty avoidance in rodents clearly point towards a role for AHR in the host defence to prevent the consumption of potentially harmful foodstuffs.

AHR is widely expressed in the organs acting as barriers of the body. In addition to liver or BBB [396, 397], it has been found in the skin, lungs, placenta and in the intestine (e.g. [396, 398-400]). In-deed, there are several findings pointing to a physiological role for the AHR in host defence. The ligand-activated AHR induces inflammatory responses [401-403] but on the other hand, recently it has been shown that a functional AHR is needed for the maintenance of the immune system in the intestine [135, 137] where AHR has a role in mediating anti-inflammatory signals (reviewed in [404]). Furthermore, the findings of this thesis support the proposal of gastrointestinal target(s) for TCDD action. At sublethal doses, the administration route affected the aversion outcome (**II**): after intraperitoneal and intragastric administration, the latter route proved to be more effective at diminishing intake of the novel food item. Although avoidance behaviour and wasting syndrome appear at a very different doses, and represent two different phenomena (type I and type II responses, respectively), it is possible that there is some common peripheral pathway or mechanism involved in the regulation. The early work Stahl and Rozman [405] suggested that in order to observe diminished feeding a peripheral administration route was needed. Further support to the proposal that there are responsive sites to TCDD within GI-tract comes from the early finding from our laboratory. TCDD appeared to increase the sensitivity of exposed rats to nutrient information arising from the GI-tract [370]. TCDD sensitises rats to postingestive satiety signals and H/W rats showed aberrant responses to metabolic challenges, for example they were unable to increase feeding after 2-deoxy-glucose treatment [274, 275]. It has also been shown that after TCDD exposure fat is the least preferred macronutrient [12, 286]. Interestingly, AHR appears to work in concert with fatty acid

translocase Cluster of Differentiation 36 (CD36) [143, 406], one of the proteins involved in 'fat taste'. Fatty-acid specific receptors are expressed within taste receptor cells in both the oral cavity and GI-tract where they participate in the regulation of fat intake [159]. It is not known whether the AHR has any role in the tasting system, but if an AHR-agonist can modify CD36 expression also in taste receptor cells, it may be directly able to modulate feeding responses within the GI-tract. Furthermore, stomach is known to be a target tissue of AHR activation in animals with a constitutively active AHR [407]. In *Ahr*$^{-/-}$ mice focal proliferative lesions were observed, suggesting that AHR regulation may be involved in cell homeostasis in gastric epithelium [121]. Moreover, also human patients have suffered from GI-symptoms in cases of severe TCDD intoxication [408, 409]. During recent years new discoveries have been made regarding the interplay of gut-brain axis. There is evidence that in the gut, enteroendocrine cells (secreting peptides) and enterochromaffin cells (secreting serotonin) use the same signalling pathways as the taste and olfaction, and they are therefore able to respond to luminal contents within stomach and intestine directly by affecting taste receptors and transmitter or peptide release (for a review, see [410]). It would be interesting to determine how the GI-tract and the tasting and odour pathways are involved in effects of TCDD. Existing data are scant, but our own short-term study showed that bilateral transection of the chorda tympani nerve prevented the decrease in feeding after TCDD (8.5 µg/kg, L-E rats) on the third day, supporting a contribution of the tasting system to the feeding responses elicited by TCDD (unpublished findings). The chorda tympani nerve is a branch of the facial nerve, mediating both the taste and trigeminal sensation of the tongue [411], thus it would be highly interesting to study whether its transection has any effect on novelty avoidance.

It is not known whether the difference between H/W and L-E in growth rate results from the difference in the AHR structure, but it seems that an interplay between AHR signaling and food intake regulation, circadian rhythmicity and energy metabolism do contribute to normal body weight level. Recent data from these rats have revealed further differences between the strains in terms of the genes involved in many metabolic pathways [86], and serum levels of leptin and insulin which were in H/W rats about two-fold higher than the corresponding values in L-E rats [295]. The circadian fluctuation in these parametres [412] was not evaluated in these experiments and it may provide some amount of variation (and confounding) to the responses. Nevertheless, it is known that AHR is involved in many physiological processes related to growth [121, 124, 126], and the early growth of *Ahr*$^{-/-}$ mice is retarded [75, 119]. It is possible that the difference in the AHR structure combined with its ability to interfere with circadian clockwork and energy metabolism contributes to the differences in phenotype and in the feeding behaviours of L-E and H/W rats. Nevertheless, in the presence of TCDD, phase-shifts in feeding and drinking were observed. As the AHR, circadian clockwork and intermediary and xenobiotic metabolism are linked together (e.g. [142, 329, 337]), the findings of the circadian activity

(**III**, **IV**) emphasize the interplay between the AHR and the circadian clockwork in feeding regulation, with and without TCDD.

These studies mainly utilised male animals, but there are known to be gender differences in responses to feeding challenges after dioxin [(**II**) and e.g. [279, 284]]. Furthermore, in the animals used in these experiments there is a contrasting gender-related sensitivity difference to TCDD lethality: male rats are more resistant than females while in mice the situation is inverted (Table 1). Many biological events or toxic outcomes have not been studied in female animals (or the estrous cycle has not been taken into account in the data analysis). Often male animals are used in order to avoid estrous cycle dependent rhythmic oscillations in several hormones, metabolism, and even in feeding [329, 412, 413]. Although it is known that TCDD affects expression of many hormones including estrogen (e.g.[414]) and this may as such evoke effects on feeding behaviour [413] it would be important to clarify the TCDD-induced feeding behavioural responses also in females.

6.5 Involvement of CNS in dioxin-induced acute responses - some possible signalling pathways

The mechanistic pathway(s) through which activated AHR modulates feeding and drinking remained obscure since the focus of this thesis was not to examine regulatory mechanisms. Thus, in this respect this thesis raises more questions than it answers, but the results presented here may help to make more targeted questions than previously possible. Now it seems clear that TCDD exposure has distinct effects on both feeding regulation and on taste memory processes. These studies showed that TCDD influenced (at least) two distinct pathways of feeding regulation: the first is involved in regulating aversive behaviour (representing the type I dioxin response [85]) and the second mediating TCDD-induced wasting (type II dioxin response). Whether the regulatory pathways of these effects overlap at any phase of dioxin intoxication will be a very interesting question to be solved in future experiments.

The CNS has multiple functions in energy homeostasis controlling meals, long-term energy balance, and locomotor and oromotor activities (Figure 5). Additionally, it handles external information originating from the ambient environment and has control over emotions, cognition, learning and memory - processes involved also in feeding behaviour. Many important sites within brain are involved in the regulation (as overviewed in Chapter 2.3 and shown in Figure 5), but hypothalamus is considered to occupy a key position to control feeding and energy balance [16, 188]. Therefore the analysis of c-Fos focused especially on hypothalamic areas where it was anticipated to find the early effects of TCDD exposure in L-E and H/W rats. In a previous study c-Fos protein levels were shown to be upregulated but only after 3 – 4 days of TCDD exposure [346]. However, in the study (**V**) the effects of TCDD (50 µg/kg) alone were minor during the daytime, in both sensitive and

resistant rat strains. This was slightly surprising since in another study with similar treatments for the rats of the same strain, there were profound alterations in hypothalamic mRNA expression of regulatory peptides [415]. C-Fos staining proved to be a valid method because leptin, a positive control in this study, appeared to induce c-Fos activity in two target areas, VMH and Arc, in both rats similarly as has been reported [416, 417]. It is likely that specific neuropeptide stainings might have resulted in more similar outcomes, and might have produced supporting data to the mRNA work. Nevertheless, the results of the activated brain areas reflect the findings of the feeding behaviour studies: during daytime H/W rats were more active than L-E rats, and more pronounced effects both to the TCDD and to the leptin treatment were found in the brains of H/W rats when they were compared with L-E rats.

During the early phase of intoxication TCDD induced more pronounced effects on drinking than on feeding (**IV**). Unfortunately, the main drinking regulatory areas (pituitary gland, NTS, circumventricular organs, or anterior cingulate cortex and IC) [418, 419] were not analysed in the c-Fos study. Moreover, hindbrain areas or subregions of cortical structures might be interesting target areas in dioxin intoxication because these extra-hypothalamic areas are known to have a regulatory role in conditioned taste aversion and novel food item avoidance [200, 201] - behaviours which appeared soon after TCDD exposure. C-Fos activity could be used to screen the areas in the brain at different stages of CTA [201], e.g. in PBN *Fos*-mediated gene transcription seems to be necessary for the CTA memory formation [420]. However, screening of these areas and their involvement in dioxin effects would need a more precise timing of the sampling, and different experimental set-ups to clarify the aversive responses and those mediating the feeding and drinking behaviours.

In order to search the regulatory pathway targeted by the TCDD, feeding effects of TCDD can be compared to those of anorectic agents or treatments to tackle obesity. Many of them have been shown to diminish meal size, i.e. they increase intrameal satiation by facilitating processes leading to meal termination. For example, gastric surgery has been claimed to cause rather similar effects as the lethal dose of TCDD in L-E rats: diminished meal size and slowing down of meal rate, leading to a permanent weight loss. Furthermore, gastric surgery caused an avoidance of fat as an energy source whereas increased energy expenditure played no role in weight loss, these effects being similar in both rats [421] and humans [422, 423]. In these effects, GLP-1 seemed to be involved (reviewed in [424]). One can also compare TCDD effects with the effects of anorectic compounds. For example, fenfluramine is an indirect serotonergic agonist and it seemed to diminish meal size and eating rate at low doses, although at 2- 4 times higher doses it also reduced meal frequency. At lower, but still anorectic doses of fenfluramine did not affect drinking at all while at higher doses it reduced selectively feeding-related, prandial drinking [376]. Although meal related regulation or circadian expression patterns of serotonergic transmission in the brains of L-E or H/W are unknown, Unkila et al. [72] showed that brain serotonergic activity was affected by TCDD in sensitive L-E but not in

resistant H/W rats. The turn-over of serotonin in several areas of the brain was elevated, and plasma free tryptophan negatively correlated with the body weight changes following exposure [425]. Furthermore, immediately postexposure (within 1 – 4 hours) activities of hypothalamic dopaminergic and serotonergic systems have been shown to be lowered in L-E rats (H/W rats were not studied) [426], although subsequently it has been confirmed that the dopaminergic system can be induced by TCDD, in different study set-ups [427, 428]. Since site-specific perturbations in serotonergic and/or dopaminergic systems have potential to modulate feeding behaviour [429, 430], it is possible that these neurotransmitters are involved in the behavioural responses induced by TCDD. As dopamine release is strongly and positively related to desire (the food reward value) and increase in the motivational aspects of feeding [15, 188], reduced dopaminergic activity may make the food less appetising after TCDD exposure. Serotonergic and dopaminergic systems are also important mediators in the taste memory encoding [201]. Moreover, in addition to the fact that increased serotonergic activity is known to be anorectic, it is also known to increase drinking [431], evidence that the increased seronergic activity may have a role in the behavioural responses in L-E rats during early phases of the intoxication following a lethal TCDD dose.

There is a study with hypothalamic blocks that provided some indications of the regulatory pathways laying behind the feeding and drinking behavioural effects of TCDD, during daytime [415]. Hypothalamic blocks were collected from the brains of L-E and H/W rats after similar treatments as in (**V**). The findings revealed that 24 hours after TCDD in L-E rats the mRNA level of the ghrelin receptor was clearly increased whereas that of the neuropeptide Y 5-receptor (receptor for neuropeptide-Y) was decreased in comparison to controls. In H/W rats, a corresponding increase in hunger signal ghrelin did not exist whereas neuropeptide Y 5-receptor mRNA expression was decreased [415]. These findings are supported by the ghrelin assays conducted in serum samples. In serum, ghrelin levels were increased in L-E rats immediately and remained high (i.e. on days 1, 4 and 10 after 100 µg/kg dose of TCDD), whereas in H/W rats a significant increase in ghrelin was evidenced only at the last measurement (10 days post exposure) [295], reflecting the increase in feeding at the start of recovery phase (**IV**). Thus, the timing of behavioural responses may reflect the timing of ghrelin signalling: in both rats the ghrelin increase indicates an emerging hunger state, but due to enhanced satiation in L-E rats and increased satiety in H/W rats, food intakes remain at low level (**IV**). Since ghrelin is a short-lived hunger signal, it is believed to regulate the latency to eat and thereby meal frequency having no effect on meal size [161]. In order to initiate feeding, ghrelin not only activates the NPY system, but also the agouti-related petide and orexin neurons. NPY activation leads to an increase in food intake (NPY increases meal size by acting in the lateral hypothalamus and meal frequency by acting in the PaVN [432]), prolonged meal duration, and delayed satiety whereas the effects of orexins are linked to increased arousal, although they also modulate hindbrain satiety signalling

[432, 433]. Thus, studies of the downstream and upstream pathways in ghrelin-signaling after TCDD would be of special interest. Research in this field is scant, in addition to the mRNA study by Linden *et. al.* [415], increased expression of NPY mRNA has been reported in Sprague-Dawlay rat hypothalami at a sublethal dose (15 µg/kg) [434]. Nevertheless, this response was detected only at six days post exposure and therefore it may reflect a secondary response to TCDD.

Another possible peptide involved in the feeding effects of TCDD is glucagon. The glucagon response differed between L-E and H/W rats [295]: glucagon levels were higher in TCDD-treated L-E rats than in controls or feed-restricted counterparts. In H/W rats, glucagon levels were lowered below controls only on the fourth day [295]. Glucagon is known to acutely reduce food intake and body weight gain, and increase energy expenditure and improve glucose homeostasis. Importantly, it is believed to have endogenous role to the control of within-meal satiety (i.e. satiation) at physiological doses, while anorectic effects occur at much higher dose levels, elicited by a range of stressors [435, 436]. Thus, at first glucagon in L-E rats may participate in the increased satiation to reduce meal size, then contributing also to the wasting. Furthermore, as it has been shown that combined administration of glucagon and GLP-1 induce 'superanorexia' at the doses by which neither of them alone is capable of reducing feeding [435], possible role(s) of glucagon and/or GLP-1 in TCDD-induced wasting provide interesting study targets.

Apparently, many feeding regulatory pathways are involved in dioxin effects. For example, the rapid increase in serum FFA level [295] is likely to induce the anorectic tone [159] in the brains of L-E rats. Interestingly, gastric peptides have been shown to participate not only in the regulation of meals but also in the regulation of long-term energy balance and in mediation of food reward and hedonic value. Ghrelin is known to have a role in signalling of reward value of meal, central amylin may act as an anorectic signal independent of metabolic state of an animal, and GLP-1 has a dual role both in meal regulation and in the regulation of energy balance, since it has target sites in both the brain and the periphery. In particular, the feeding regulatory role of ghrelin has been a target of intensive research during recent years. Ghrelin appeared to interfere with dopamine release in NAcc where dopamine release is related to the reward value of meal, and it has been postulated that an intact dopaminergic signalling is necessary in order to achieve ghrelin's effect on mediating the food-reward value (reviewed in [410, 424]). Ghrelin is thought to be involved in saccharin preference, too ([410, 424]). In rats, it has a role in olfaction by increasing olfactory sensitivity and stimulating exploratory sniffing to identify and select foods, being therefore an important regulatory component transducing cues from the environment and nutritional status to the brain [437]. Furthermore, ghrelin enhances food-seeking behaviours such as hoarding [381]. Collectively, all these findings indicate that ghrelin-signalling pathways may be involved in mediating not only the TCDD-induced high dose effects on feeding, but it might also have a role in other behavioural effects that are initiated at sublethal doses of TCDD.

Vagal pathways have an important role in the control of food intake and transducing bidirectional metabolic and food- and nutrient-derived signals between the brain and periphery [16, 170, 188]. In the view of the fact that TCDD exposure modulates many gastrointestinal responses (Chapter 6.4), it was an interesting and surprising finding that hepatic vagotomy did not prevent wasting and affected feed intake only marginally in H/W rats (and not at all in L-E rats) [438]. However, this finding point towards a possible role of the BBB in the TCDD-induced feeding responses. In order to gain access to the CNS from the bloodstream, compounds need to cross the BBB. They can either use non-saturable transport (such as PYY and GLP-1) or saturable transporters at the BBB (insulin, leptin, ghrelin, amylin, PP) [439]. Peripheral regulators may also alter the normal functions of the endothelial cells in the BBB, and in that way modify the transport systems (e.g. the permeability) of the BBB. Additionally, they can alter the secretion of cytokines, nitric oxide or prostaglandins from the endothelial cells [440, 441], which subsequently have their own effects on energy balance and feeding [442, 443]. The BBB seems to play a role in illness and aging induced anorexia (reviewed in [444]). As the function of BBB is sensitive to oxidative stress and inflammatory responses [445], in the acute phase of immune reaction, activation of cytokines can inhibit feeding by acting either at endothelial and perivascular cells of BBB or by directly affecting neurons and receptors to induce anorectic effects [444]. Since dioxin exposure modulates expression of gastrointestinal hormones [295] and causes an increase in the release of inflammatory cytokines [446], these compounds might induce direct effects on BBB *per se*. In rodent models, activation of AHR has been shown to up-regulate (*in vivo* and *in vitro*) expression and transport activity of cellular ATP-driven efflux transporter proteins, such as P-glycoprotein in the BBB [447]. Unfortunately, saturable or non-saturable transport systems were not studied. At present nothing is known about the role of BBB in TCDD-induced diminished feeding, or on the effects of TCDD exposure on ghrelin, leptin or insulin transport into the brain.

6.6 Future challenges for the mechanistic studies and risk assessment of dioxins

There is lack of data on how the molecular clockwork fluctuates and how its ticking can regulate metabolism in either H/W or L-E rats. The crosstalk among AHR, circadian players and key metabolic proteins has been established (reviewed in [448]), as exemplified by the close interplay between the expression of AHR, BMAL1 and peroxisome proliferator-activated receptor-α [142]. Thus, although AHR is a known mediator of toxic responses, it might be that its roles in adaptive, housekeeping and developmental processes are evolutionarily of critical importance [325, 449]. In that sense it can be hypothetised that the aversion to food xenobiotics represents one of the housekeeping roles of AHR. Moreover, its constitutive role in feeding behaviour might be related to environmental adaptation, a proposal that is further supported by

our recent data regarding AHR protein and mRNA levels in starvation: in comparison with controls or TCDD-treated counterparts, feed restricted animals had a high hepatic AHR protein level and a low mRNA level after ten days of progressively diminished food availability [295]. The situation was reversed in lethally TCDD-treated L-E rats at the same time point [295]. Furthermore, at sublethal doses, AHR activation modifies feeding in many ways (**I**, **II**, **IV**). During evolution, it may have been beneficial that an organism is capable of assessing the properties of food at many levels: its taste, energy content and nutritional value. The interplay between AHR and circadian clockwork may ensure that the behavioural activity, along with metabolism of nutrients and xenobiotics, occur in an appropriately controlled manner throughout the day. In recent years, the crosstalk between the clockwork system and the cell's metabolic state together with behavioural rhythms has been shown to be an important contributor for health and longevity (for reviews, see [233, 450]). There is evidence of increased appetite after sleep loss, e.g. shift-work has been associated with a variety of diseases and metabolic disorders. In addition, the converse is true: in many obese or diabetic animal models the diurnal rhythmicity of molecular clockwork and/or behaviour is impaired (reviewed in [233, 234, 254, 451]). An interesting finding supporting the tight interplay and importance of diurnal rhythmicity on body weight control and metabolism was published in 2012 by two groups: despite similar caloric intake with ad libitum fed controls, timed availability prevented a variety of adverse effects of consumption of a high-fat diet. Thus, the coordinated control between the circadian clockwork and components of intermediary metabolism seemed to function appropriately only if the switch to the higher energy intake was restricted to the nighttime, the normal active period of these rodents [264, 266]. These findings highlight the importance of identifying the players involved in coupling the circadian clockwork to other biological processes. On the other hand, the contribution of circadian clockwork and fluctuation of genes, proteins, and behaviour are important issues to be considered in biological experiments, although it is all too often neglected.

In addition to environmental contaminants such as dioxins, there are many other AHR ligands or their precursors that are able to induce AHR mediated signaling pathways (as reviewed e.g. [9, 110]), or activation may occur even without ligand binding (reviewed in [10]). Many AHR ligands are present in food, and humans are commonly exposed to them when they ingest such foods, e.g. cruciferous vegetables. These AHR ligands do not induce AHR mediated toxicity apparently due to their rapid metabolism as they are substrates for the induced xenobiotic metabolising enzymes. In contrast, it seems that AHR is necessary for the maintenance of many normal physiological events, such as cell cycle control [452] and in the gut, AHR activation is needed for the expansion of intestinal lymphoid cells. Furthermore, activation of AHR by dietary compounds has been claimed to inhibit release of proinflammatory cytokines and to attenuate the symptoms of colitis – in mice and humans [404]. The use of AHR ligands as therapeutic agents to treat

inflammatory or autoimmune diseases is nowadays attracting increasing research interest [453]. However, the role of AHR in inflammation seems to be a double-edged sword as the TCDD-induced inflammatory pathway in adipose tissue [454] has been linked with metabolic disorders and obesity. In obesity, inflammation of adipose tissue contributes to the resulting metabolic disorders, thus by affecting adipose tissue (via inflammatory and many other pathways) endocrine disrupting environmental pollutants, such as TCDD, have been suggested to act as obesogens [455]. Thus, in the future, the understanding of the positive and negative, dose-dependent, and chronic vs. acute effects of AHR activation by different ligands in different tissues will be a great challenge for studies of AHR biology as well as for dioxin risk assessment.

In addition to disturbances in the energy balance, TCDD induces different types of alterations in feeding behaviour that are both immediate and persistent with low-dose levels, as it was shown here. Whether the mechanisms of the two effects, perturbed body weight set-point and novelty avoidance, intersect warrants further dose-response studies. In particular, extremely low dose experiments are needed to study the mechanistic pathways involved in the aversive responses. Solving the mechanism mediating these effects would provide clues to the mechanisms involved in disturbed food intake in humans, such as in acute illness anorexia, appetite loss in aging, or cancer-related cachexia. Novel therapies for treating unnatural food intake in disease states would result in better nutrition, and it would improve health and prolong lifespan. Hence, further studies to determine the role of AHR in the regulation of neophobic responses, food intake and energy balance could represent promising therapeutic targets, as well as advancing our understanding of AHR biology and dioxin toxicity.

7 Conclusions with future perspectives

Findings of this thesis characterise effects of TCDD-exposure on ingestive behaviours, namely on feeding and novel food aversion. The results highlight the role of the AHR in the regulation of food aversion and feeding behaviour. It appeared, that dioxin-exposure induces two kinds of effects on food intake: novel food aversion and diminished food intake, but only the latter of these leads in a dose-dependent manner to wasting and death. Single dosing is needed to induce both effects but their dose-responses are very different. The sensitivity of a rat strain to aversion of novel food items is independent from that of acute toxicity of TCDD (type I dioxin response), whereas the severity of diminished feeding is clearly dependent on the animal species and strain (type II dioxin response). Minor differences in the activated hypothalamic areas were detected in the brains of L-E and H/W rats during the daytime, at 24 hours post exposure.

More precisely:

* Important novel finding was that dioxin-exposure induces a rapid and sensitive behavioural response in adult animals. Novel food aversion emerges within hours. Importantly, it occurs after a very low dose of TCDD that does not affect the energy intake of an animal. ED50 values for chocolate aversion in differently dioxin sensitive adult rats were 0.34 - 1.07 µg/kg.

* The emergence of food aversion is linked to the simultaneous presentation of the novel food item with the TCDD exposure while it does not depend on the actual properties of foodstuff.

* Novelty aversion was detected also in mice. Although it was more severe in rats than in mice, in AHR-deficient mice aversion was not present.

* Aversion to novel food items was affected by the administration route of TCDD: the effect emerged earlier after intragastric dosing than after intraperitoneal injection. This implies that there are responsive sites modulating food intake within GI-tract, and/or in close contact with the GI-tract.

* Rat strains with different AHR receptor structures display distinct differences in their feeding behaviour, even without TCDD exposure. The most pronounced differences were detected in the feeding activity patterns: while in the sensitive L-E strain the times of lights-on and lights-off were the active feeding and drinking periods, in the resistant H/W strain consummatory activies were rather evenly distributed throughout the day. Differences in circadian feeding patterns in combination with a faster eating rate may contribute to enhanced weight gain and

growth in H/W. Also TCDD-exposure caused phase-shifts in activity patterns of feeding and drinking, indicating the the interplay of circadian clockwork, AHR, and feeding regulation.

* High, lethal dose of TCDD seems to override control of satiation (diminished size of a meal or a drinking bout) in rats, while sublethal doses adjust feeding regulation and processes that affect satiety (meal frequencies decreased).

* A week after the exposure, resistant rats started to recover: they increased both meal size and duration (above the controls). Diminished meal frequency kept the total food intake below the controls throughout the observation period (two weeks). Thus, factors related to satiety regulation in H/W rats seem to be the most important determinants in maintaining the body weight level of exposed rats below that of controls.

FUTURE PERSPECTIVES:

* The findings of distinctive meal and drinking patterns, with and without dioxin exposure, suggest that the differential AHR structure of L-E and H/W rats does contribute to the regulation of circadian feeding and drinking patterns. Although there is evidence for the interplay of circadian clockwork, AHR, and metabolism (reviewed in [448]), the possible direct or indirect impacts of AHR status and activation on feeding regulatory genes and proteins, and importantly, on their circadian fluctuation, deserve further scrutiny.

* The ligand-activated AHR seems to act to prevent further ingestion of harmful compounds, thus this acute effect (at low doses of TCDD) can be considered to promote enhanced survival of the organism. It is not known whether the activation of AHR by ligands other than TCDD would exert similar aversive effects as TCDD. Furthermore, it would be interesting to study if this finding is restricted to feeding or can it be extended to other novelties, and whether the AHR-mediated behavioural response can be observed in different animal species and in both genders.

* The AHR-activated pathways regulating the aversive behaviour remain to be elucidated, but this finding provides additional support for the proposal that activated AHR has an acute house keeping role extending to behaviour.

* Further experiments will be needed to determine if there is any common component in the pathways regulating aversion and feeding responses to TCDD.

* Further experiments to elucidate the role of AHR in the regulation of aversive feeding behaviour might be of importance for the development of medical treatment of anorectic diseases, such as cancer cachexia.

* Based on the findings of the meal and drinking patterns, it is concluded that timing is critical for the detected outcome at different phases of dioxin toxicity. Thus, to elucidate dioxin-targeted, site-specific pathways in the brain and in the periphery, further dose-and time-response studies will be of importance.

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