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TCDD-induced changes in the expression of selected hypothalamic feeding-regulatory genes and mRNA quantification using reverse transcription–qPCR

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ACADEMIC DISSERTATION

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Lauralle ja Martille

"*Give Me a Place to Stand and I will Move the Earth*"
A remark of Archimedes quoted by Pappus of Alexandria

ABSTRACT

Dioxins (polychlorinated dibenzo-p-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls) are pervasive and biomagnifying environmental contaminants, to which people are generally exposed through foods of animal origin; in Finland, a major source is some fatty fish species of the Baltic Sea. The dioxins, especially the most potent of them, the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are often called “super poisons”, based on their extreme acute toxicity to laboratory animals and feared long-term effects in humans. Children, before and after birth, are especially susceptible to this toxicity, possibly induced by picogram daily exposures, while the risks to adults are less certain and appear, for example in Finland, to be outweighed by the beneficial effects of fish consumption. Furthermore, the most important endpoints of dioxin toxicity have not been fully untangled, and even the mechanisms of the most sensitive developmental effects have not been thoroughly elucidated.

After gaining access to the body, the dioxins bind to a specialised intracellular aryl hydrocarbon receptor (AHR), directly influencing the expression of a substantial number of genes. The AHR, a transcription factor with no established endogenous ligand, first “converges” the dioxin effects, but then diverges them depending on the affected species and even the strain, as well as the gender, age and developmental stage of the individual. This divergence is exemplified in the dramatic variability of the acute toxicity of TCDD even within one species: Han/Wistar (H/W) rats tolerate over 1000-fold larger dioxin doses than the Long–Evans (L–E) strain, almost solely depending on a minor structural difference in the ARH. In addition, TCDD toxicity develops slowly and is manifested differently in various laboratory animals. A unifying feature of acute TCDD intoxication in many species, however, is a dramatic feed intake reduction and weight loss, termed the wasting syndrome, which seems to be related to direct or indirect derangement of the central regulation of feeding. Clarification of the mechanisms behind the wasting syndrome would thus yield information on an important facet of acute dioxin toxicity and open the way to using TCDD as a molecular tool to study the physiology of feed intake and body weight regulation.

In this research, a straightforward approach was selected to tackle the patho-physiological challenge of wasting. The aforementioned strain difference was utilised to address the AHR function, and gene expression measurements were targeted at the hypothalamus, the essential food intake and body weight regulator in the brain. In the first part of the thesis research, alterations in the AHR signalling cascade and some related proteins as well as food intake-related neuropeptide neurotransmitters and their receptors were measured using quantitative reverse transcription PCR (RT-qPCR). In the latter, methodological part, some intricacies of the RT-qPCR technique were considered: The reproducibility of the poorly controllable reverse transcription (RT) of RNA to cDNA is a major determinant of the reliability of expression analysis, and toxicological studies involving TCDD have particular difficulty in discovering treatment-unaffected stable mRNA transcripts, reference genes, that can especially be used to reduce RT variation. In the two expression studies, the mRNA levels of AHR-related proteins and feeding regulatory factors were measured from hypothalamic blocks at 6, 24 or 96 hours after TCDD doses that generated only a minor reduction in feed intake in the resistant H/W rats, but induced a marked reduction in feed intake and body weight (and would have

eventually led to lethality) in the sensitive L–E strain. In the two methodological experiments, a set of stable reference genes was first sought among the extensive and divergent expression changes induced by AHR activation, and then the identified reference genes were employed in a study comparing the robustness of various RT and qPCR enzymes and mapping the RT-qPCR variation sources.

There were small constitutive differences in the hypothalamic mRNA expression of some AHR signalling cascade molecules between L–E and H/W rats, but these were most likely not causally related to the development of the wasting syndrome. A functional AHR signalling cascade seems to be present in the hypothalamus, as shown by the pronounced induction of cytochrome oxidases and Ahrr, an AHR signalling repressor protein. However, the magnitude and importance of cytochrome induction in neurones should be determined, employing anatomically more detailed sampling. The lack of any drastic up- or downward changes in hypothalamic neuropeptide or receptor mRNA following TCDD treatment and the stability of hypothalamic reference gene expression speak against a severe cytotoxic effect on, or permanent hyperexcitation of the cells taking part in eating regulation. A late elevation of some orexigenic (feeding inducing) factors brought about by TCDD seems to be a compensatory reaction to body weight loss.

Notably, the employed hypothalamic block sampling may cause expression changes confined to a localised point and/or circadian time to elude detection, and the complex neurophysiology and anatomy of the hypothalamus may lead to reciprocal cancellation or dwindling of alterations. In addition, the somewhat divergent feeding and energy balance regulation between L–E and H/W rats also warrants further research, and as a more general question, the importance of the hypothalamic neuropeptides in relation to other systems in eating regulation has not been definitively resolved.

The number of genes displaying an acceptable steadiness of expression in the face of lethal TCDD toxicity is small; four transcripts (Actb, Gapdh, Pgk1 and Sdha) were satisfactory for the hypothalamus, and besides the expression constancy, RT stability was found to have a strong influence on the usability of the potential reference genes. Hence, the use of only one steadily expressed but unstably reverse transcribing reference gene (Actb) has most likely not induced marked bias in the results attained in the hypothalamic expression studies, but it may have inadvertently increased variation. Furthermore, RT variance markedly exceeded qPCR variance, stressing the importance of replication at the RT level, while the practical consequences of differences in the reproducibility of the individual qPCR enzymes appear to be of little significance. On the contrary, salient differences were noted between replicate PCR runs, and these should be taken into account in the design and data analysis of RT-qPCR experiments. Finally, the use of linear hierarchical models and Bayesian inference was found to offer the possibility to build a coherent statistical model of the whole RT-qPCR experiment with normalisation over all expression measurements, thereby maximising the use of the data.

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This thesis has been in the making for a number of years, which means that there are a large number of people who have helped and guided me during the more than 20 years spent hanging around dioxin toxicology. I have been privileged to have been able to work in both Helsinki and Kuopio and to get to know many people from two different laboratories. The kindness and help that I have received in both places has been heartening and the large palette of skills and knowledge I have witnessed staggering.

Finally, I would like to thank my dear wife, Riikka, for her enduring love and support (and necessary prodding) through all these years, and my parents Raija and Unto for their patience, encouragement and help in all matters.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred in the text by their Roman numerals:

I Korkalainen M., Lindén J., Tuomisto J. and Pohjanvirta R. (2005) Effect of TCDD on mRNA expression of genes encoding bHLH/PAS proteins in rat hypothalamus. Toxicology 208: 1-11.[a]

II Lindén J., Korkalainen M., Lensu S., Tuomisto J. and Pohjanvirta R. (2005) Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and leptin on hypothalamic mRNA expression of factors participating in food intake regulation in a TCDD-sensitive and a TCDD-resistant rat strain. Journal of Biochemical and Molecular Toxicology 19: 139-148.

III Pohjanvirta R., Niittynen M., Lindén J., Boutros P.C., Moffat I.D. and Okey A.B. (2006) Evaluation of various housekeeping genes for their applicability for normalization of mRNA expression in dioxin-treated rats. Chemico-Biological Interactions 160: 134-149.

IV Lindén J., Ranta J. and Pohjanvirta R. (2012) Bayesian modelling of reproducibility and robustness of RNA reverse transcription and quantitative real-time PCR. Analytical Biochemistry 428: 81-91.

[a] Merja Korkalainen has previously used publication I in her Doctoral thesis entitled "Structure and expression of principal proteins involved in dioxin signal transduction and potentially in dioxin sensitivity". Publications of the National Public Health Institute KTL A11/2005.

LIST OF ABBREVIATIONS

Agrp[a]	Agouti related protein homolog (mouse)
Ahr	Aryl-hydrocarbon receptor
ARC	Arcuate nucleus
Arnt	Aryl hydrocarbon receptor nuclear translocator
Arnt2	Aryl hydrocarbon receptor nuclear translocator 2
Ahrr	Aryl-hydrocarbon receptor repressor
Aip	Aryl-hydrocarbon receptor-interacting protein, also XAP2 or ARA9
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
BST	Bed nucleus of the *stria terminalis*
bHLH/PAS	Basic Helix-Loop-Helix / homologous region of PER, ARNT and SIM proteins
BLA	Basolateral amygdala
CART	Cocaine- and amphetamine-regulated transcript; gene *Cartpt*
Cartpt	CART prepropeptide; peptide CART
CV	Coefficient of variation
cDNA	Complementary DNA
CTA	Conditioned taste aversion
CRF	Corticotropin releasing factor
Cck	Cholecystokinin
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2
Cyp1b1	Cytochrome P450, family 1, subfamily b, polypeptide 1
2,4-D	2,4-Dichlorophenoxyacetic acid
DA	dopamine
2DG	2-deoxy-glucose
DMH	Dorsomedial hypothalamic nucleus
DRE	Dioxin response element
EROD	Ethoxyresorufin O-deethylase (activity)
GABA	γ-aminobutyric acid
GD	Gestational day
GLUT1	Glucose transporter type 1; gene *Slc2a1*
Ghsr	Growth hormone secretagogue receptor, ghrelin receptor
HSP90	Heat shock protein 90
5-HT	5-hydroxytryptamin, serotonin
H/W	Han/Wistar rat strain
IC	Insular cortex
Insr	Insulin receptor
LHA	Lateral hypothalamic area
LD_{50}	Median lethal dose
L–E	Long–Evans rat strain
LOAEL	Lowest observable adverse effect level
MCH	Melanin-concentrating hormone; gene *Pmch*

Mc4r	Melanocortin 4 receptor
Mc3r	Melanocortin 3 receptor
M-MLV	Moloney murine leukaemia virus
α-MSH	α-melanocyte-stimulating hormone; gene *Pomc*
Npy	Neuropeptide Y
Npyr5	Neuropeptide Y receptor Y5
NTS	*Nucleus tractus solitarii*
NAc	Nucleus accubens
p23	A co-chaperone in the unligated AHR complex; gene *Ptges3*
PBN	Parabrachial nucleus
Per1	Period homolog 1 (Drosophila)
Per2	Period homolog 2 (Drosophila)
Pmch	Pro melanin concentrating hormone; peptide MCH
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
Pomc	Proopiomelanocortin; propeptide for several derived peptides e.g. α-MSH
POP	Persistent organic pollutant
PVN	Paraventricular nucleus
qPCR	Quantitative real-time PCR
RT	Reverse transcription
RT-qPCR	Quantitative real-time reverse transcription PCR
SCN	Suprachiasmatic nucleus
SD	Sprague–Dawley rat strain
sd	Finite-population (sample) standard deviation
Sim1	Single-minded homolog 1 (Drosophila)
SMR	Standardised mortality ratio
SON	Supraoptic nucleus
TAD	Transactivation domain in the carboxyl-terminal of the AHR
TCDD	2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin
TEF	Toxic equivalency factor
TEQ	TCDD equivalent quantity
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TSH	Thyroid-stimulating hormone, thyrotropin
VMH	Ventromedial hypothalamic nucleus
VTA	Ventral tegmental area

[a] Equivalent gene and protein symbols, e.g. *Ahr*/AHR, are written here in their gene symbol form; italicised and capitalised. Conventional protein designations (generally uppercase letters) have not been modified.

Symbols and gene nomenclature: Rat Genome Database Web Site, Medical College of Wisconsin, Milwaukee, Wisconsin. WWW (URL: http://rgd.mcw.edu/). Retrieved October 2012.

1 INTRODUCTION

Dioxins (polychlorinated dibenzo-p-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls) are known by name and reputation as “super poisons” based on their acute toxicity and teratogenicity to laboratory animals [1, 27, 270], irrefutable adverse effects on humans after occupational or accidental exposures, and feared long-term effects of picogram doses [400]. Historically, understanding of dioxin risks and the control of human exposure has walked hand in hand with the understanding of their mechanistic toxicology, the development of analytical methodology and the “chemical awakening” of the society.

Dioxins emerged as industrial contaminants, extremely potent but mainly related to workplace accidents and chloracne in the chemical industry (and to a massive chicken feed contamination in the southern USA) [89, 111, 325]. In the early to mid-1970s, however, they came to be of more general concern due to their effects on people following accidents, especially after a chemical plant explosion in Seveso, Italy (1976), as well as environmental pollution and human exposure through dioxin-containing chemicals and products or industrial waste. An example is the notorious defoliant Agent Orange used in the Vietnam War and a waste oil accident at Times Beach, Missouri [135, 320]. Finally, during the last 20 to 30 years (in conjunction with the decreasing releases), the dioxins have generally been regarded as pervasive, trace-level environmental contaminants with feared long-term effects and unknown limits of safe exposure, and have also transpired as model molecules to investigate the physiological processes of environmental adaptation.

Mechanistically, the most intriguing aspects of dioxin action are the specialised aryl hydrocarbon receptor (AHR) conveying and combining the effects of dioxin-like substances [252], and the subsequent divergence of these effects, depending at least on the structure of the receptor of the affected species and strain, as well as the gender, age and developmental stage of the individual [270]. AHR is a cytoplasmic transcription factor of the basic helix-loop-helix (bHLH)/PAS receptor family, which upon binding an agonist, e.g. a dioxin molecule, is transferred to the nucleus and attaches to specific DNA sequences, regulating a diverse set of genes and interacting with numerous other transcription factors [349]. Remarkably, the AHR appears to have a similar role in all eutherian mammals [119] with an astonishingly wide repertoire of functions and promiscuity towards various environmental and endogenous substances, but without a known true endogenous ligand [65]. Furthermore, although the dioxin signalling cascade up to DNA is generally well understood, little is known of the intervening cellular and physiological steps between the DNA binding of the activated AH receptor and the endpoints of toxicity.

The discovery of the AHR has had profound consequences for both regulatory toxicology and toxicological research: It has enabled the development and use of the toxic equivalency factors (TEF) in dioxin risk assessment and regulation [364], and consolidated the utilisation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent dioxin congener, as the reference substance in experimental studies and in regulatory toxicology [124]. Importantly, it also has shifted the focus of mechanistic studies from TCDD toxicity to ARH-relayed toxicity and AHR-related physiology in the species, strain, age and tissue context [97].

The diverging effects of dioxin are exemplified in the dramatic variability and distinctive features of acute toxicity of TCDD in experimental animals: Hamsters tolerate over 1000-fold larger doses than guinea pigs [273], and strikingly, there is an equally extensive difference between two rat strains, Long–Evans and Han/Wistar, depending on the ARH structure [283]. Besides the genetic background, gender influences TCDD susceptibility, even affecting it in an opposite way in different species [272]. Characteristically, acute TCDD toxicity develops slowly and death does not ensue until 1 to 8 weeks after even a large TCDD dose. In many species it is preceded by a dramatic weight loss, the wasting syndrome, which might be related to direct or indirect derangement in the central regulation of feeding [212]. Nonetheless, while wasting is the major contributor to death in TCDD toxicity studies in rats, it is not necessary for lethality, and the critical target tissues and physiological systems for the acute lethal effects of TCDD thus remain to be established.

Untangling the mechanisms of wasting syndrome provides information on an important facet of acute dioxin toxicity and sheds light on some of its processes, from transcription to pathology. An additional benefit is the possibility to use TCDD as a toxicological tool in physiology to study feed intake and body weight regulation. The straightforward approach to tackle this pathophysiological challenge, starting from transcription alterations, is to use the differences between rat strains to address AHR functioning and quantify altered gene expression by measuring mRNA transcripts of the factors relating to feed intake and body weight regulation. These preliminary surveys at the mRNA level would then pave way to more focused or functional experiments, for example expression measurements at the protein level, chemical analysis of neurotransmitters or studies concerning nerve cell activation. However, much of the value of these more advanced analyses is lost if they cannot be targeted at the right molecules and CNS nuclei based on reliable mRNA expression data.

Quantitative real-time reverse transcription PCR (RT-qPCR) is currently the most agile, cost-effective and sensitive method to reproducibly measure a limited number of RNA targets from a large number of tissue samples [398]. In RT-qPCR, RNA is first reverse transcribed to cDNA and then quantified using real-time PCR [43], and this two-step process induces some challenges to the precision of the measurements: The fidelity of the cDNA synthesis step is a major determinant of reliable expression analysis [46, 185], and although normalisation of the genes of interest to internal reference genes improves reliability, it still requires that the relative efficiencies of the RT reactions for the genes of interest and the normalisation genes are consistent from reaction to reaction [46]. Toxicological studies involving TCDD have an additional difficulty in finding stable reference genes among the extensive and divergent expression changes induced by AHR activation.

In the first part of this thesis, the AHR-dependent sensitivity difference between Long–Evans and Han/Wistar rats was employed to study TCDD-induced wasting syndrome and the changes in hypothalamic mRNA levels of factors relating to the AHR signalling cascade or participating in feed intake and body weight regulation. In the second, methodological part, potential RT-qPCR reference genes for dioxin studies were sought, and the robustness of various RT and qPCR enzymes was examined.

2 REVIEW OF THE LITERATURE

2.1 DIOXINS AS ENVIRONMENTAL TOXICANTS

2.1.1 DIOXIN CHEMISTRY AND TCDD

"Dioxin" is an inexact umbrella-term for a group of chlorinated (chemically, the organic frame could contain also bromine) chemicals among a larger family of persistent organic pollutants (POPs). The dioxins, or "dioxin-like compounds" – including some polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) – are linked by their persistence and accumulation in the food chain, and by the planar (flat) molecular structure of their two connected benzene rings with a symmetrical lateral position of at least four chlorine atoms, facilitating high affinity for the aryl hydrocarbon receptor (AHR) [6, 124, 285, 313, 403]. All dioxins thus convey their effects through a single receptor and share the same basic toxicity mechanism [270] (see 2.2). The prototype of the dioxin group is its most thoroughly studied and most toxic member, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), and each of the toxicologically relevant PCDD, PCDF and PCB congeners has been assigned a toxicity equivalence factor (TEF) value in relation to it [391]. TCDD was itself the key molecule in the discovery [284] and characterisation [253] of the AHR in the laboratories of Allan Poland and Allan Okey in the 1970s [97, 252].

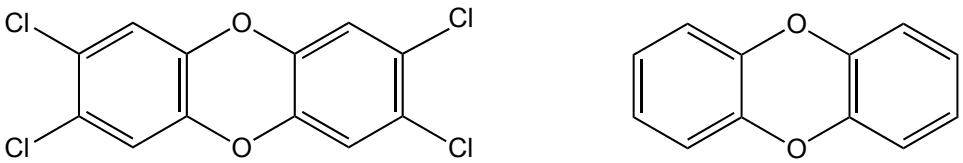

Figure 1. Structures of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dibenzo-p-dioxin. The TEF of TCDD is 1.

Notably, of the possible 75 PCDD and 135 PCDF compound structures, only 7 dibenzo-p-dioxins and 10 dibenzofurans bind to AHR with sufficient affinity to elicit marked AHR-mediated biochemical and toxic responses and thus be considered as dioxins [393, 394]. Each chlorine substitute in excess of the four decreases the potency, but the toxic effects generally remain the same. Of the 209 PCBs, 12 are deemed to be dioxin-like [394]: The four non-ortho compounds, having no chlorine substitute in any position next to the inter-ring C–C-bridge (positions 2, 2', 6, 6'), are the most potent as a group, while the eight mono-ortho PCBs have some activity, but roughly thirty thousand times weaker than TCDD (Figure 2). None of the other PCBs induce dioxin-like effects due to their three-dimensional structure; only the non-ortho compounds are freely rotating along the C–C-bridge, and more than one chlorine in any of the o-positions beside the C–C-bridge makes it impossible for the molecule to assume a planar conformation required for AHR binding.

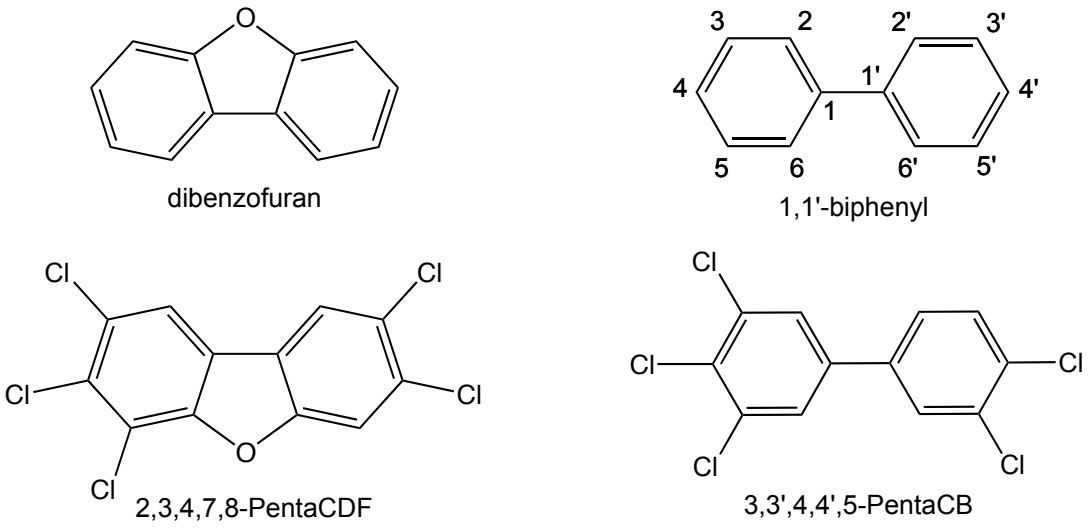

Figure 2. Structures of dibenzofuran, 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF), biphenyl and 3,3',4,4',5-pentachlorobiphenyl (3,3',4,4',5-PentaCB; PCB 126). The TEF of 2,3,4,7,8-PentaCDF (the most potent furan) is 0.3 and that of 3,3',4,4',5-PentaCB (the most potent PCB) is 0.1.

The operative definition of dioxin-like compounds also excludes a number of other coplanar aromatic halogenated hydrocarbons (polychlorinated azobenzenes and azoxybenzenes, polychlorinated naphthalenes, or polybrominated and mixed [both chlorine- and bromine-containing] dioxins, furans and biphenyls) that bind to the "promiscuous" AHR [65, 287, 315, 394]. However, data on human exposure and the toxicity of these compounds is still scarce, and by and large the inclusion of some of them in the dioxin group would not markedly alter the logic of the dioxin definition and TEF assignment [366]. On the other hand, the consideration of a large number of natural and ubiquitous [64] non-halogenous AHR agonists and partial agonists [65, 316] would make the definition of dioxin-like compounds very complex [366].

2.1.2 SOURCES AND EXPOSURE

Chlorinated dibenzo-p-dioxins and dibenzofurans (collectively PCDD/Fs) have never been intentionally manufactured except for research purposes, but are generated as by-products or impurities in chemical syntheses, metal smelting, refining and processing, as well as in various forms of combustion, and during the heating or burning of PCBs [146, 187, 384]. The best known contaminated products are surely the herbicide 2,4,5-T (2,4,5-Trichlorophenoxyacetic acid) and Agent Orange (a mixture of 2,4,5-T and 2,4-D). In contrast, the PCBs, technical mixtures with variable amounts of dioxin-like PCBs and PCDD/F contaminants, were produced in high volumes, totalling over 1.3 million tonnes, and extensively used from the 1930s to the 1980s (no known production after 1994) for a vast number of functions, e.g. as hydraulic, heat transfer and lubricating fluids; as dielectric and coolant fluids in capacitors and transformers; and as plasticisers or additives in plastics, paints, rubber products, building caulk and building sealants, among other products. [33, 35].

Generally, the releases and environmental levels [303] of PCDD/Fs have markedly decreased in industrialised countries during the last three decades; there was an

approximately 90% reduction in their releases in the US between 1987 and 2000 [384], and emissions from the most important sources were estimated to have decreased by roughly the same extent in the EU from 1985 to 2005 [298]. The reduction of PCCD/Fs is mainly attributed to the abatement of releases from municipal waste incineration and from other large-scale incineration facilities, cessation of the use of chlorine gas in pulp bleaching, and the banning or strict restriction of the use of products containing PCCD/F impurities, e.g. chlorophenols, chlorophenoxy herbicides and PCBs. Examples of the remaining dioxin sources, in addition to old-fashioned incinerators, iclude steel and iron production [162], forest fires and the burning of solid fuels, principally wood, and of agricultural residue, mainly straw [81, 187, 298], as well as the recycling of electrical devices [400]. Common, unintentional fires in landfills also produce dioxins [78, 81]. Since PCBs are no longer produced, their atmospheric release is mostly related to emissions from dumpsites and landfills (including fires), the burning of PCB-containing material, and releases from large capacitors in fires and at disposal. Consequently, the releases of PCBs have rapidly decreased, and have levelled off during the last 10 years, with an expected gradual disappearance in 20 to 40 years, probably sooner, in western countries [34, 69].

Dioxin-like compounds are almost solely broken down in the environment by ultraviolet radiation and biodegradation and remain stable for decades, even millennia, in deep soil and sediments [337]. Being extremely poorly soluble in water, deposited PCDD/Fs and PCBs are considered not to markedly accumulate in most terrestrial plants [308, 404] or to be widely released to the aquatic biota [402], but after gaining access to the biosystem they readily bioaccumulate and biomagnify in the food chain due to their supreme lipophility and slow metabolism in mammals [392]. Humans are exposed to dioxin-like chemicals through food of animal origin (and nowadays rarely through industrial exposure), while the main sources (meat, milk or fish) vary depending on the country and composition of the diet [209].

The most recent studies from western Europe, exemplified by reports from Sweden [382], France [338] and Belgium [410], estimate the mean daily intakes of PCDD/Fs and dioxin-like PCBs to be around 0.7 pg/kg (TEQ_{WHO-98}; TCDD equivalent quantities calculated using TEFs), and to have been reduced by a factor of 2 to 3 in 5–10 years. This appears to be in line with an earlier Finnish study (sampling in 1997–9), in which the mean daily intake was found to be 1.5 pg/kg TEQ [177]. Similarly to food samples, human breast milk samples have shown a dramatic reduction in the dioxin and PCB body burdens since the 1970s [193, 250, 385], although the doses received by the infants (15–150 pg/kg TEQ_{WHO-98} per day during six months of breast feeding [385]) are still large in comparison to food basked-based daily intake estimates of adults. Human blood samples attest to an increase in PCDD/F and dioxin-like PCB concentrations with increasing age [178], explained by the long half-life of the compounds [392], and stress the importance of diet in the exposure; Baltic Sea fishermen consuming fish at least twice a week exhibit over five times larger plasma concentrations of PCDD/Fs than the average Finnish population [179]. The amount of food consumed in relation to the body weight is also important, as shown in children, who ingest relatively larger doses of environmental contaminants than adults. In a recent Finnish study (food sampling from year 2002 to 2005), the average intakes by 1-year-old children were 1.1 pg/kg TEQ_{WHO-98} per day, but almost doubled, and

exceeded the previous estimate in Finnish adults, in 3- and 6-year-old children, being 1.9 and 1.8 pg/kg TEQ, respectively [163].

Dioxins are strictly regulated. The recent chronic oral exposure reference dose (RfD; "not likely to cause harmful effects during a lifetime"; tolerable daily intake [TDI]) for TCDD assigned by the United States Environmental Protection Agency (US EPA) is below 1 pg/kg per day [383], and acceptable levels of dioxins in foodstuffs imposed by the European union are extremely low [82]. In Finland, where fish is the main source of dioxins [177], the Finnish Food Safety Authority Evira has given special recommendations for the use the most dioxin-burdened Baltic herring and wild Baltic salmon to ensure the protection of the most vulnerable part of the population, i.e. children before and after birth [83]. Notably, also much more drastic recommendations concerning fish consumption have also been given based on pg/g concentrations of these chemicals [136]. On the other hand, the risks imposed by dioxin intake appear to be outweighed by the beneficial effects of fish consumption, at least in adults (as recently considered in the population of Finnish fisherman in ref [380]), and the "excessive precautionary advice" on dioxins based on epidemiological data has been seriously questioned [370]. Furthermore, the most important endpoints of dioxin toxicity have not been fully untangled [274], and even the mechanisms of the most sensitive developmental effects have not been thoroughly elucidated [1, 405], placing additional demands on both scientific risk assessment and mechanistic toxicity studies [367].

2.2 AHR IN DIOXIN TOXICITY

2.2.1 BIOLOGY, STRUCTURE AND RELATED PROTEINS

The AH receptor (Figure 3) is a ligand-activated transcription factor belonging to the bHLH/PAS (see below) protein family, the members of which have important roles in environmental adaptation; they sense endogenous or xenobiotic small molecules, respond to hypoxia, take part in the regulation of neural development, participate in the generation and maintenance of circadian rhythms, and many act as transcriptional partners and co-activators [174, 225]. While firmly attributed to conveying the toxic effects of dioxins and induction of various xenobiotic metabolising enzymes [287], the AHR itself has an astonishingly wide repertoire of important physiological functions, from liver development [147] and cell cycle control [70] to the maintenance of gut-associated lymphatic tissue [199]. Furthermore, it is capable of binding a diverse array of "classical" coplanar and "nonclassical" non-coplanar exogenous and endogenous ligands functioning as agonists and antagonists, suggesting the existence of multiple endogenous ligands producing unique cell- or tissue-specific responses [65]. The diversity of AHR responses to xenobiotic stimuli is illustrated by the vast array of gene expression changes radically differing between species and even between rat strains after TCDD administration [29, 429]. Dioxin toxicity is thus intricately bound to the position of the AHR as a physiological and developmental regulator, perhaps at some point transforming the TCDD from an environmental "super toxicant" into a physiological tool [97].

The 20 proteins of the bHLH/PAS family combine motifs from two parent super families, bHLH and PAS (Per-Arnt-Sim), making them structurally capable of both DNA

binding and protein interaction [3, 225]. Proteins of the large bHLH (basic Helix–Loop–Helix) super family often function as transcription factors and share two highly conserved and functionally distinct domains, making up a region of approximately 60 amino-acid residues. At the amino-terminal end of the HLH region is the basic b domain, which binds the transcription factor to DNA, while the HLH domain is needed for heterodimerisation of two bHLH/PAS proteins [3, 156]. The PAS acronym stems from the names of three genes: *period* (*per*), a Drosophila melanogaster clock gene; human a*ryl hydrocarbon receptor nuclear translocator* (*ARNT*), a component of the dioxin signalling pathway; and *single-minded* (*sim*), a neurodevelopmental regulator in *D. melanogaster*, which were noted to share sequence homology in a domain of 250–300 amino acids [225]. Currently, PAS refers to two (A and B) homologous amino acid regions in the original domain that are important for the specificity of the dimerisation and other molecular interactions, including DNA and ligand binding, relating to signal transduction [174, 219, 225]. In addition to the bHLH and PAS domains, the carboxyl-terminal transactivation domain (TAD) is vital to AH-receptor function. In the extremely TCDD-resistant H/W rat, the binding of TCDD to the AHR and the binding of the receptor complex to the DNA occur normally, but a mutation in the Ahr gene leads to alternative splicing of RNA, finally resulting in two types of deletion in the AHR protein TAD [270, 285]. The resultant receptor proteins seem to be ineffective at initiating the transcription of some genes (and probably repressing others), thus making the animals exceptionally resistant to some aspects of dioxin toxicity (see 2.3.2).

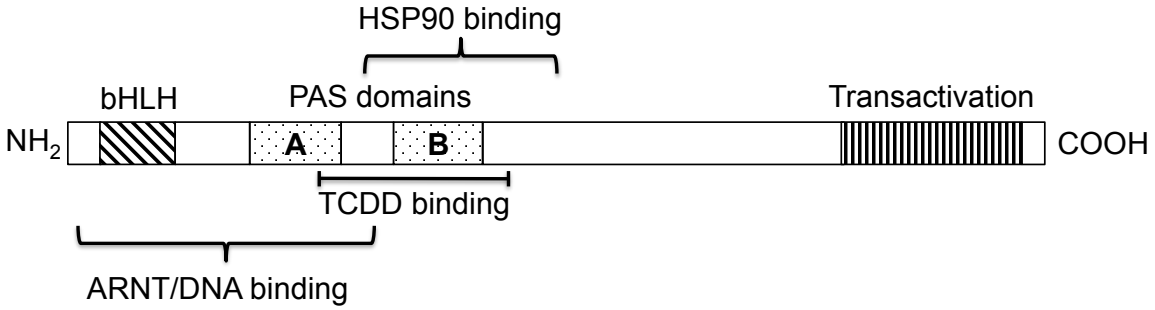

Figure 3. Major functional domains of the AHR. The approximate locations of the regions responsible for DNA binding, ligand binding, HSP90 binding and heterodimerisation, as well as transactivation, are shown. The PAS domain comprises two subdomains, A and B. The transactivation domain has at least three subdomains (not depicted). Modified from [213, 219].

Besides the AHR signalling cascade molecules (elaborated on below), one member of the bHLH/PAS family is worth mentioning. SIM1 (single-minded homolog 1 [Drosophila]) is a critical regulator of neuronal differentiation, expressed at the highest levels in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [230] and at lower levels in the amygdala and lateral hypothalamic area (LHA) [137]. Homozygous Sim1 mutants (Sim1 –/–) die perinatally lacking PVN and SON [230], because of failure of terminal migration and differentiation of the SIM1 neurons [423]. Intriguingly, Sim1-heterozygous mice survive, and develop hyperphagic obesity [137], which is even enhanced in conditional postnatal Sim1 homozygotes, both exhibiting a marked decrease in hypothalamic oxytocin and PVN melanocortin 4 receptor (Mc4r) mRNA [362].

2.2.2 AHR RECEPTOR SIGNALLING

The AHR is indispensable for dioxin action, most definitively attested by studies with AHR knockout mice, which are unresponsive to all the major effects of TCDD [85, 231, 272, 429]. In addition, there is a good correlation between the toxicity of the classic dioxin congeners and their binding affinity to the AHR [287, 315], and in C57BL/6J mice, congenic at the Ah locus, a 10-fold difference in the binding affinity of the AHR to TCDD is associated with a sensitivity difference of an approximately similar magnitude to most of the toxic and biochemical effects of TCDD [26]. Moreover, Ahr-mutated H/W rats are up to 1000-fold more resistant to TCDD lethality than sensitive rat (sub)strains (see above). Most of the effects of dioxins are conveyed through a "canonical" AHR signalling cascade, as demonstrated in a series of transgenic mutation experiments in Christopher Bradfield's laboratory, targeting all the major steps of the transduction: the translocation of the ligand-activated receptor from the cytosol to the nucleus [41], heterodimerisation with ARNT [391] and DNA binding at specific sites, the dioxin response elements (DREs) [40]. However, besides the canonical pathway, the AHR has been found to employ alternative signalling routes. For example, in certain cell lines, ligand-activated AHR is alone able to repress oestrogen-regulated transcription [192], and activate protein kinases and ultimately cyclo-oxygenase 2 [222]. In addition, DRE binding has been shown not to be involved in the AHR-related induction of acute-phase response genes *in vitro* [259], or in the suppression of genes involved in cholesterol synthesis *in vivo* [357].

At the molecular level, canonical AHR signalling (Figure 4) is best characterised by the induction of phase I xenobiotic-metabolising enzyme CYP1A1 (recently reviewed in [18, 213, 272]). The unbound AHR resides in the cytosol in a protein complex also containing a dimer of heat shock protein 90 (HSP90), aryl hydrocarbon interacting protein (AIP; also XAP2 or ARA9) and p23, which enhance ligand binding, stabilise the AHR, reduce spontaneous nuclear translocation of the receptor and regulate the signalling in a complex manner [59, 245]. Upon ligand binding, the AHR undergoes a conformational change exposing nuclear localisation signal peptide sequences, and the protein complex is actively transported into the nucleus [148]. The AHR then dissociates from the chaperones and heterodimerises with another bHLH/PAS protein, ARNT, and the dimer binds to the DNA at DREs [351]. AHR activity is terminated by nuclear export and ubiquitin-mediated degradation of the receptor [219, 288], and the transcriptional activity of AHR is constitutively and inducibly inhibited by AHR receptor repressor (AHRR). This competes with the AHR to form a heterodimer with ARNT and directly represses gene expression while binding to DREs [94]. Notably, the mRNA transcription of the *Ahrr* gene is generally markedly induced by AHR receptor stimulation, while TCDD exerts only a minor influence on *Ahr* and *Arnt* mRNA abundance [94, 143, 144, 274]. Brauze et al., however, reported a threefold increase in *Ahr* mRNA in female SD rat liver after a TCDD dose of 25 µg/kg. In contrast, TCDD exposure accelerates AHR protein degradation (see before), and concurrently AHR is drastically reduced *in vitro* [105, 219, 288]. *In vivo*, AHR protein is initially diminished but then displays a tendency towards recovery, the speed and extent of which depend on the rat strain and TCDD dose [92, 288]; recent findings show the depression to persist for up to 6 days in mouse liver [274].

Transactivation, the launch of mRNA transcription after DNA binding, involves interactions of the AHR/ARNT dimer with a large number of transcriptional coactivators and other transcription factors, depending on tissue and even on single cells [18, 123].

However, little is known of the molecular mechanisms of AHR activation-induced repression of gene expression, although in adult mice and rats it does not appear to involve micro-RNAs [234, 430]. The complexity and importance of the transactivation in AHR-mediated dioxin toxicity is best exemplified by the extreme variability in the responses of H/W rats and hamsters to TCDD, recently thoroughly discussed by Pohjanvirta et al. [272]. H/W rats harbour two forms of deletion in their AHR protein transactivation domains (see before), but intriguingly some of their TCDD responses, termed type I, are not changed at all (typically the induction of cytochromes, thymus atrophy and foetolethality), while other, type II responses (e.g. lethality, liver toxicity and wasting syndrome) are drastically weakened [275, 335, 378]. The molecular mechanism of this dual outcome is hypothesised to be related to the structural alterations in the mutated H/W AHR: The truncated TAD might be less suited to interplay with the coregulators or other general transcription factors [272]. Alterations in crosstalk with other signalling pathways, especially oestrogen receptor signalling, which is known to cross ways with activated AHR even before transactivation [38], are also possible mechanistic explanations for the selective TCDD resistance of H/W rats [272].

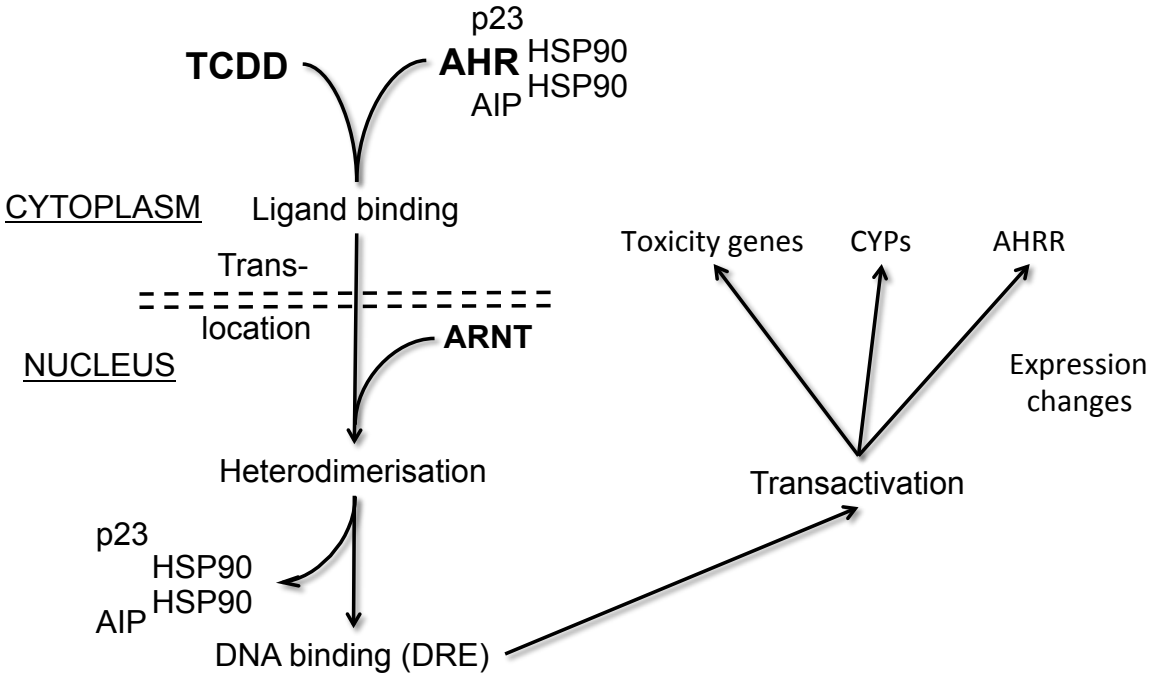

Figure 4. A schematic and simplified diagram of the key steps of the canonical AHR signalling pathway. Dioxin binding to the AHR leads to its translocation into the nucleus, heterodimerisation with ARNT and binding to the DNA at DREs, with ultimately modulating expression levels of target genes. One of the gene products elevated by this mechanism is AHRR, which forms a feedback loop by inhibiting AHR action. Modified from [272].

2.2.3 SIGNALLING PATHWAY IN THE RODENT CNS

Studies in rats and mice suggest the AHR and other key proteins of the canonical AHR signalling pathway to be expressed in the developing and adult mammalian CNS. In prenatal mouse, AHR and ARNT mRNA and protein were present in high concentrations in the neuroepithelium of the developing brain around gestational days (GD) 10–12, but

then started to diminish with only regional, generally lower levels being expressed after that; in the hypothalamus, a strong *Arnt* mRNA signal was recorded at GD11 and GD15 [2, 7, 151]. In comparison, AHR immunoreactivity was only marginally detected in human foetal brains at 4 to 7 months of gestation, however, which is far past the embryonic phase terminating at 8 weeks [153]. After birth, AHR protein expression was detected on postnatal day 1 in primary cortical astrocytes and in primary cortical endothelial cells from weanling mice [88], and in another study cerebellar AHR protein was shown to be present at all time-points from birth to adulthood and ARNT from post-natal day 3 onwards [407].

In rats, *Ahr* and *Arnt* mRNA has been found pre- and postnatally in the hypothalamus [292], and *Ahr* mRNA has been detected in virtually all GABAergic (γ-aminobutyric acid) neurons in the preoptic area on postnatal day 3 [125]. The adult rat brain exhibits wide *Ahr* and *Arnt* mRNA expression, with especially high levels occurring in the caudal part of the arcuate nucleus (ARC) in the hypothalamus [144, 159, 266], and there is also pronounced DRE binding in the hypothalamus [189]. On the other hand, in adult mice, *Ahr* mRNA levels in the brain were about 10-fold lower than in the lungs, the tissue displaying the highest expression [208]. The data regarding AHRR are somewhat conflicting. Huang et al. [143] noted the basal *Ahrr* mRNA abundance to be high in the mouse pituitary, but low in the cerebellum and cortex, and negligible in the hypothalamus, whereas Bernshausen et al. [21] found most *Ahrr* mRNA, among the nine tissues examined, in the brain and heart, and the expression diminished by two orders of magnitude in *Ahr*-deficient mice, indicating a key regulatory role for the AHR [21]. Furthermore, strong AHRR immunoreactivity was seen in the mouse hippocampus and cortex, and staining was recorded in nuclei of several neurons throughout hypothalamus [86].

The aryl hydrocarbon nuclear translocator 2 (*Arnt2*) is a paralog of *Arnt* whose expression is primarily confined to the brain in adult mice [75, 134]. During mouse development, the two genes show a high level of overlap in most organ systems, while *Arnt2* mRNA is especially intensively expressed throughout the CNS [7]. In the rat hypothalamus, the highest expression of *Arnt2* mRNA has been found in the SON and in the PVN, but low or moderate levels have been detected in most other hypothalamic regions [266]. The function, if any, of ARNT2 in the classical (CYP1A1) AHR signalling pathway is debated, since it has the ability to dimerise with the liganded AHR *in vitro* and is influenced by the activating ligand, yet appears to be limited in its ability to influence AHR-mediated signalling in cell culture [74, 122, 331]. On the other hand, ARNT2 appears to function in close connection with SIM1 in neuronal development, since homozygous *Arnt2* mutants die perinatally and exhibit an identical hypothalamic defect to that observed in homozygous *Sim1* mutant (see before) mice [170]. It is postulated that heterodimerisation with ARNT2 is required for SIM1 function in the developing hypothalamus [140, 170, 229], and accordingly, expression of *Sim1* has been shown to be regulated by AHR–ARNT2 and react to TCDD *in vitro* [428].

Induction of phase I and II xenobiotic-metabolising enzymes has been shown to occur in various regions of the rodent brain at mRNA, protein and enzyme activity levels, attesting to the presence of a functional AHR signalling cascade in the CNS [52, 139, 143, 144, 386]. In Sprague–Dawley (SD) rats, a non-lethal dose of 10 μg/kg TCDD brought about a fairly uniform induction of *Cyp1a1* mRNA throughout the brain up to 4 weeks after exposure, but the response was much weaker in the CNS than in the liver, and no

CY1A1 or CYP1A2 protein was detected in the brain with Western blotting [144]. On the other hand, in L-E and H/W rats at 10 days after 5 or 50 μg/kg TCDD, certain regions of the brain exhibited induction of CYP1A1 activity (measured as EROD [ethoxyresorufin O-deethylase] activity), whose magnitude was almost comparable to that measured in the liver [386]. Treatment of SD rats with another AHR agonist also led to the induction of both *Cyp1a1* and *Cyp1a2* mRNA in various regions of the brain [323], while CYP1A1 immunoreactivity and catalytic activity appeared to largely localise in choroid and arachnoid membranes [240]. Increased CYP1A1 and CYP1B1 protein (as well as three xenobiotic efflux pumps) was detected in brain capillaries from SD rats 2 days after 1 or 5 μg/kg (i.p.) TCDD administration [397]. As to individual cell types in the CNS, AHR agonist administration induced CYPA1A1 protein expression in endothelial cells of veins in mouse brain and leptomeninges, and in the capillary endothelium of the choroid plexus, whereas CYP1B1 protein was constitutively expressed in smooth muscle cells of brain and leptomeningeal arteries [110]. The CYP1A1 and CYP1B1 induction response to TCDD has been reported in mouse primary cerebellar granule neuroblasts [407] and in mouse cerebral vascular endothelial cells, while in astrocytes, CYP1B1 but not CYP1A1 was induced [88].

2.3 ASPECTS OF DIOXIN TOXICITY IN HUMANS AND ANIMALS

The toxicity of dioxins, principally TCDD, has been addressed in a vast number of epidemiological investigations in man and experimental studies in animals. In humans, unequivocal acute or subacute dioxin toxicity has been noted in several workplace exposures (subacute to chronic) and accidents in the chemical industry, in an industrial accident in Seveso in 1976 and in two incidents of direct food (rice oil) contamination: “Yusho” in Japan 1968 and ”Yucheng” in Taiwan 1979, each affecting approximately 2000 people [84, 141, 257, 262, 353, 403, 431]. Notably, few signs of acute or subacute toxicity were noted in military personnel applying dioxin-contaminated phenoxyacetic-acid herbicides, mainly Agent Orange, in Vietnam during operation Ranch Hand [150], possibly explained by the length of time between exposure and assessment [403].

Epidemiological data and animal experiments both attest the developing foetus and children (combined with elevated exposure through breast milk) to be very susceptible to the acute and long-term harmful effects of dioxins [84, 115, 141, 365, 403, 427]. The sensitivity to cumulative high-dose prenatal exposure was most gravely demonstrated in the Yusho and Yucheng catastrophes, where intrauterine growth retardation, low birth weight, hyperpigmentation, neurological dysfunction, dental anomalies and alterations in sexual development were noted [84]. In the latter case, even infant mortalities were recorded (8 deaths among 39 infants severely exposed *in utero* [141]). Sensitivity to prenatal and infant exposure is especially well characterised in tooth development defects at minute chronic doses [9, 408]. Accordingly, the latest authoritative TCDD risk assessment by the US EPA [383] relies heavily on the lowest observed adverse effect levels (LOAELs) supplied by epidemiological studies relating foetal dioxin exposure to altered neonatal thyroid function [12] and acute pre- and postnatal (up to puberty) exposure to lowered adult sperm quality [232, 233]. On the other hand, the relationship and dose response between adult chronic dioxin exposure and the manifold suspected long-term

alterations and health effects – especially carcinogenicity – are debated [1, 28, 145, 149, 212, 322, 370, 381, 403].

2.3.1 ACUTE AND SUBACUTE HUMAN TOXICITY

Adult humans generally appear to be relatively resistant to the toxic effects of dioxins after single or short-term exposure. In three documented cases of accidental or deliberate poisonings with very high measured internal doses, 26 000–144 000 pg/g serum lipid (corresponding to an external dose of 6–25 μg/kg) of TCDD, the main clinical signs were persistent chloracne and gastrointestinal symptoms, but no permanent organ damage was reported [104, 321]. However, in the case of Mr. Yushchenko, followed from the start of the intoxication, acute to subacute gastrointestinal inflammation, hepatitis and pancreatitis as well as neuropathy and arthritis lasting over a year were detected [321]. Concurring findings of chloracne and gastrointestinal ailments, without other apparent early or mid-term health effects, were detected, mainly in children, in Seveso following a single (short-term) exposure, with the highest internal measured dose of 56 000 ppt in serum [23, 50]. Typically, chloracne has been detected after cutaneous exposure and with back-calculated concentrations of over 8000 pg/g serum lipid [322] – but sometimes even below 1000 pg/g [157, 353]. These estimates are, however, riddled with various uncertainties and greatly variable exposure lengths.

In Yusho and Yucheng ("oil disease" in Japanese and Chinese, respectively) poisonings, caused by rice oil contaminated with technical PCB fluid, the main finding in adults was chloracne accompanied by cutaneous and mucosal hyperpigmentation and conjunctival discharge from the Meibomian glands [141, 190]. Besides these, a number of symptoms and objective clinical alterations, including inconstant abdominal pain, were recorded for Yusho, but no mortalities were directly attributed to acute or subacute toxicity [190, 431]. However, an increase was noted in standardised mortality ratios (SMR) of diabetes mellitus and cardiovascular disease shortly after exposure in a recent epidemiological study [165]. In contrast, few clinical signs or symptoms besides cutaneous changes were recorded (or reported) in adult Yucheng patients, while 11 of them died from hepatoma, liver cirrhosis or liver diseases during a 4-year follow-up period [141], and a significant increase in SMR of chronic liver disease and cirrhosis during the first seven years after exposure was detected in males [364]. Notably, both Yusho and Yucheng patients were exposed to complex mixtures of PCBs and PCDFs, and estimated to have consumed roughly 1 g of PCBs and 3–6 mg PCDFs [114, 141, 190] during the incident. Thus, non-planar PCBs have also been suggested to have taken part in the toxicity [115], although dioxin-like chemicals, mainly 2,3,4,7,8-penta-CDF (back-calculated concentration in 5 Yusho patients about 20 000 TEQ pg/g serum lipid) [220, 221], are generally considered to have been the principal toxicants [165, 166, 188, 257, 431].

2.3.2 ACUTE TOXICITY IN LABORATORY ANIMALS

In laboratory animals, the most conspicuous features of the acute toxicity of TCDD are delayed lethality, drastic and long-term changes in body weight and eating, and huge inter- and intra-species differences in susceptibility stemming from AHR structure (see 2.2.) [274, 280]. Even high acute dioxin doses do not kill animals immediately, but death occurs in one to several weeks, and in many species (e.g. rats, mice, hamsters and guinea

pigs) after substantially reduced feed intake and prominent weight loss [99, 128, 171, 256], called the wasting syndrome. Lethal levels and those inducing wasting vary widely among species and strains: The LD50 value of TCDD for guinea pigs is 1–2 μg/kg, and for most rat strains 20–50 μg/kg, but the most resistant H/W rat strain may tolerate more than 10000 μg/kg. In mice, the LD50 for most strains ranges from 150 to 300 μg/kg, but for a resistant strain, DBA/2, it is about 10 times higher. The most resistant mammalian species is the hamster, with an LD50 of 1000–5000 μg/kg. Gender also affects sensitivity, and in a species-specific manner. Female rats are about twice as sensitive to the acute lethality of TCDD as males, but in contrast to rats, female mice are the resistant gender, the divergence being over 10-fold in one tested substrain [274]. Body weight loss due to hypophagia seems to be the principal reason for death in wasting syndrome-affected species [272]. However, additional factors seem to be at play, since parenteral nutrition does not prevent lethality [98, 142].

The functional and morphological alterations in experimental acute dioxin toxicity are numerous and tightly coupled with the species, strain, gender and age of the affected animal [27, 272, 275, 287, 327], recently summarised by Pohjanvirta et al. in [274]. Notably, and in contrast to humans, chloracne is a very unusual finding in experimental animals, excluding rhesus monkeys [224], and has only been induced in hairless mice and rabbits, usually by topical administration [293, 327]. In brief, TCDD is immunotoxic [172] and a potent endocrine disruptor, lowering and/or altering the circadian rhythmicity of several hormones and interfering with their receptors through AHR interaction [38]. The underlying mechanism of the disturbances in hormonal levels may also be accelerated metabolism (thyroxine, melatonin), impaired biosynthesis (testosterone, corticosterone) and adaptive or compensatory reactions and/or a mixture of mechanisms (TSH, insulin) [272]. There are also a number of changes in clinical chemistry parameters, many related to liver function, but the changes are often species specific [272, 275].

Thymus, liver, gastrointestinal tract and testis are among the tissues showing morphological alterations in acute TCDD toxicity in young adult animals; however, the changes are again, excluding thymic atrophy [93], highly dependent on the species studied [223, 275]. Surprisingly, after 30 years of intensive research, the critical target tissue for the acute lethal toxicity of TCDD remains to be found [272]. The drastic adaptive CYP induction brought about by minuscule dioxin exposure does not explain the toxicity, as shown by the similar CYP1A1 response in H/W and L–E rats and *Ahr*-transgenic mice [184, 270, 271], and the dissociation of the persisting CYP1A (EROD activity) induction from the diminished acute toxicity in ARNT hypomorphic (having a low-expressing Arnt allele) mice [391]. In the mouse liver, *Cyp1a1* and *Cyp1a2* induction through the DRE cluster even appears to offer protection against acute dioxin-induced hepatocellular necrosis and hepatic inflammation [252]. There might, however, be a slight indirect effect of *Cyp1a2* induction, since the CYP1A2 protein accumulates TCDD in the liver of rats and mice [168, 390], and a deficiency of its gene leads to a lower hepatic TCDD concentration [72, 73], affording partial protection against hepatotoxicity in mice [340]. In addition, in the mouse, CYP1A2 appears to play a causal role in dioxin-induced hepatocellular hydropic degeneration, but not in inflammatory or necrotic changes [252].

The liver is a central and almost invariably affected organ in acute TCDD toxicity, suggested to show the most promise in elucidating the fundamental biochemical and molecular mechanisms of dioxin action [29], and hepatopathies have been related to high

dose exposure in humans (see above). The hepatotoxicity also serves as an excellent, and the most studied, example of the species-, strain- and even gender-specific effects of TCDD and AHR physiology at the organ level. Generally, the prominent morphological liver alterations in dioxin intoxication are hepatocellular hypertrophy, multinucleated hepatocytes (in rats and hamsters), steatosis and inflammatory cell infiltration, as well as scattered necrotic foci, and are accompanied by respective serum transaminase elevations depending on lesion severity [275]. The liver lesions, however, differ quantitatively and qualitatively among species, and at least between the sensitive L–E and resistant H/W rat stains [273, 368]. Among mammals, severe liver necrosis might be directly attributed to lethality only in rabbits [223]. In the most TCDD-sensitive species, the guinea pig, lesions are conspicuously mild [116], and rats generally exhibit substantially less liver affection than mice [275]. Recently, a drastic difference between sexes in TCDD liver toxicity was also shown by histology in mice; abundant necrotic foci and a purulent inflammatory reaction were observed in males, while little necrosis accompanied by a granulomatous reaction was recorded in females [274]. Microarray studies concur with the crude histopathological findings in attesting to a surprisingly different response to TCDD in the mouse and rat liver [30, 32]. Furthermore, the number of hepatic genes affected by TCDD is lower in H/W rats than in TCDD-sensitive strains [91], and different rat strains/lines exhibit dramatic transcriptional heterogeneity in their hepatic responses to TCDD, excluding some 10 classic AHR-regulated genes, such as *Cyp1a1*, *Cyp1b1*, *Nqo1* and *Tiparp* [31, 235, 429].

2.4 WASTING SYNDROME AND EATING REGULATION IN THE BRAIN – AN OVERVIEW

Derailment of the body weight and food intake regulation in laboratory animals, known as the wasting syndrome, is a highly characteristic effect of dioxins (mostly studied in TCDD) but a rare outcome of chemical exposure in general. In addition, although firmly established as being mediated by canonical AHR signalling, the biochemical and pathophysiological basis of the ailment have remained elusive. Below, I will address the salient features of dioxin-induced changes in food intake in connection with the central regulation of energy balance. This selective discussion is based on our recent in-depth review on the subject [213].

2.4.1 GENERAL ORGANISATION OF FOOD INTAKE REGULATION

Animals and humans adjust the amount of food eaten according to the caloric content of the nourishment, their energy expenditure and long-term energy stores, striving to maintain an energy balance. However, this homeostasis seems to be biased towards an upward shift in favour of excess adiposity, resulting in a new balance that is then defended [169, 241, 319, 333], and some models of intake regulation even discard the notion of an actively defended homeostasis (see 2.4.3). An opposite shift in energy balance towards reduced body fat and weight seems much less favoured [326], and is seldom observed in a physiological context, except for seasonal animals. When ample, uniform food is constantly available, regular eating patterns integrated with other behaviours are

established (the major "zeitgeber" being the light–dark cycle), and so-called distal cues (smell, taste, texture, etc.) as well as preparatory physiological alterations strongly regulate the size of meals [348, 416, 418]. In contrast, in "natural" conditions this regulation is mixed with, or even dominated by, environmental influences and internal non-homeostatic functions and processes, such as food reward, satisfaction, learning and cognition [241, 333]. Fittingly, human food intake regulation is suggested to partly consist of energy homeostasis and partly of "reward homeostasis" [344].

The central neuroanatomical system regulating eating is generally accepted to reside in hypothalamic and brainstem nuclei, amongst a myriad of connections with other brain areas. However, the primacy of this system as the integrator of (long-term) energy balance, as opposed to a more decentralised regulation, is debated [87, 241, 333]. In satiation-based food intake regulation, the energy balance sets the general sensitivity of the hypothalamus – caudal brainstem ("hindbrain") system to satiety signals affecting the meal size; an abundance of stored and circulating nutrients enhances sensitivity to the meal-terminating signals [241, 249, 417]. On the other hand, the incentive (reward) value of palatable food can override satiety signals and promote excess eating, aptly termed non-homeostatic food intake regulation. Here, the reward itself contains several psychological components (liking, wanting and learning) that correspond to distinguishable neurobiological mechanisms [22], with partly overlapping and only partially known CNS circuits and signals [87, 191, 249, 333].

2.4.2 WASTING SYNDROME

The reduction of body weight in dioxin-induced wasting syndrome has been shown to primarily result from hypophagia in rats, mice and guinea pigs [171], and neither gross malabsorption nor increased energy expenditure seem to contribute substantially to the wasting [291, 328, 330]. TCDD-treated rats also do not appear to suffer from nausea [280], although recent data regarding neophobic food aversion, induced by TCDD doses not affecting food intake [204, 205], has raised the possibility of some visceral ailments. Typically, a single lethal TCDD dose leads to a dramatic reduction in feeding and a consequent decline in body weight of up to 50%, while a persistently stunted growth and decreased feeding results from a single high but sublethal dose [275, 283, 328, 329]. In rats, the most studied species, food intake typically progressively decreases during the first few days after TCDD exposure, leading to weight loss, but the animals do not usually show a total refusal of feeding (an exception being H/W rats at doses over 100 μg/kg [278]). At sublethal doses, rats generally increase their feed intake and start gaining weight in 1–2 weeks, but their body weight lags behind that of control rats [275, 328, 329], and some alterations in feeding rhythm, feeding behaviour and feed choice remain [202, 204, 283, 377].

An alteration in the circadian pattern of feeding after a (lethal) dose of TCDD was first noticed in TCDD-sensitive L–E [279] and SD rats [58], and the H/W strain was later found to exhibit a slowly developing but persistent increase in proportional feed intake during the light hours of the day after exposure to a high sublethal dose (1000 μg/kg) of TCDD [276, 277]. A recent feeding and drinking microstructure study in L–E and H/W rats employing a sublethal (10μg/kg) and a lethal (100 μg/kg) and two sublethal doses (100 and 1000 μg/kg), respectively, partly confirmed previous findings [202]. In L–E rats,

the lethal dose of TCDD induced a precipitous drop in feed intake accompanied by a reduced meal size in the evening, night and morning, but not in the daytime, and eating suppression peaked during the morning, while at the sublethal dose only a slight decrease in the number of meals during the daytime was observed. In contrast, in H/W rats the hypophagia remained moderate at both doses, stemming from reduced meal frequency, and the main effects were seen during the constant light or dark phases. In conclusion, the AHR may play a modulating role in the circadian rhythms of feeding, but different regulatory pathways seem to underlie the feeding responses in L–E and H/W rats [203].

Feeding (as many other phenomena) has circadian rhythms controlled by clock genes belonging to the bHLH/PAS protein superfamily [174], and the neuronal circuits of feed intake regulation and circadian rhythmicity are closely connected (see 2.4.6). Expression of the AHR protein fluctuates in several tissues of mice and rats in the course of the day [242, 309], and although the physiological role of the AHR in circadian rhythms is still debatable, some findings support a role for the clock genes Per1 and Per2 in the modulation of AHR-mediated responses to TCDD in different organs *in vivo* [242, 295, 296] and *in vitro* [96, 428]. Both genes are strongly expressed in the rat suprachiasmatic nucleus (SCN; the master circadian clock) [332], and a low dose of TCDD has been shown to induce a rapid and transient phase shift in the circadian expression of mouse PER1 protein in the SCN and liver [207]. Another system that bears on the circadian feeding rhythms and food intake is the histaminergic tone in the hypothalamus [118]. Accordingly, a slight increase was recorded in histamine concentrations in the whole hypothalamus of L–E rats at 28 h after a low but generally lethal TCDD dose of 50 μg/kg [369], and in the median eminence of outbred Long–Evans rats at 25 h after a high lethal (1000 μg/kg) dose [376]. These changes, however, were minor and do not support the role of histamine as a key mediator in TCDD-induced hypophagia.

Exposure of rats and mice to TCDD not only reduces total feed consumption and alters the feeding rhythmicity, but also results in a peculiar, rapidly-emerging and long-lasting aversion to novel food items, originally observed as a neophobic avoidance of a tasty food item (chocolate) in TCDD-exposed rats [377]. Later, the neophobic food aversion was found not to be strictly confined to any specific food type, although exceptionally pronounced to chocolate, but requiring temporal proximity to the TCDD exposure [204], and findings from *Ahr*-deficient and wild-type mice implied the involvement of the AHR [205]. Intriguingly, the latter study also showed the similarity of the ED50 values for novel food item aversion to those for CYP1A1 induction in the liver, suggesting the aversion to be a means to restrict exposure to potentially toxic dietary substances causing the hepatic induction of xenobiotic-metabolising enzymes. The relationship between the aversion reaction and the wasting syndrome is, however, unclear. Mechanistically, the novel food avoidance could be explainable by taste (gustatory) neophobia (innate fear of new foods) or conditioned taste aversion (CTA; learned aversion to a novel taste associated with transient visceral illness); see 2.4.7 for discussion of the neurophysiology of these reactions.

Experiments combing TCDD with other food-intake and body weight change-inducing treatments have yielded variable results, while the animals generally defend their lowered body weight level against feeding challenges [275, 277, 328-330]. The type of diet affects the severity of the body weight loss and lethality, and fat appears to be an unfavourable source of energy after TCDD exposure [246, 247, 372, 377]. However, an unexpected

outcome regarding the effect of diet appeared in a study in female TCDD-sensitive mice, which on a high-fat diet significantly increased their weight gain after biweekly exposure to 100 μg/kg TCDD for a period of 8 weeks [435]. In comparison, in a recent study employing a high-fat and low-fibre (Western) diet but no dioxin exposure, TCDD-susceptible mice with a high-TCDD-affinity AHR gained significantly more weight and more fat tissue (in absolute terms) than congenic 10-fold more TCDD-resistant mice with a low-affinity AHR. The regular diet had no significant differential effects [173].

2.4.3 WEIGHT SET POINT AND WASTING

To explain the apparent energy homeostasis, a theoretical "set-point" towards which body mass (or its correlate) is regulated was proposed [129, 344], contested by a "settling-point" hypothesis contending that body weight and adiposity simply represent a freely fluctuating settled level formed by energy intake (inflow) and expenditure (outflow) [411]. Later, a refined, simulation-based "general model of intake regulation" akin to the settling-point hypothesis was put forward to specifically address human behaviour and disposition to obesity [62], and finally a "dual intervention point model" was recently devised, combining the active regulation of the set-point model, outside of the upper and lower intervention (set-) points, with the settling-point model of passive regulation operating in between them [344]. Notably, the last two models are abstract and address weight regulation, especially obesity, mainly in humans. In contrast, a number of studies in laboratory animals (and in humans in certain settings) have demonstrated that body weight seems indeed to be defended [169, 344], and this system can be actively regulated and dynamically adjusted [48, 237].

There is compelling evidence of the avidity of dioxin-treated rats to defend their lowered body weight level (see 2.4.2) and react to lesions of the hypothalamic nuclei (see 2.4.6), in a way consistent with the maladjustment of a pursued body weight set-point. In addition, rats made diabetic (showing simultaneous hyperphagia and underweight) before TCDD administration continued to exhibit increased eating relative to non-diabetic control rats, suggesting that reduction of feeding is a secondary rather than the primary effect of TCDD [284].

The regulated primary variable for a weight set point is, however, still elusive. Initially, blood steroids were suggested as the principal correlate of body weight [129], and this idea was later modified by hypothesising that the concentration of hypothalamic corticotropin releasing factor (CRF) would serve this function, so that a decrease in this concentration would stimulate food intake [48]. On the other hand, studies in seasonal mammals have pinpointed thyroid hormone as a cause of the rhythmic alterations in energy balance [79], the ultimate regulator in this case conceivably being the thyrotropin-releasing hormone neurons [197]. Finally, a structural basis for the set point might be formed by synaptic or neuronal plasticity with a perpetual struggle between orexigenic and anorexigenic tones [4, 71]. Intriguingly, plasma ACTH and corticosterone levels are elevated in rats treated with TCDD [25, 236], suggestive of increased hypothalamic CRF. One study also detected increased mRNA levels of CRF in the hypothalamic PVN (a critical site for the effects of CRF on food intake [185]) and ARC nuclei 7 and 14 days after TCDD administration, while there was a transient reduction in CRF expression in some related nuclei at 2 days [236].

2.4.4 PERIPHERAL ADIPOSITY AND SATIETY SIGNALS

The long-term adjustment of body weight and the regulation of energy balance in mammals is based on keeping the body fat stores constant with the aid of two adiposity signals, leptin and insulin (Figure 5); for recent reviews, see e.g. [241, 319, 417]. Leptin, originating from adipocytes, and insulin, produced by the pancreas, convey information on body adiposity to the CNS [16, 17], while insulin also has a second, equally important and partly overlapping role in constant regulation of plasma glucose [103, 194]. Depletion of leptin or insulin, or their receptors, generally results in increased food intake and obesity mediated by the brain [103, 319]; type 1 diabetes represents a pathological state of insulin depletion leading to weight loss due to severely impaired glucose utilisation. Administration of exogenous leptin or insulin (at low doses not inducing hypoglycaemia) brings about marked reductions in food intake and body weight [113, 120, 191, 317], but after about two weeks of leptin administration food intake normalises while body weight remains repressed [120, 317, 318]. Resistance to leptin and insulin occurs in obesity and type 2 diabetes [87, 120, 239], as well as in inflammation in the CNS [289, 359].

At lethal doses, TCDD reduces circulating insulin levels in rats most likely through diminished feed intake [108, 109], while at a sublethal dose it seems to directly reduce insulin secretion [108]. Impairment of glucose-stimulated insulin secretion was also reported in islets isolated from rats treated with a low (1μg/kg; no effect on feed intake) TCDD dose [251]. On the other hand, various findings imply that TCDD improves insulin sensitivity (see also 2.4.4) at the whole organism level, at least in rats and mice [213], prompting further research regarding the transport of insulin from the blood to the CNS and the central insulin action in TCDD-exposed animals in relation to wasting syndrome pathogenesis. There is also a paucity of data on the possible influence of dioxin on leptin. A small-scale experiment with TCDD doses affecting feed intake showed an initially elevated plasma leptin concentration (measured with mouse leptin antibody) on day 1 after TCDD exposure, but then an identical downhill course in both TCDD-treated L-E rats and their pair-fed controls [371]. In comparison, unchanged plasma leptin levels were reported 24 h after a low (1μg/kg) dose of TCDD in SD rats [251].

In lieu of the long-term adiposity signals, there are a number of messengers that suppress appetite in response to food and energy ingestion and act as satiation signals (Figure 5) [417]. These small peptides are produced in the intestine (e.g. cholecystokinin [CCK], glucagon, oxyntomodulin, peptide YY, enterostatin) or in the pancreas (amylin and pancreatic polypeptide), and most of them act primarily or partially via vagal afferent fibres [20, 39, 53, 76, 164, 183, 313]. Traditionally, the satiation signals were considered to be short acting, but at least some of them seem to have also a role in longer-term energy balance regulation and interact both with each other and with insulin and leptin [24, 218, 419]. In regard to TCDD and energy ingestion, a long-lasting change in consummatory behaviour, interpreted as augmented sensitivity to post-ingestive satiation, was detected some 2 weeks after exposure to a high (1000 μg/kg) but sublethal dose of TCDD in H/W rats [276, 282]. However, the feeding response to peripherally administered CCK concomitantly remained unaffected [277, 282], and vagotomy had an additive, not an alleviating effect on feed intake and body weight in TCDD-treated L-E or H/W rats [368].

2.4.5 PERIPHERAL SIGNALS INDUCING FOOD INTAKE

Peripheral hormonal signals directly inducing food intake are conspicuously few (ghrelin being the sole generally accepted example), but several neurons in the CNS and peripherally are capable of sensing the levels of glucose, fatty acids and amino acids (Figure 5) [194, 319, 360]. In addition, the brain indirectly receives information on (at least) peripheral glucose levels through the vagus nerve, insulin and ghrelin [194, 342, 360, 426]. Direct nutrient sensing of glucose, in particular, is important in maintaining the short-term energy stores at physiological levels and also intricately intertwined with the long-term regulation of energy balance. As an example of this constant regulation, a decrease in blood glucose below the euglygemic level triggers an immediate release of glucagon and elicitation of feeding [194, 360], although this glucostatic/glucoprivic feeding initiation is generally assumed to come into play in non-physiological, acute energy depletion states [77]. Ghrelin seems to act both as an "acute" hunger signal and as a longer-term augmenting factor of feeding [24, 71, 154, 294, 419]. It is produced both peripherally and centrally in the ARC and appears to increase food intake by direct CNS receptor (growth hormone secretagogue receptor; *Gshr*) stimulation and/or through the vagus nerve, with the possible involvement of hypothalamic *de novo* synthesis [24, 61, 71, 158, 182, 294, 419].

No studies have hitherto addressed the possible role of ghrelin in the wasting syndrome, but glucose-depletion (glucoprivic) challenges have been imposed on TCDD-treated rats. A high but sublethal dose of TCDD (1000 μg/kg) in H/W rats rapidly abolished the normal feeding response to glucoprivation induced by 2-deoxy-glucose (2DG) or by a high dose of insulin [283], and the response seemed to be persistent [277]. Thus, the inability of TCDD-exposed rats to respond by eating to an acute energetic crisis may be the key reason for their sensitivity to insulin lethality [109, 277, 283]. In comparison, in mice a low TCDD dose (not affecting body weight gain) induced a swift and long-lasting reduction (20–30%) in glucose transport to the brain and a comparable reduction in GLUT1 (*Slc2a1* gene) protein expression [214]. As noted earlier, vagotomy did not markedly modify the effect of TCDD on feed intake or body weight [368], arguing against a critical role for vagal signals in the wasting syndrome. However, vagotomy had an additive diminishing impact on body weight, resembling that of a DMH lesion (see 2.4.6).

2.4.6 THE HYPOTHALAMUS IN FOOD INTAKE REGULATION

The hypothalamus, particularly the ARC, as well as the ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), LHA and PVN are believed to play a pivotal role in the integration of feeding-regulatory signals, especially insulin and leptin [191, 194, 248] (Figure 5). As a basic mechanism, insulin and leptin modulate in tight integration the two populations of neurons acting as the cardinal regulators of energy balance in the ARC [87, 194, 319]: the food intake increasing agouti related protein/ neuropeptide Y (AgRP/NPY) neurons reduce their activity and neurotransmitter expression following leptin and insulin stimulation, whereas the anorexigenic neurons, expressing pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART), function in the opposite way. However, the organisation of the system, even in the ARC, is far more complex, as exemplified by the differing electrophysiological

effects of insulin and leptin [133, 241, 319], constant suppression of POMC/CART neurons by AgRP/NPY neurons [95, 228], the considerable regional variability in leptin responsiveness of POMC neurons [325], and the non-monotonous effects of exogenous leptin (see 2.4.4). The ARC also receives and projects various inhibitory and excitatory contacts to other areas in the hypothalamus [4, 241, 319], and the synaptic or neuronal plasticity in- and outside the ARC (also shown to be modulated by ghrelin and leptin) is even suggested to be a structural basis for the body weight set-point [4, 71]. Notably, plasma nutrient levels, at least glucose and fatty acids, also influence the activity of orexigenic and anorexigenic neurons in the ARC [195, 290, 319].

Beyond the ARC, AgRP/NPY and POMC/CART neurons innervate the VMH, PVN, LHA and DMH, as well as several other hypothalamic and extra-hypothalamic nuclei, e.g. the parabrachial nucleus (PBN) [4, 36]. POMC/CART neurons even extend their connections to the brainstem, including the *nucleus tractus solitarii* (NTS) [339]. Of the hypothalamic nuclei, the "satiety centre" VMH, restricting eating and body weight gain, is assumed to have an independent role in the promotion of food intake, responding to at least glucose and leptin [249, 319], while the anorexigenic PVN and the orexigenic LHA are important relay sites of information for autonomic and neuroendocrine effects and eating, with afferent connections to the brainstem. The LHA also communicates with cortical neurons and integrates energy balance and reward circuits [87, 241, 249]. The orexigenic DMH is innervated by NPY/AgRP-positive fibres from the ARC, and, akin to the VMH, autonomously adjusts the energy balance in response to glucose and leptin [19, 180, 436]. The VMH–SCN connection has recently been shown to affect experimental food anticipatory activity, attesting to the role of the VMH in the link between circadian rhythms and eating regulation [5].

Studies combining TCDD administration with targeted hypothalamic lesions have revealed some hypothalamic nuclei to be connected with the wasting syndrome, and offer support to the notion that TCDD somehow adjusts the pursued body weight level. A VMH lesion inducing hyperphagia and metabolic obesity aggravated TCDD-induced wasting syndrome in both TCDD-sensitive L–E and TCDD-resistant H/W rats when it preceded TCDD treatment [373], while a lesion of the orexigenic DMH induced an additive reduction of food intake with TCDD in H/W rats [375]. Respectively, findings after a lesion of the anorexigenic PVN (causing an increase in food intake and body weight, especially with a palatable diet) were slightly different from the VMH lesion [374]. The PVN lesion led to an increased feed intake and overweight in comparison to the sham-operated controls only after the rats were offered palatable chocolate in addition to the standard diet. After TCDD administration, the lesioned rats reduced their feed consumption more than the sham-operated counterparts, so that 3 weeks after TCDD exposure the body weights of the lesioned and sham groups were indistinguishable. In addition, the TCDD-exposed rats clearly reduced their energy intake and changed their dietary composition, especially by reducing chocolate (fat and carbohydrate) intake, suggesting an aversion to energy-rich food and an altered taste preference. Intriguingly, this and the exaggerated anorexia after a PVN lesion might be mechanistically explainable by an effect of TCDD on a system or centre regulating body weight "before" the PVN, resulting in a similar set point in both lesioned and sham-operated animals. Notably, the PVN and DMH lesion experiments were conducted with H/W rats employing high but sub-lethal TCDD doses of 1000 µg/kg.

2.4.7 BRAINSTEM, REWARD CIRCUITRY AND FOOD AVERSION

The caudal hindbrain is the main site receiving information from peripheral energy metabolism, chiefly by way of the vagal afferent fibres (Figure 5). It is particularly involved in the control of meal size via signals arising from the mouth and gastrointestinal tract, and the NTS functions as an important integrator in energy balance regulation [24, 95, 112]. Caudal brainstem circuits also initiate glucoprivic feeding, contain systems for satiating signal and leptin interaction, and have multiple afferent connections to the hypothalamus [112, 310]. As to the impacts of TCDD on energy homeostasis, the hindbrain has not been addressed, but the prominent effects of TCDD on glucoprivic feeding, meal size and aversion to novel food items encourage further study. In addition, the caudal brainstem has been suggested to be an important target for the anorexigenic action of leptin in rats [113], possibly explained by the leptin-induced inhibition of serotonin synthesis and release from brainstem neurons, as shown in mice [424, 425]. This paradoxical effect of a reduced serotonin (a generally anorexigenic transmitter) level in the hypothalamus is thought to be mediated by specific serotonin HTR1A autoreceptors in the ARC (see 2.4.8).

The complex reward-based regulation of eating involves interactions between various CNS centres relying, for example, on dopamine (see 2.4.8.) and on opioid and cannabinoid systems in signal transmission [333, 355, 422]. The midbrain mesolimbic dopaminergic system contains a set of ventral tegmental area (VTA) neurons that innervate the striatum, amygdala and prefrontal cortex [249, 333], and leptin and insulin act directly on VTA neurons, reducing dopamine release and diminishing food seeking and intake [87], whereas food restriction and ghrelin induce an opposite effect [191, 333] (Figure 5). LHA orexin neurons project to the VTA, and stimulation of the LHA strongly promotes food seeking [87, 249, 333]. A subconscious “liking reaction” to food can be elicited by hindbrain neural circuits, and seems to be related to the striatal nucleus accumbens (NAc) and ventral pallidum [333], while the NAc has an inhibitory feed-back loop with melanin concentrating hormone (MCH) neurons in the LHA [320, 347]. The aversive reactions to food (see 2.4.2), taste neophobia and CTA, are closely correlated and are both mediated at the cognitive level by the basolateral amygdala (BLA) and insular cortex (IC) [211, 307, 312]. However, in CTA the required malaise stimulus is transmitted via the vagus nerve or through the area postrema to the NTS, and from there to the PBN, before reaching the BLA and IC [253]. Loss of GABAerigic (γ-aminobutyric acid; the main inhibitory neurotransmitter in the CNS) inhibition of PBN (see 2.4.8) even leads to anorexia and starvation in mice, possibly due to nausea [420].

The reward circuitry does not seem to have a direct critical role in the wasting syndrome, but the available mechanistic data are very scant. Increased neuronal activation was detected in several hypothalamic nuclei, the central amygdala and bed nucleus of the stria terminalis (BST) some days after TCDD (50 μg/kg) administration to rats [57], and later the same group reported an increase in methionine–encephalin immunoreactivity in various forebrain nuclei, such as the central amygdala, PVN and BST 2 weeks after similar TCDD treatment [56]. However, the authors suggest the latter to be a compensatory reaction to body weight loss and hypophagia. As an indirect finding regarding TCDD and food reward, H/W rats treated with a high dose of TCDD 3 months earlier exhibited a blunted reaction to the opioid antagonist naloxone, which reduced the 24-h fast-induced feeding in control animals [277].

2.4.8 THE NEUROTRANSMITTERS OF FEEDING REGULATION

In hypothalamus, the strongly orexigenic NPY signals through several (redundant) receptor types [161, 302], and the LHA seems to be the principal site of its action, even though it also inhibits neuronal firing in the PVN and VMH [4, 54]. The orexigenic effect through the LHA is generally believed to be mediated by MCH, with a shorter-term influence of orexins (hypocretins), although the exact mechanism of NPY-induced stimulation of MCH secretion is unclear [36, 117]. Another layer of complexity is exemplified in neuropeptide Y receptor Y5 (*Npy5r*) knock-out studies, in which unexpected obesity has resulted despite inhibition of the NPY system inhibition, probably due to a compensatory change in POMC/AgRP gene expression [130]. The anorexigenic melanocortin system is based on melanocyte-stimulating hormone (α-MSH), produced from POMC in POMC/CART neurons and secreted together with CART [4, 71]. The principal second order nucleus in melanocortin signaling is the PVN, which contains high levels of melanocortin receptors 3 and 4 (MC3R, MC4R) stimulated by α-MSH [4]. It also appears to be an important convergence point of anorexigenic and orexigenic signalling, since AgRP, the second product of AgRP/NPY neurons, is an antagonist of melanonocortin receptors, and CRF exerts its anorexic effect in the PVN.

The classical monoamine neurotransmitters serotonin (5-hydroxytryptamine, 5-HT), noradrenaline and dopamine, as well as histamine (see 2.4.2.), also take part in the control of energy balance. Serotonin generally has a suppressive effect on food intake and body weight, for example in the PVN, VMH and DMH, mainly acting through 1B (HTR1B) and possibly 1C (HTR1C) receptors [301], while autoreceptor HTR1A mediates hyperphagia [60]. It has even been proposed that leptin may exert its action on food intake by inhibiting the production of serotonin in brainstem neurons (see 2.4.7.). This would then curtail the appetite through reduced HTR1A activity in the ARC, leading to increased POMC expression and decreased NPY and AgRP expression [424, 425]. Noradrenaline may have dual effects on food intake: in the PVN, activation of α2 receptors stimulates feeding, whereas α1 agonists suppress it [301]. Dopamine (DA) appears to be more influenced by food intake rather than influencing it [301]. Intriguingly, however, mice that lack dopamine (DA), exhibit fatal hypophagia as part of a generalised hypoactivity and respond differently to the restoration of DA activity in individual nuclei, depending on the targeted area [355]. Restoration of DA production within the caudate putamen restores feeding and nest-building behaviour, whereas restoration of DA production in the NAc restores exploratory behaviour. Intervention in either region restores the preference for sucrose or a palatable diet without fully rescuing coordination or the initiation of movement, the classical alterations in dopamine deficiency.

Few studies have directly addressed the effect of TCDD on hypothalamic neuropeptides, dopamine or noradrenaline, while the serotonergic system has received considerable attention. A sublethal TCDD dose (15 μg/kg) in SD rats brought about an increased expression of *Npy*, *Pomc* and CART prepropeptide (*Cartpt)* mRNA in the ARC at a 6 days, and in the LHA, pro-melanin-concentrating hormone (*Pmch*) expression was also elevated [86]. In line with these findings, a dose (50 μg/kg) close to the LD50 value of the same strain of rat increased POMC mRNA levels in the ARC at 7 and 14 days; at the latter time-point, a significant increase was also found at lower doses [236]. Serotonin turnover was found to be uniformly accelerated after a single dose of 50 μg/kg in L-E (lethal) but not in H/W rats (sublethal) in several dissected brain areas, including the

hypothalamus [387]. However, the reason for the increased turnover was shown to be an increased level of plasma-free tryptophan [389], and it does not appear to be causally related to the wasting syndrome, because specific depletion of brain serotonin did not affect the TCDD-induced reduction in body weight [346]. In support of the reasoning above on the role of monoamines in TCDD toxicity, intervention with dopamine antagonists, an α-adrenoceptor blocker or a serotonin synthesis inhibitor did not modulate TCDD-induced wasting syndrome in L-E rats [279]. In conclusion, there is thus limited evidence for the direct interference of TCDD in the action of regulatory neuropeptides or monoamine neurotransmitters. However, more information is needed regarding the neuropeptides, especially on the early stages of dioxin intoxication, and possible changes at the level of single nuclei.

Recent findings underline the importance of GABAergic and glutamatergic (the dominant excitatory transmitter in hypothalamic neuroendocrine regulation) transmission in the regulation of feeding, especially in the LHA. Glutamate elicits an intense feeding response in satiated rats when injected into the LHA [228], and is currently considered as a major orexingenic factor, functioning especially through the various NMDA receptor subtypes [347]. In contrast, GABA appears to function in the LHA as a mediator of satiety via $GABA_A$ receptors [347, 379], while the loss of GABAergic transmission of AgRP/NPY neurons to the PBN leads to the activation of postsynaptic neurons and starvation, which is proposed to be related to the activation of circuits that normally promote nausea-induced anorexia [420, 421]. GABA is also orexigenic by way of its inhibitory action on POMC/CART cells in the ARC, where both $GABA_A$ and $GABA_B$ receptors have been demonstrated [228].

To combine the actions of NPY, glutamate and GABA, Stanley et al. [347] proposed the LHA to be the major integrator of moment-to-moment feeding, which to a large extent reflects the balance of glutamatergic and GABAergic inputs to LHA neurons, and that this balance modulates and is modulated by peptidergic actions. There is evidence from prenatally exposed animals that dioxins may affect GABAergic and glutamatergic neurotransmission in the CNS. For example, prenatal TCDD exposure in rats postnatally induced glutamate receptor subunit mRNA alterations in the cortex and hippocampus [138, 160], while in mice it was found to compromise the differentiation of GABAergic neurons in the ventral telencephalon [107]. However, hardly anything is known of the possible modulation of GABAergic or glutamatergic neurotransmission by dioxins in adult animals.

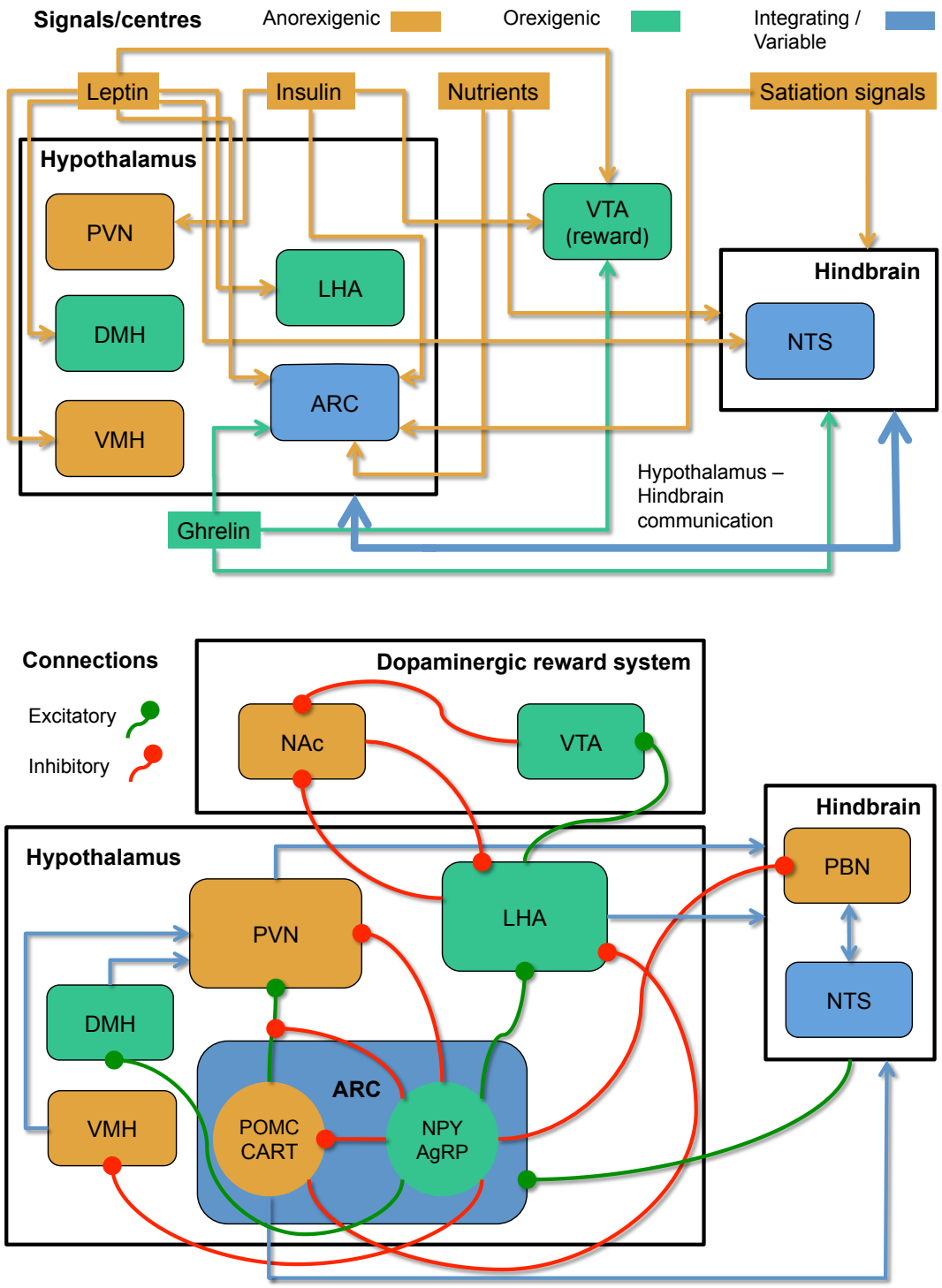

Figure 5. A highly simplified, schematic representation of some major centres in the CNS involved in food intake regulation and in the reception of peripheral signals mediating information related to adiposity, nutrients and satiation. The CNS nuclei and signals are designated anorexigenic (amber), orexigenic (light green) or integrating (blue) according to their primary role based, for example, on lesion experiments. Directly or indirectly excitatory connections are depicted by bright green links and directly or indirectly inhibitory connections by bright red links. PVN paraventricular nucleus, DMH dorsomedial hypothalamus, VMH ventromedial hypothalamic nucleus, LHA lateral hypothalamic area, VTA ventral tegmental area, ARC arcuate nucleus, NTS nucleus tractus solitarii, PBN parabrachial nucleus, NAc *nucleus accumbens*. Modified from [213, 241, 319].

2.5 QUANTITATIVE REAL-TIME RT-PCR IN GENE EXPRESSION ANALYSIS

Quantitative real-time RT-PCR is the most cost-effective and sensitive method to chart TCDD-induced gene expression alterations at various time points after exposure and a with limited amount of RNA. However, this methodology is not without challenges: The precision of the cDNA synthesis step is a major determinant of reliable expression analysis, and toxicological studies involving TCDD have an additional difficulty in finding stable reference genes among the extensive and divergent expression changes induced by AHR activation. The quantitative real-time RT-PCR technique is briefly reviewed here, since two studies of this thesis assessed the reproducibility of the reverse transcription step, the fidelity of the RT enzymes and the applicability of some control genes for dioxin studies.

2.5.1 DEVELOPMENT OF RT-qPCR

Two-step reverse transcription quantitative real-time PCR (RT-qPCR) is a technique to measure the abundance of mRNA transcripts (gene expression) by first reverse transcribing extracted RNA to cDNA in one reaction, and then quantifying the cDNA stretch corresponding to the mRNA of interest in a separate quantitative real-time PCR (qPCR) reaction [43, 44, 186]. The first step, reverse transcription (RT), has been used in molecular biology from the beginning of the 1970s [345], and the first studies (conducted at the end of the 1980s) that used reverse transcription and PCR to measure mRNA employed an RT step that was similar to the one used today. However, the PCR step was much different, as it relied on conventional end-point PCR and densitometric analysis of ethidium bromide (EtBR)-stained gels or radioactively labelled primers to quantify the amplified product (amplicon) [55, 304, 305, 396].

Using end-point PCR for quantitative analysis is inherently problematic [42], and the use of internal (reverse transcribed together with the target RNA) heterologous reference (housekeeping) genes – as was often done – did not amend this, although it allowed the normalisation and comparison of the measured raw target gene values. An alternative method, “competitive PCR”, offered a means to greatly improve the reliability of the quantification by using an added synthetic or cross-species nucleic acid standard that competed for the same primers as the target sequence in the same PCR reaction [334]. After PCR, the amplicon of interest and the standard amplicon were separated in an agarose gel based on their lengths, or according to sequence differences using other methods, and quantified as above. Furthermore, the synthetic nucleic acid standards could be also made of RNA (and can thus be referred to as internal for the RT reaction), enabling the normalisation of both RT and PCR reactions and a simple (in principle) way of multiplexing [396]. Incidentally, competitive PCR, with various quantification protocols and strategies, has remained and developed over the years as an alternative to qPCR, especially for mRNA quantification, as it is claimed to be exceedingly accurate [433], while not as adaptable as RT-qPCR.

Real-time monitoring of the PCR reaction for quantification of the initial template copy number was first suggested and developed by Higuchi et al. using EtBR [131, 132]. Some three to four years later, in the mid-1990s, the technique was refined to use a threshold

cycle C_q (C_t) for quantification and dual-labelled fluorogenic hybridisation probes as reporters [106, 126], and the first dedicated rapid cycle qPCR instrument was described [413]. In addition, another system of fluorogenic probes and the currently most employed dsDNA-specific dye (SYBR Green I) were exploited as reporters, and melting curves suggested as a means to measure the specificity of the amplification [412]. Finally, in the following 10 years, the qPCR technique established itself as the most powerful and agile tool for quantitative nucleic acids analysis [186, 398]. It has shown burgeoning growth in the last few years, attested for example by the exponential increase in the annual PubMed citations retrieved with the keyword "qPCR", and by the large number of reviews dealing with the subject [45, 176, 186, 244, 361]. There is also an active and opulent website dealing with various aspects of RT-qPCR (Gene-Quantification.info WWW [URL: http://gene-quantification.info]).

2.5.2 HYBRIDISATION-BASED mRNA ANALYSIS TECHNIQUES

A number of hybridisation techniques are capable of directly detecting and quantifying mRNA. These methods offer the definitive advantage over RT-qPCR of not requiring the poorly controllable RT step, although they have a number of their own shortcomings. Below, the four most widely used of these methods are discussed in collation to RT-qPCR.

The first widely employed technique to directly detect specific mRNA species was Northern blotting, in which RNA is separated by gel electrophoresis, transferred to a membrane and identified by hybridisation with a labelled (often the radioisotope ^{32}P) nucleic acid probe [10, 11]. This method is still employed in expression analysis [15], and quite useful in the study of RNA degradation and transcript size [398]. The RNase protection assay is another method used to quantify and characterise RNA species. In this technique, isolated RNA is first hybridised with isotopically-labelled antisense RNA probe(s), protecting target sequence–probe hybrids from digestion in the following single strand-specific RNase treatment. The remaining target and labelled probe nucleotides are finally separated in a polyacrylamide gel and visualised by autoradiography [49, 198]. The RNase protection assay is currently most often used in studies involving analysis of the RNA production rate and decay or mapping of post-transcriptional RNA modification [8, 343], while it is generally less often employed for simple expression measurements. In contrast to Northern blotting, it offers the possibility of multiplexing (by adjusting probe lengths), and is more sensitive and accurate in low abundance mRNA quantification. However, both of these methods are technically complicated and multiphase, not as sensitive as RT-qPCR, not very straightforward to multiplex or extend, and are also hampered by quantification and normalisation difficulties.

A new hybridisation-based method, the nCounter gene expression system, using colour-coded probe pairs has been developed by NanoString Technologies [100]. This combination of capturing and reporting probes with colour-coded tags of several fluorophores provides a unique detection signal for up to several hundreds of mRNA species at the same time. The nCounter is claimed to be similar in sensitivity and accuracy to RT-PCR, and, being a streamlined commercial assay system, is uncomplicated to use (NanoString Technologies WWW [URL: http://nanostring.com]. Retrieved November 2012). On the other hand, the system is generally intended for the analysis of more than one hundred mRNA transcripts at a time with several pre-designed gene sets. Moreover,

although the desired mRNA targets (assuming their sequence is known) can be freely selected, the actual colour-coded probe sets are designed and synthesised by NanoString. The cost and time needed for the probe synthesis and the expense of the hybridisation and imaging unit thus make the system less well suited for rapid and affordable analysis of variable mRNA transcripts.

In situ hybridisation (ISH) enables the combined accurate anatomical localisation and detection or quantification of specific nucleic acid sequences either in tissue sections or in whole cell preparations, based on the complementary binding of a nucleotide probe (with a radioactive or non-radioactive label) to a specific target sequence of DNA or RNA inside cells [226, 399]. Accordingly, the ISH has become one of the principal techniques in neurophysiological and -anatomical research [86, 152, 200], and has evolved, with the advent of fluorescent and microscopic techniques, into a group of methods with abilities to provide extremely detailed chromosomal analysis and subcellular localisation of single mRNA molecules [206, 399]. The major drawbacks of the IHS and FISH (fluorescence *in situ* hybridisation) techniques are the difficult RNA probe synthesis, the inability to use more than a few probes simultaneously on one tissue section and the relatively poor reproducibility between experiments. These obstacles can be surmounted, as demonstrated by the genome-wide mouse brain expression atlas constructed using ISH with non-isotopic digoxigenin-labelled riboprobes [200]. However, this involved an industrialised and automated ISH and data-analysis platform and ample resources, perhaps best suited for generating basic physiological data.

2.5.3 RT REACTION AND REVERSE TRANSCRIPTASES

The reproducibility of qPCR has generally proven to be very good and the RT reaction step is believed to contribute most of the variation to the determination of mRNA quantities [349], making the quality of the cDNA and the reproducibility of the cDNA synthesis major, if not the most important, determinants of reliable gene expression analysis [46, 90, 186, 265, 401]. Especially when no stable reference genes or synthetic spiking RNA (see before) are utilised, both absolute and relative (comparative) quantification rely on the premise that the efficiency of RT and qPCR reactions are similar across all samples to be compared. Normalisation of the genes of interest to some internal reference gene, or preferably to a set of reference genes [363, 395], improves reliability, but still requires that the relative efficiencies of the RT reactions for the genes of interest and the reference genes are consistent from reaction to reaction; quantification without external qPCR standards extends this requisite to the qPCR [46]. If a single gene is selected for normalisation, it should always be pre-validated for the treatment or condition to be examined [68, 175, 324].

The importance of the quality (and homogeneity of quality) of the RNA to unbiased RT-qPCR analysis has been demonstrated in several studies (see above), and an analytical framework to assess the quality of RNA using several parameters has recently been conceived [401]. The effects of different types (gene-specific, random oligomer or oligo-dT) of primers used for cDNA synthesis have also been assessed in several studies [47, 201, 349, 434], but the performance of various RT enzymes has received little attention [47, 350].

Retroviral reverse transcriptases are RNA-dependent DNA polymerases that are capable of using RNA as their template. These enzymes were first discovered by Temin and Mitzutani [358] and by Baltimore [14] in 1970 and have become widely used in molecular biology [102]. The retroviral RT enzymes are capable of using both RNA and DNA as a template and have a peculiar partial endonuclease activity called RNAse H, enabling the same enzyme to cleave and degrade the RNA template from the synthesised DNA/RNA hybrid while producing complementary DNA [51, 167]. They are also much more error-prone than DNA-directed DNA polymerases, which (among other factors) enhances the genetic variability of retroviruses [258, 311].

The main types of reverse transcriptase originate from avian myeloblastosis virus (AMV), and Moloney murine leukemia virus (M-MLV), with possible modifications to improve their performance [102]. (To be precise, the AMV RT enzyme should be called AMV/MVA RT, since it does not originate from AMV but from myeloblastosis-associated virus [MAV], a natural helper virus present in AMV stocks [264].) Besides these two agents, other retroviruses and even bacteria may also be utilised as sources of RT enzymes [269, 336], but are generally much less used in practice. The best-characterised AMV- and M-MLV-derived enzymes differ somewhat in structure and function, both requiring divalent cations for activity [269]. The functional unit of AMV is a heterodimer consisting of two subunits (smaller α and larger β), the smaller one containing reverse transcriptase and RNAse H activity. In contrast, the M-MLV enzyme contains just one polypeptide comprising both domains [102]. Compared with M-MLV, native AMV has been shown to be more efficient [37], and to function at higher temperatures [102, 181], but to possess significantly greater RNAse H activity [66]. The main modifications to improve RT enzymes are aimed at increasing their processivity (amount and length of cDNA produced) and the fidelity of transcription of low abundance mRNA species. To achieve these aims, better enzymes are painstakingly being sought and the old ones modified.

2.5.4 PRECISION OF THE QUANTITATIVE REAL-TIME PCR

The absolute accuracy of gene expression measurements using RT-qPCR is questionable, since the true RT efficiency cannot be known, and the methods employing synthetic control RNA are cumbersome for routine analysis and riddled with some technical difficulties (see before). However, in assays measuring genomic DNA (e.g. copy number profiling), excellent accuracy and reproducibility can be achieved by careful optimisation and meticulous data analysis [67, 155]. The error induced by qPCR manifests itself on the one hand in systematic differences in reaction efficiencies among different templates or different amplicons (genes), and on the other in random tube-to-tube (well-to-well) variations. The former systematic (template-to-template and/or amplicon-to-amplicon) deviations in reaction efficiencies can be minimised by primer and reaction optimisation, experimental planning and repeated measurements. In quantification relying on a standard or calibration curve, the reaction efficiencies are assumed, or adjusted, to be equal in the standards and samples; this method may be called absolute or relative depending on whether the standard curve is based upon known units or some relative quantities [67, 196, 306, 354].

Relative quantification without external standard curves was originally based on an assumption of identical amplification efficiencies of both the gene of interest and the

reference (calibrator) gene [216], but has been refined to compensate for systematic efficiency dissimilarities between the gene of interest and the reference or calibrator gene or genes [127, 267]. In addition, relative quantification without external standards but with efficiency correction can be performed without the use of any internal reference or calibrator genes by calculating theoretical fluorescence and thence copy numbers at the first PCR cycle [261]. This method allows a formal separation between quantification and normalisation, and results in practically identical normalised results after either absolute or relative quantification. Considerable effort has indeed been invested in searching for and perfecting various quantification strategies, with or without external standard or calibration curves [127, 196, 354, 415].

Random variation in the tube- or well-specific efficiencies can be determined by modelling and fitting the real-time fluorescence curve [215, 300], but this approach has been questioned due to its mathematical complexity and introduction of systematic errors, increased noise and/or variance [261, 314, 432]. Currently, there seems to be a consensus on omitting the sample-specific PCR efficiencies and using a fixed or mean efficiency per amplicon for quantification [314]. Perhaps, therefore, individual tube-specific efficiencies are not employed in most of the automated software tools for mRNA expression quantification, normalisation and data analysis [127, 243, 267].

In conclusion, the basic technical determinants of the fidelity of expression analysis are largely the choice of equipment, primers and enzymes: Specialised (often high-throughput) instruments are used, and the RT and PCR enzymes employed are nearly always commercial, their manufacturer thus supplying a specific buffer concentrate for the reaction setup and providing protocols for the temperatures and reaction times to be used in both reaction steps. However, despite its apparent ease and rapidity, the current RT-qPCR technique is not without many intricacies, and its careless use has led to some high-profile failures [13]. In response to the lack of consensus on how best to perform and interpret qPCR experiments, a set of MIQE guidelines has recently been devised to promote better experimental practice, leading to more reliable and less equivocal interpretation of qPCR results [44].

3 AIMS OF THE STUDY

The objectives of the present thesis research were twofold. In the first two studies (I, II), the goal was to employ the sensitivity difference of the L–E and H/W rats to assess the relationship between TCDD-induced wasting syndrome and the changes or basal mRNA levels of factors relating to the AHR signalling cascade, or participating in feed intake and body weight regulation. In the second set of studies (III, IV), potential RT-qPCR reference genes for dioxin studies were sought and the robustness of various RT and qPCR enzymes was examined. The specific aims of the studies were as follows:

1. To assess the constitutive mRNA levels and effects of TCDD treatment on mRNA expression of *Ahr* and some other bHLH/PAS proteins, as well as two cytochromes in the H/W and L–E hypothalami (I);

2. To determine the influence of TCDD on hypothalamic mRNA levels of a number of selected factors involved in feed intake and body weight regulation (II);

3. To compare the effects of TCDD on the aforementioned feed-intake factors with those of leptin and feed restriction (II);

4. To examine the stability of the expression levels of a number of commonly applied reference genes in various tissues, including the hypothalamus, after an acutely toxic dose of TCDD or reduced feed intake (III);

5. To compare various RT and qPCR enzymes and the behaviour of four common reference genes in RT and qPCR reactions in a practical RT-qPCR setting (IV); and

6. To gain a better understanding of the factors contributing uncertainty to the RT-qPCR technique (IV).

4 MATERIALS AND METHODS

4.1 ANIMALS AND HOUSING

The studies were mainly carried out in inbred TCDD-susceptible Long–Evans (*Turku/AB*; L–E) and random-bred TCDD-resistant Han/Wistar (*Kuopio*; H/W) rat strains (for resistance characterisation see [281, 388]). Some intermediately resistant line B animals (derived from crosses between L–E and H/W rats [378]) were used in study III. The rats were obtained from the breeding colonies in the SPF barrier unit of the National Public Health Institute, Kuopio, and were 10–15 (L–E and H/W; males) or 15–20 (line B; females) weeks old at the time of the experiments.

The animals were housed singly (line B rats in groups of two to four) in suspended stainless steel wire-mesh cages in artificially illuminated animal rooms with a constant temperature of 21.5 ± 1.5 °C, humidity 55 ± 10% and a 12/12 h light/dark rhythm (lights on at 7.00 am). They had free access to tap water and to pelleted (Lactamin R36, Stockholm, Sweden) or powdered feed (Ewos R36, Ewos, Södertälje, Sweden), except for some feed-restricted L–E groups (studies II and III), for which the amount of feed given was gradually restricted after corn oil administration.

The Animal Experiment Committee of the University of Kuopio and the Kuopio Provincial Government approved the procedures and study plans. The studies had the license numbers 03-53 (18 August 2003) and ISHL-2004-04429/Ym-23 (13 December 2005).

4.2 CHEMICALS

TCDD was >99% pure as determined by gas chromatography-mass spectrometry. It was first dissolved in diethyl ether for storage. For dosing, an adjusted volume of this solution was re-suspended in corn oil (Sigma Chemicals, USA), allowing the ethyl ether to evaporate. The dosing solution was mixed in a magnetic stirrer and sonicated for 20 min before administration. The TCDD concentration was adjusted to lead to an identical dosing volume of 4 ml/kg in all experiments.

Lyophilised mouse recombinant leptin, CAS 181030-10-4 (Lot B36665, Calbiocem, San Diego, USA), was first dissolved in 15 mM HCl and then 7.5 mM NaOH was added according to the manufacturer's instructions. The final concentration of leptin was adjusted to 1 mg/ml using 0.9% NaCl.

4.3 EXPERIMENTAL DESIGN

The first two studies (I and II) of the thesis were designed to measure the effect of TCDD on the hypothalamic gene expression of some AHR-related bHLH/PAS proteins and hypothalamic factors known to take part in the regulation of feeding and metabolism. For

the rationale of the measured gene products see I and II, and sections 2.2.2, 2.2.3 and 2.4.8 of the literature review. The third study (III) was designed to examine the effect of TCDD on the expression of a large number of potential RT-qPCR control genes in various tissues, and the final one (IV) to analyse the RT and qPCR stability of four control genes and to compare RT and qPCR enzymes using a pooled RNA sample from rat hypothalami.

Table 1. Study and experimental outlines

Study	Experiment	Tissue	Treatment	Strains	Sampling
I		Hypothalamus	TCDD 50 µg/kg	L–E and H/W	6 h, 4 d
			TCDD 100 µg/kg	L–E and H/W	5 d[a]
II		Hypothalamus	TCDD 50 µg/kg	L–E and H/W	6 h, 24 h, 4 d
			TCDD + leptin	L–E	4 d
			Feed restriction		
			Feed restr + leptin		
III	1	Liver[b]	TCDD 100 µg/kg	L–E and H/W	4 d
			Feed restriction	L–E	
	2	Liver, spleen	TCDD 100 µg/kg	L–E	10 d
			Feed restriction		
	3	Hypothalamus	TCDD 50 µg/kg	L–E	10 d
	4[c]	Liver	TCDD 300 µg/kg	Line B	2 d, 7 d, 14 d, 32 d
IV	A “RT”	Hypothalamus	None	H/W	
	B “qPCR”				

a After RNA isolation, mRNA was further enriched from the total RNA (see 4.4).

b The expression measurements were carried out using microarrays.

c The results of experiment 4 with female line B rats are not discussed further in the thesis.

In studies I–III the animals were given a single dose of TCDD (50, 100 or [300 µg/kg – line B]) by intragastric gavage (ig) or an equal volume (4 ml/kg) of the corn oil vehicle and killed for sampling at various time points, from 6 hours to 10 days, after the treatment. Leptin (1.3 mg/kg; shown to reduce feed intake within 4 h in rats [237]) was intraperitoneally administered to some of the animals 2 hours before sampling in study II, and the feed intake of some control animals was gradually restricted for 4 or 10 days (until sampling) in studies II and III to mimic TCDD-induced wasting. Notably, the TCDD doses employed are well above the LD_{50} value (18 µg/kg [378]) of the sensitive L–E strain, leading to a pronounced reduction in feed intake and body weight, while they induce only minor effects on H/W rats. The timing of the sampling in studies I and II was considered to enable the detection of both early (acute) expression changes at 6 hours, “intermediate” changes at 24 hours and more advanced changes at 4 or 5 days. The expected secondary gene expression changes induced by feeding reduction and the resultant negative energy balance were addressed by employing feed-restricted control groups in studies II and III.

There were no specific animal treatments in study IV, in which the hypothalamic RNA of four H/W rats was pooled for later RT and qPCR reproducibility experiments. The RT experiment (A) started with the pooled RNA, which was reverse transcribed with eight enzymes (enzyme 7 was used twice with different RT primers; see below). Five parallel RT reactions were carried out with each enzyme, and the cDNA levels of four control genes of

each RT reaction were quantified with real-time PCR in two replicate PCR runs. Diluted cDNA from the previous RT experiment was used in the qPCR experiment (B), and the same four genes were again measured from each of the five RT reactions. The measurements were performed with six real-time PCR enzymes and there were three repeats of each RT in each replicated PCR run. See the original publications (I–IV) for more detailed descriptions of the study designs.

4.4 SAMPLING, RNA ISOLATION AND REVERSE TRANSCRIPTION

The rats were killed by decapitation and the brain, liver and spleen were rapidly removed. Hypothalami were dissected using a metal brain mould (RBM-4000, ASI Instruments, Warren, MI), making the rostral incision immediately in front of the of the optic chiasm, the caudal incision to the caudal border of the mamillary body, the dorsal incision to the ventral border of the anterior commissure and lateral incisions to the medial borders of the tuber cinereum and mamillary body complexes [260]. Pieces weighing approximately 200 mg were sliced from the left lateral lobe of the liver and samples of a similar size were taken from the spleen (III). The tissue samples were flash-frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses.

Total RNA from the hypothalamic samples (I–IV) was isolated using TRIzol Reagent (Life Technologies, Eggenstein, Germany; I, II), RNeasy kits (Qiagen Nordic, Crawley, UK; III) or RNeasy Lipid Tissue Mini kits (Qiagen; IV). The isolated RNA was treated with DNAse I (Ambion, Cambridgeshire, UK) in study II, and in studies III and IV, employing the RNeasy kits, DNase treatment (Qiagen) was performed on the elution columns. Total RNA for RT-qPCR from the liver and spleen samples (III) was isolated with a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO; liver in experiment 4 and spleen) or with the TRIzol Reagent (Invitrogen, Paisley, UK; liver in Experiments 1 and 2) and DNAse treated with the DNA-free kit (Ambion). For microarray analysis (Experiment 1), total RNA was extracted from liver using RNeasy kits (Qiagen, Mississauga, Canada) and, as before, DNase treatment was carried out on the elution columns. In study I, poly(A)+ RNA (“mRNA”) was further enriched from total RNA of the day-5 samples (for SIM1 analysis) with the MicroPoly(A)Purist kit (Ambion, Austin, TX). The RNA yield was always quantified and purity checked by UV spectrophotometry, and RNA integrity was verified in some samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Reverse transcription (RT) reactions to produce cDNA for qPCR in studies I–III were conducted using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) with random hexanucleotides (Roche, Mannheim, Germany) or (for hypothalamic samples in study III) a 10:1 mixture of hexanucleotides (Roche) and oligo-dT (Sigma) as primers. cDNA was synthesised from 1.2 µg or 0.6 µg of RNA in a 20-µl or 10-µl reaction volume, respectively, and the reaction mixture was incubated at 37 °C for 60 minutes for hypothalamic samples or for 90 minutes for liver and spleen samples. In study IV, several RT enzymes were used, but the same amount of RNA, 1.2 µg, was added to each 20-µl reaction and an identical concentration of unspecific primers (excluding some of DyNAmo RT repeats), a 10:1 mixture of hexanucleotides (Roche) and oligo-dT (Sigma). In addition, an equal incubation time of 60 minutes was employed (see IV for details regarding individual RT enzymes).

4.5 REAL-TIME PCR (I–IV) AND MICROARRAY (III) METHODS

The real-time PCR quantification was based on external standard curves generated from linearised plasmid DNAs (I, II) or isolated and purified PCR products (I, III, IV). Plasmids were produced by amplifying the cDNA of selected animals and tissues (see above) by conventional PCR with the qPCR primers, and cloning the PCR products into pCR-Script SK(+) Amp plasmids (Stratagene, La Jolla, CA). The PCR product standards were directly amplified from cDNA by conventional PCR and purified from preparative agarose gels. Concentrations of the plasmids and amplicons were measured by spectrophotometry, and standard curves were produced from them with a ten-fold dilution series in duplicate or triplicate ranging from 10^{-1} down to 10^{-14}, depending on the transcript. See publication I and [184] for more detailed descriptions of the preparation of the cloned standards, and publication III for the preparation of the PCR product standards and construction of the standard curves.

Quantitative RT-PCR analyses were performed with three real-time PCR instruments, employing various qPCR enzyme premixes. The Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Mortlake, NSW, Australia) and QuantiTect SYBR Green PCR kit (Qiagen) were used in studies I and III, the LightCycler instrument (Roche) with the Fast-Start DNA Master SYBR Green I kit (Roche) or Dynamo SYBR Green I Mastermix (Finnzymes, Espoo, Finland) in studies I–IV, and the Rotor-Gene 3000A Real-Time PCR instrument (Corbett) with six qPCR enzyme premixes in study IV. The PCR reaction volume in all instruments and enzyme premixes was 20 µl, and it contained cDNA originatin°g from 15 ng (I–III; Rotor-Gene), 20 ng (I–IV; LightCycler) or approximately 7 ng (IV; Rotor-Gene) of total RNA (or mRNA in I; see RNA isolation). See Table 2 for details of the genes measured from the hypothalamus in studies I–IV.

The PCR reactions in the Rotor-Gene 2000 instrument were initiated with a 15-min incubation step at 95 °C, followed by 45 cycles of denaturation at 94 °C for 20 s, annealing at 61–64 °C (first cycle) to 55–58 °C for 20 s (usually with a "touchdown" during the first six cycles) and elongation at 72 °C for 20 s. In the LightCycler, there was an initial incubation at 95 °C for 10 min, followed by 45 cycles each consisting of denaturation at 95 °C for 15 or 10 s, annealing at 59 °C for 5 or 20 s and elongation at 72 °C for 12 s or 10 s. The cycling programs in the Rotor-Gene 3000A instrument with the six qPCR enzymes compared (IV) were adjusted according to the manufacturer's instructions. In general, the cycling programs of 45 cycles were composed of denaturation at 94–95 °C, annealing with touchdown from 63 °C to 57 °C for 20–30 s and elongation at 72 °C for 20–30 s. A melting curve was generated in all instruments after amplification to verify the specificity of the PCR reaction. The cycling protocols are described in detail in the original publications. Finally, the standard curve–based absolute abundances of the genes of interest were divided by beta-actin values of the same samples to normalise the amount of cDNA in PCR reactions (I and II) or expressed as cDNA concentrations in the qPCR sample (III, g/l; IV, ng/l), thus indirectly normalising them in the latter studies to the amount of total RNA.

The microarray analysis in study III was performed with Affymetrix RAE230-2 arrays at the Centre for Applied Genomics of the Hospital for Sick Children (Toronto, Canada) following standard manufacturer's protocols. After hybridisation, the array data were loaded into the *R* statistical environment [299], preprocessed (normalised) using specific packages of the Bio-Conductor project [101], and annotated using an "in-house" algorithm partly developed by Paul C. Boutros at the Department of Pharmacology, University of Toronto, Canada. The data preprocessing and annotation are detailed in (III).

Table 2. Gene information and sizes of transcripts measured from the hypothalamus in studies I–IV

Gene	Symbol	References	Size (bp)	Study
Aryl hydrocarbon receptor[a]	*Ahr*	621557[a], NM_013149[b]	199	I
Aryl hydrocarbon receptor nuclear translocator	*Arnt*	2153, NM_12780	162	I
Aryl hydrocarbon receptor nuclear translocator 2	*Arnt2*	2154, NM_12781	299	I
Aryl-hydrocarbon receptor repressor	*Ahrr*	1559857, NM_001024285	161	I
Single-minded homolog 1 (Drosophila)	*Sim1*	1320568, NM_011376[c]	158	I
Period homolog 2 (Drosophila)	*Per2*	61945, NM_031678	183	I
Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	2458, NM_012540	361	I
Cytochrome P450, family 1, subfamily a, polypeptide 2	*Cyp1a2*	2459, NM_012541	473	I
Growth hormone secretagogue receptor (ghrelin receptor)	*Ghsr*	621397, NM_032075	199	II
Neuropeptide Y	*Npy*	3197, NM_012614	188	II
Neuropeptide Y receptor Y5	*Npy5r*	3199, NM_012869	318	II
Agouti related protein homolog (mouse)	*Agrp*	2068, NM_033650	191	II
Cholinergic receptor, muscarinic 3	*Chrm3*	2343, NM_012527	449	II
Pro-melanin-concentrating hormone	*Pmch*	3358, NM_012625	494	II
Syndecan 3	*Sdc3*	621486, NM_053893	382	II
Hypocretin (orexin)	*Hcrt*	2786, NM_013179	200	II
Hypocretin (orexin) receptor 2	*Hcrtr2*	2788, NM_013074	180	II
Leptin receptor[d]	*Lepr*	3001, NM_12596	495	II
Insulin receptor	*Insr*	2917, NM_017071	497	II
Proopiomelanocortin	*Pomc*	3366, NM_139326	247	II
Melanocortin 4 receptor	*Mc4r*	3057, NM_013099	510	II
CART prepropeptide	*Cartpt*	2272, NM_017110	343	II
Neurotensin	*Nts*	621612, NM_001102381	241	II
Urocortin	*Ucn*	3929, NM_019150	245	II
Histidine decarboxylase	*Hdc*	2790, NM_017016	405	II
Histamine receptor H1	*Hrh1*	2830, NM_017018	396	II
Actin, beta (beta-actin)	*Actb*	628837, NM_031144	298	I–IV
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	2661, NM_017008	170	III, IV
Phosphoglycerate kinase 1	*Pgk1*	619878, NM_053291	180	III, IV
Succinate dehydrogenase complex, subunit A	*Sdha*	621557, NM_130428	160	III, IV

a Gene names, symbols and RGD IDs: Rat Genome Database Web Site, Medical College of Wisconsin, Milwaukee, Wisconsin. WWW (URL: http://rgd.mcw.edu/). Retrieved October 2012.

b NCBI Reference Sequences (RefSeq): National Center for Biotechnology Information Web Site (URL: http://www.ncbi.nlm.nih.gov/). Retrieved October 2012.

c The RGD ID and RefSeq are for the mouse. The mouse sequence was used to design the primers for *Sim1*, because at the time of contriving the primers, the rat sequence (NM_001107641) was unknown. The primers subsequently proved to bear homology rates of 19/20 (F) and 23/23 (R) nucleotides to the rat sequence, and efficiently amplified a single product of the expected size.

4.6 STATISTICAL ANALYSES (I–III)

In study I, differences between control and TCDD-exposed groups and between corresponding groups of the strains at each time point were assessed using two-tailed t-tests for independent samples. The basal mRNA levels of CYP1A were below the detection limit in most of the control samples, and these were assigned the value of the lowest measurable sample before analysis. In study II, analysis of variance (ANOVA) for repeated measures followed by t-tests were used to evaluate the effect of TCDD on daily feed consumption and body weight change. Two-way ANOVA were used to determine the statistical significance of TCDD and leptin treatment effects on the hypothalamic mRNA levels at each time point and for each strain, followed by t-tests between control and either TCDD- or leptin-treated groups. At 96 h in L–E rats, a similar approach was used to evaluate the effects of restricted feeding and leptin. In both studies, effects or differences were considered to be statistically significant if the p-value was <0.05.

In study III, the quantitative RT-PCR data were first checked for variance homogeneity across groups to be compared using Levene's test. Data with homogeneous variances and more than two groups were analysed within each strain/line by one-way ANOVA followed post-hoc ($p < 0.05$) by Duncan's multiple range test to detect the deviating group(s). If non-homogenous variances were found, a log10-transformation was performed on the data and Levene's test run afresh, and if this procedure did not homogenise the variances, non-parametric Kruskal–Wallis ANOVA tests followed by Mann–Whitney U-tests for group-wise comparisons were employed. In experiment 4, the controls were pooled from two time points after vehicle treatment, since there were no significant differences between the time points for any variable. The microarray data were analysed by fitting the following linear model for the normalised expression value of each individual ProbeSet of the arrays:

$$\text{Expression} = (\text{H/W}) + (\text{L–E}) + (\text{TCDD}) + (\text{feed}) + (\text{feed:L–E}) + e,$$

where (H/W) and (L–E) are the separate effects of each strain, (TCDD) and (feed) are indicators (effects) of TCDD and feed-restriction treatments, (feed:L–E) is an interaction term and e the error term.

After empirical Bayes moderation of the standard error [339], model-based t-tests based on a predetermined contrast matrix were employed to extract the *M* (differential expression) and *p* values for each contrast and gene after a false discovery rate adjustment for multiple testing [80]. The statistical analysis of the microarray experiment is detailed in (III).

4.7 MODELLING OF RT AND qPCR ROBUSTNESS (IV)

In the statistical modelling of study IV, the RT and qPCR reactions were treated as a series of multiplicative components with unknown efficiencies and variances. The multiplicative efficiencies were converted into additive effects by logarithmic transformation of the measured expression values leading to a log-normal distribution of the various efficiency parameters [209]. This also resulted in a reasonable scaling of the expression levels of the measured genes and RT and enzyme efficiencies. Separate linear multilevel (hierarchical) models were fitted to RT and qPCR experiment data with OpenBUGS software (version

3.2.1, rev 781) [217] using Bayesian methodology, and both infinite-population (σ) and finite-population sample (sd) standard deviation parameters were used to extract all relevant information from the models. Where applicable, the infinite and finite-population log-normal standard deviations were converted to coefficients of variation (CV) on a natural scale [209] to be more comprehensible. The uncertainties of the parameters were expressed as Bayesian credible intervals. The model construction is explained in detail and the mathematical formulas for calculation of the CVs and finite population sd parameters are given in (IV).

4.7.1 RT REACTION MODEL

In the RT model, the RT enzymes constituted the highest hierarchical level, inside which nested the five replicate RT reactions (tubes) for each enzyme with enzyme-specific variance components. The enzyme parameters (E) were set as fixed effects without distribution parameters, treating the RT reaction replicates as five trial runs of nine "enzyme machines" producing cDNA with pre-set, unknown identical settings (the true abundances of the RNA transcripts) and with enzyme-specific fixed unknown efficiencies. The four studied RNA transcripts, reverse transcribed among a myriad of other RNAs in each tube, comprised the lowest level of the model with gene-specific variances. The duplicate qPCR reactions were omitted from the model, allowing the qPCR effect to sink into the error variance term.

For each (log-transformed) measurement Y_i:

$$Y_i \sim \text{Normal}(\mu_i,\sigma^2) ,$$

where σ^2 is the measurement error variance component with prior $\sigma \sim U(0,20)$, and the mean is

$$\mu_i = ETG_{e(i)t(i)g(i)} ,$$

where prior $ETG_{etg} \sim \text{Normal}(E_e, \sigma^2_{eg})$. The nine enzyme effects are fixed with priors $E_e \sim U(-100,100)$, e = 1,...,9 and variances $\sigma^2_{eg} = \sigma^2_e + \sigma^2_g$, e=1,...,9 and g = 1,...,4, with priors $\sigma_e \sim U(0,20)$ and $\sigma_g \sim U(0,20)$. Hence, the variance components of reaction tubes (between tube variability) within enzymes are assumed to be common within each enzyme, and the variance components of genes (between-gene variability) within reaction tubes within enzymes are assumed to be common for each gene.

4.7.2 qPCR MODEL

In the qPCR model, the highest level was the RT reactions (tubes, T), inside which nested the four studied genes (TG), and inside these the six studied qPCR enzymes (TGE). The fourth level (GER) was constructed by nesting the two qPCR runs inside genes and enzymes, omitting the tube effects, since each qPCR run is unique only for each enzyme and gene combination. The highest level of the PCR model, comprising the five RT reaction repeats, was modelled as random effects using hierarchical centring. Here, the μ value of parameter T corresponds to the mean cDNA concentration of the four transcripts produced by RT enzyme 5.

For each (log-transformed) measurement Y_i:

$$Y_i \sim \text{Normal}(\mu_i,\sigma^2) ,$$

where σ^2 is the measurement error variance component with prior $\sigma \sim U(0,20)$, and the mean is

$$\mu_i = T_{t(i)} + TG_{t(i)g(i)} + TGE_{t(i)g(i)e(i)} + GER_{g(i)e(i)r(i)} ,$$

where the five RT reaction tube effects are modelled using the hierarchical centring technique as random $T_t \sim \text{Normal}(\mu_{tube}, \sigma^2_{tube})$, $t = 1,...,5$ with general mean μ_{tube} and variance component σ^2_{tube} setting priors $\mu_{tube} \sim \text{Normal}(0, 10^4)$ and $\sigma_{tube} \sim U(0,20)$. Within each reaction tube, the effects of the four measured genes are modelled as random $TG_{tg} \sim \text{Normal}(0, \sigma^2_g)$, $g=1,...,4$, with gene-specific variance components with prior $\sigma_g \sim U(0,80)$. Within each gene, and within each reaction tube, there are six enzymes whose effects are modelled as random $TGE_{tge} \sim \text{Normal}(0, \sigma^2_e)$, $e=1,...,6$, with enzyme-specific variance components with prior $\sigma_e \sim U(0,20)$. The random effects T, TG and TGE are hierarchically nested, but assume a common variance component of T for all reaction tubes, common variance components of TG within genes and common variance components of TGE within enzymes. Additionally, random effects were also assumed for the runs within enzymes within genes $GER_{ger} \sim N(0, \sigma^2_{run})$, with a single variance component σ^2_{run} with prior $\sigma_{run} \sim U(0,20)$.

5 RESULTS

5.1 THE EFFECTS OF TCDD ON SELECTED bHLH/PAS PROTEINS AND CYPS

In study I, the mRNA expression of five bHLH/PAS proteins, *Ahr*, *Ahrr*, *Arnt*, *Arnt2* and *Per2*, as well as *Cyp1a1* and *Cyp1a2* (Table 2) was measured from the hypothalami of untreated and TCDD-treated (50 μg/kg) H/W and L–E rats after 6 h and 4 days. The detection of *Sim1* was an exception: the analysis was performed using cDNA samples derived from hypothalamic mRNAs of both strains, treated with 100 μg/kg of TCDD 5 days before. The measured dioxin signalling related proteins, *Ahr*, *Ahrr*, *Arnt* and *Arnt2*, attested to minor strain-specific variation in constitutive expression and reaction to TCDD treatment. The amply present *Ahr* mRNA was about two- to three-fold more abundant in L–E than in H/W rat hypotalami, and TCDD did not affect its levels. In contrast, the constitutive levels of *Ahrr* mRNA were very low, but TCDD increased them considerably in both strains, amounting to an approximately 10-fold increase in L–E rats and 5-fold in H/W rats. The relative constitutive levels of *Arnt* mRNA were about a 1000-fold lower than those of *Arnt2* mRNA, but except for this difference in basal apparent concentrations, the overall expression patterns of *Arnt* and *Arnt2* were somewhat similar; the L–E rats expressed about two-fold higher levels of *Arnt* mRNA at each time point than H/W rats, while in *Arnt2* the difference between the strains was smaller. However, there was no detectable effect of TCDD in either strains or time points.

Constitutive expression of the other measured bHLH/PAS proteins *Sim1* and *Per2* was very low; *Sim1* was even analysed from cDNA reverse transcribed from enriched mRNA (see before). TCDD treatment did not significantly affect the expression of either of them, while there was a slight downward tendency of SIM1 and upward tendency of *Per2* in L–E rats. Akin to *Sim1* and *Per2*, the basal mRNA abundances of cytochromes were low (mRNA levels of *Cyp1a2* were below the detection limit in most of the control samples), but contrary to *Sim1* and *Per2* their expression was drastically increased after TCDD treatment. The TCDD-induced levels of *Cyp1a2* mRNA were significantly higher in L–E than in H/W rats.

5.2 TCDD AND HYPOTHALAMIC FEEDING-REGULATORY FACTORS

In study II, the mRNA expression of 18 hypothalamic factors (Table 2), known to take part in the regulation of eating and metabolism, was measured by quantitative RT-PCR at 6 h, 24 h and 4 days after a single dose (50 μg/kg) of TCDD or corn oil. The effects of TCDD were compared with those of leptin (1.3 mg/kg; given 2 h before sampling) and with feed restriction. TCDD alone mainly modified the expression of orexigenic factors and, as a general rule, it initially suppressed mRNA levels, but later there was a shift to increased expression. This was seen in both strains, although TCDD-sensitive L–E rats responded at a faster rate. In anorexigenic factors, there was a similar general pattern, except that the

late increase was missing in L-E rats. Moreover, the number of significant changes was clearly fewer and their magnitude smaller than in the case of orexigenic factors.

At 6 h, TCDD reduced *Npy*, *Npyr5* and *Pmch* in L-E rats, and hypocretin (orexin) receptor 2 (*Hcrt*) in both strains, while at 24 h decreases were mainly recorded in H/W rats. In L–E rats, the expression of *Npyr5* was still low, even as the other factors had returned to normal or exhibited already elevated (e.g. *Pmch)* levels. At 96 h, only increases were recorded, with *Agrp* being strongly up-regulated in both strains. *Npy* was increased in L–E rats, roughly to a similar degree as described after a 48-h feed deprivation [352].

Leptin influenced the expression of both orexigenic and anorexigenic factors in a more even manner, but the statistically significant effects were conspicuously clustered at the first time point, especially in L–E rats. At 6 h after TCDD or corn oil exposure, leptin almost solely brought about suppressive changes; *Pmch*, *Pomc* and *Cartpt* displayed similar alterations in expression in both strains at this time point, whereas several anorexigenic factors were down-regulated only in the L–E strain. A decline in *Pomc* expression after leptin administration was detected in both strains at 6 h and in L–E rats at 24 h. At later time points, the most prominent leptin effects were decreases in *Agrp* and *Pmch* expression seen in H/W rats, and increased expression of insulin receptor (*Insr*) detected in L-E rats. Four-day progressive feed restriction, mimicking the action of TCDD, induced a statistically significant reduction in *Cartpt* and histamine receptor H1, and an increase in *Ghsr* (ghrelin receptor) and *Agrp* mRNA levels, while there was a non-significant increase in *Npy*.

5.3 REFERENCE GENES IN TCDD-TREATED RATS

In experiment 1 of study III, hepatic mRNA levels of 18 commonly employed normalisation genes were measured by microarray on day 4 after TCDD (100 μg/kg) or corn oil administration. Of these, eight (44%) appeared to be responsive to TCDD in L–E and four (22%) in H/W rats. In addition, five genes (28%) exhibited altered expression after feed restriction. Three of these latter genes were also affected by TCDD, with one being modulated in opposite directions by TCDD and feed restriction. In experiment 2, a selected subset (14) of the housekeeping genes analysed with microarray was verified by qPCR, and expression of the same battery of genes was further determined at a later time point (10 days) in the L–E strain. The data generated by the two independent methods proved to be astonishingly convergent with regard to both the occurrence and direction of changes and the degree of induction or repression; only in two cases was there a distinct difference in outcomes. Furthermore, the expression patterns remained fairly constant at 4 and 10 days post-exposure, and strikingly, whenever an mRNA concentration was affected by either treatment (TCDD or feed restriction), there were statistically significant departures from control levels on both occasions. In most cases, the direction of change also remained the same.

Control genes *Gapdh*, *Pgk1* and *Sdha*, which had exhibited the most stable expression levels in livers of L–E, H/W and line B rats, were further explored in the hypothalamus and spleen of L–E rats (experiments 2 and 3). *Actb* was also included to the testing, as it was (at the time) the most commonly used reference gene in RT-qPCR, and was also employed in studies I and II. In the hypothalamus, all four reference genes retained their

basal levels at 10 days after TCDD (50 μg/kg) administration, while in the spleen a TCDD dose of 100 μg/kg induced a two-fold increase in *Sdha* expression without any apparent influence on the other three genes examined. However, no such effect was discernible in feed-restricted control rats. Notably, TCDD has been shown to be a fairly strong inducer of *Gapdh* transcription *in vitro* [227], while we did not record statistically significant departures from control levels in any of the tissues examined. The fact that feed restriction brought about a repression of up to 50% in *Gapdh* mRNA expression in the liver and that no such impact occurred in TCDD-exposed rats with a comparable body weight loss also argues for a moderate induction capability also in rats in vivo. In this case, the induction simply appears to be masked by an equally powerful opposite force.

5.4 RT ENZYME EFFICIENCY AND REPRODUCIBILITY

In the first part of study IV (A), equal amounts of RNA from a pooled hypothalamus-derived RNA sample were reverse transcribed in parallel reactions (in five replicates) with eight RT enzymes, one enzyme with and without oligo-dT primers. The four control genes (*Actb*, *Gapdh*, *Sdha* and *Pgk1*; Table 2) found in study III to be stably expressed in hypothalamus after TCDD administration were then measured with qPCR from the resulting RT reaction products in 9×5 cDNA batches. Based on raw data, the RT enzymes generally exhibited reasonable coherence in their gene-specific reaction efficiencies. However, enzyme 8 was much less productive than the others and formed 2–4 $\log_{10}$ units less cDNA. According to the Bayesian multilevel model for the RT reactions (model 1), the source (AVM, M-MLV or unknown) or RNAse H activity of the enzymes 1–7 had only a slight effect on the general efficiency of the RT reaction. Nevertheless, the only (known) AMV-based enzyme yielded the highest parameter efficiency value, and conversely the RT-reactions from which the oligo-dT primers were omitted exhibited the lowest efficiency.

The infinite-population sd parameters measuring general enzyme-specific reproducibility of the reverse transcription fell into two loose groups: Enzymes 4 to 8 had their parameter medians, expressed as coefficients of variation (CVs) on a natural scale, between 13% and 20%, and 1, 2 and 3 between 29% and 56%. The known AMV-based enzyme showed the worst enzyme-specific RT reproducibility, but RNase H activity did not seem to have a definite effect. Analogous finite-population sds, measuring variation among RT reactions within enzymes, also did not reflect an effect of RNase H activity, while pinpointing enzymes 1, 2 and 8 as the least precise. Notably, the equivalent infinite- and finite-population variance components of the qPCR model (model 2), employing RT enzyme 5, were smaller than those of the RT model.

The RT enzymes reverse-transcribed *Gapdh* and *Pgk1* in line with their general RT precision, with median CVs of enzymes 4 to 8 being 15% to 20% and 16% to 23%, respectively. In contrast, the RT efficiency precision of the *Actb* and *Sdha* genes was more variable, with considerably worse overall CV median values from nearly 40% to more than 100% (excluding enzyme 8 and the *Sdha* transcript). The most consistent enzymes in *Actb* transcription were (in order of worsening precision) 7, 8, 4, 5 and 1, enzymes 2, 3 and 6 exhibiting larger variation. The most reproducible enzymes in *Sdha* transcription turned out to be enzymes 5, 4 and 6, while enzymes 1 and 8 (CVs 103% and 183%) were substantially less precise.

5.5 qPCR REPRODUCIBILITY AND RT REACTION ROBUSTNESS

In the second experiment (B) of study IV, the five cDNA batches produced by one of the RT enzymes (enzyme 5) were used afresh to compare six qPCR enzymes. The four previously used control genes were again quantified with the qPCR enzymes, and a second Bayesian multilevel model (model 2) was constructed for the expression values, enabling the comparison of the posterior distributions of analogous parameters of the two models. The raw data from the qPCR experiment demonstrated considerable heterogeneity, and accordingly, there was a marked difference in the average (general) reproducibility of the qPCR enzymes: The infinite-population qPCR precision parameter medians (expressed as CVs) of qPCR enzymes 1, 2, 4 and 6 were 3%–5%, while those of enzymes 3 and 5 were 11%. These infinite-population qPCR variance components of individual enzymes thus varied from values smaller than *Gapdh* and *Pgk1* finite-population RT sd parameters to the same magnitude as *Actb* and *Sdha* RT sd parameters. However, similarly to the RT model, the gene-specific finite-population qPCR sd parameters (measuring gene-specific qPCR reproducibility over all qPCR enzymes) were almost identical. The reproducibility of the qPCR step thus varied very little among different transcripts in both models.

The finite-population gene- and enzyme-specific sd parameters attached to *Actb* and *Sdha* RT were 2 to 4 times larger than *Gapdh* and *Pgk1* sds in both RT and qPCR models, whereas the difference was 2 to 3 orders of magnitude in the corresponding infinite-population parameters. This led to infinite-population gene-specific RT variation CV medians of 9% for *Gapdh* and 11% for *Pgk1* according to the RT model, and approximately 5% for both genes according to the qPCR model – and enormous, non-meaningful CVs for *Actb* and *Sdha* in both models. Consequently, the intrinsic RT efficiencies of the "stable" *Gapdh* and *Pgk1* transcripts seem to closely follow the general transcription efficiency in each RT reaction and have little variation, while the "unstable" *Sdha* and *Actb* transcripts appear to have their own independent intrinsic RT efficiencies and exhibit somewhat less stability from one RT reaction to another. Finally, there were salient differences between replicate runs in the qPCR experiment (enzymes 2 and 3 generally exhibiting the smallest differences). However, this variation did not seem to be gene or enzyme related, but might be related to the use of external standard curves.

6 DISCUSSION

Extensive research efforts have been focused on elucidating the mechanisms of obesity and the physiological regulation of energy homeostasis, while the dioxin-induced wasting syndrome has attracted surprisingly little research interest, despite the fact that TCDD is an extremely potent compound in lowering body weight and reducing appetite. Thus, the resolution of the pathogenesis of the wasting syndrome would be important not only toxicologically but also physiologically, and as a continuation to this reasoning, the hypothalamus, the major centre of food intake and body weight regulation, seems an area worth scrutiny as a participant in the derailment. Moreover, the simplest approach to tackle a pathophysiological process, starting out from AHR-mediated transcription alterations, would be to employ the sensitivity difference between L–E – H/W rats in mechanistic studies and use RT-qPCR as a means to measure the gene expression changes of the factors relating to energy balance. However, the selection of the CNS target and the technique of measurement both induce a number of predicaments that render the inferences less certain and limited in scope. Below, the findings of the thesis studies will be discussed in this context.

6.1 HYPOTHALAMIC bHLH/PAS GENE EXPRESSION ALTERATIONS AND WASTING

The constitutive hypothalamic expression levels of *Ahr*, *Arnt* and *Arnt2* were moderately higher in the sensitive L–E than in resistant H/W rats, and concordantly TCDD produced an approximately two-times larger induction of *Ahrr* in L–E than in H/W strain. These differences, however, are most likely not causally related to the development of the wasting syndrome. The DRE binding of the AHR/ARNT heterodimers is known to occur similarly in both rat strains, and their sensitivity difference is firmly established to be caused by the difference in the transactivation domain (see 2.2.2). In addition, the abundance of *Ahr* mRNA has been shown not to affect TCDD sensitivity in female and male C57BL/6 mice livers [274], and finally, the astonishingly uniform increase in the expression levels between the 6 h and 96 h time points, regardless of strain or treatment, suggests time to have an independent effect on their mRNA abundance. The time dependence of expression could be explained by technical reasons (elaborated further in 6.3) and/or be related to circadian rhythmicity and the anatomical level of sampling. *Ahr* mRNA has been demonstrated to exhibit circadian variation in SCN, with peak expression occurring at light–dark transition [241], while *Arnt* or *Arnt2* rhythmicity has not been studied in the brain. We did not pay particular attention to the similar circadian adjustment of the sampling in study I, which may have induced the variation. Targeting the whole hypothalamic block has the advantage of measuring a “total” effect, but it has the definitive disadvantage of diluting and adding up individual, possibly conflicting, alterations at the level of single nuclei.

In previous studies, *Ahr* and *Arnt* mRNA have been shown to be widely expressed in the adult rat brain, with especially high levels occurring in the caudal ARC [144, 159, 266]. In comparison, expression of the *Ahrr* gene has not been investigated in rat the CNS, and

the two reports in mice are partly conflicting. Huang et al. [143] noted the basal abundance of *Ahrr* mRNA to be high in the pituitary, but low in the cerebellum and cortex, and negligible in the hypothalamus, while Bernshausen et al. [21] found especially high levels of *Ahrr* mRNA throughout the brain, and also reported the expression to diminish by two orders of magnitude in *Ahr*-deficient mice. At the protein level, strong AHRR immunoreactivity was seen in the mouse hippocampus and cortex, and staining was recorded in the nuclei of several neurons throughout the hypothalamus [86]. The expression of *Arnt2* is primarily confined to the brain in adult mice [75, 134], and in the rat hypothalamus the highest expression of *Arnt2* mRNA has been found in the SON and the PVN, whereas low or moderate levels have been detected in most other hypothalamic regions [266]. The effect of TCDD on AHR signalling cascade molecules in the brain has previously been addressed in only two studies: In SD rats, a single low dose of TCDD (10 μg/kg) did not alter *Ahr* expression in the hypothalamus, although a slight increase in *Arnt* emerged 28 days after treatment [144]. Concordantly, in 129/SV-C57BL/6 cross mice hypothalami, a low sublethal (50 μg/kg) TCDD dose did not significantly modify either *Ahr* or *Arnt*, while *Ahrr* mRNA was sharply increased [143], in keeping with several other tissues [94].

The other bHLH/PAS family genes, *Sim1* and *Per2*, did not show any marked alterations in response to TCDD, and there was an expected strong rise in the *Cyp1a1* and *Cyp1a2* expression (see 2.2.3 and 2.3.2), the latter exhibiting a larger induction in L–E rats. Similarly the AHR-related proteins, these findings speak against a decisive role of these factors in the development of wasting syndrome, but again stress the importance of timing and targeting of the sampling. SIM1 is involved in hypothalamic PVN and SON development and food intake regulation (see 2.2.1), and ARNT2 appears to be required for its function [140, 169, 229]. Accordingly, the expression of *Sim1* mRNA has been shown to be regulated by AHR–ARNT2 and to react to TCDD *in vitro* [428], while no other studies have addressed this *in vivo*. The clock genes *Per1* and *Per2* are strongly expressed in the rat SCN [332], and some findings support a role for them in the modulation of AHR-mediated responses to TCDD in peripheral tissues [241, 295-297]. However, in a recent study employing *in vitro* TCDD treatment of explanted tissues, including SCN, from PER2::LUCIFERASE mice, no TCDD-induced alterations in PER2 rhythms were found, suggesting that the mammalian circadian system is resistant to TCDD [263]. The induction of *Ahrr* and the cytochromes attest to a functional AHR signalling cascade being present in the hypothalamus. However, there is compelling evidence of the xenobiotic metabolism mainly being operational in vascular and other non-neural tissue of the brain (see 2.2.3), thus prompting anatomically more detailed sampling to establish the role of CYP induction in neurons *in vivo*.

6.2 NEUROPEPTIDE CHANGES

In study II, both TCDD and leptin influenced the expression of a wide variety of orexigenic and anorexigenic mediators, but two major features distinguished their effects, especially in the TCDD-sensitive L–E rats. The great majority of alterations elicited by TCDD were in the orexigenic side, while leptin affected in a fairly uniform manner both orexigenic and anorexigenic peptides and their receptors. Also, the effects of leptin generally took place at

the first time point of measurement, whereas they were more evenly distributed in the case of TCDD. In fact, in H/W rats the bulk of significant TCDD-induced alterations occurred at the two last time points, and furthermore, there was a conversion in the direction of change from decreased to increased expression at the last time point (4 days). Resembling the effect of TCDD, the imposed mild feed restriction also brought about an increase in two orexigenic factors and a reduction in two anorexigenic factors. Of the individual factors, *Pmch* was increased at 48 h and *Npy* at 96 h after TCDD administration in L–E rats. The latter increase was of a roughly similar degree to that described after a 48-h feed deprivation [352].

Collectively, TCDD did not completely abolish mRNA production or induce a marked and consistent over-expression of any of the studied neuropeptides or receptors, and there was a conspicuous lack of an effect on the hypothalamic reference genes in studies I, II and III. A severe cytotoxic effect on, or permanent hyperexcitation of the cells taking part in eating regulation in the hypothalamus thus seems unlikely. Rather, these findings suggest that the late elevation of orexigenic mediators such as *Npy* and *Agrp* may be a compensatory reaction to ongoing body weight loss, and the increased *Agrp* expression appears to be a very sensitive response to lowered metabolic fuel availability. The clustering of leptin's effects at the first time point and unexpected reduction of anorexigenic factors, especially in L–E rats, might be caused by the marked surplus in day-time energy from the corn oil vehicle, possibly coupled with handling stress. The L–E rats exhibit prominent feeding peaks at dark-light and light-dark transition phases, but generally consume a smaller proportion of their total daily ration during the light hours than H/W rats [202, 203].

Notably, two other studies that have directly addressed the effect of TCDD on hypothalamic neuropeptides at the level of single nuclei using *in situ* hybridisation have demonstrated some TCDD-specific alterations. A sublethal TCDD dose (15 μg/kg) in SD rats brought about an increase in *Npy*, *Pomc* and *Cart* mRNA in the ARC at a 6 days, and in the LHA *Pmch* expression was also elevated [86]. In line with these findings, a dose (50 μg/kg) close to the LD_{50} value of the same strain of rat increased *Pomc* mRNA abundance in the ARC at 7 and 14 days; at the latter time point, a significant increase was also found at lower doses [236]. Differences in methodology, rat strain, TCDD dose, and the time of sample harvesting impede direct comparisons between the experiments, but for *Npy* and *Pmch*, similar changes were found in study II in L–E rats at 24 or 96 h.

The straightforward experimental regime of measuring gene expression by RT-qPCR in hypothalamic blocks at fixed time points after TCDD and/or leptin administration is well suited to determining general impacts on a given transmitter or receptor, but has some technical (see 6.3) and methodological limitations. In keeping with the bHLH/PAS proteins, changes confined to a localised point may elude detection, and the possibility of reciprocal cancellation or dwindling of effects seems even more probable considering the complex neurophysiology and anatomy of the hypothalamus. Moreover, dissociation of the primary dioxin effects from the secondary outcomes of wasting calls for focusing on the early stages of intoxication and/or meticulous employment of pair-fed controls. The issues relating to the anatomical sampling accuracy and possible subtle interactions between treatments and unforeseen confounding factors are exemplified by the unexpected leptin effects. The complexity of the feeding regulation has also been well

demonstrated in *Npyr5* knock-out studies, in which obesity has surprising resulted despite inhibition of the NPY system [130].

The inter-strain differences in the physiology of energy balance regulation may even extend beyond the AHR-mediated sensitivity to the wasting syndrome, as evinced by the above-described notable divergence in responses to leptin and circadian feeding patterns, and the temporal differences in expression responses. Furthermore, the H/W and L–E strains vary in metabolic parameters and feeding responses. Male H/W rats have higher serum levels of thyroxine, free fatty acids, triglycerides and insulin, but lower serum TSH and corticosterone concentrations than male L–E rats [273], which also appear to have slightly higher levels of plasma neutral amino acids, including tryptophan [388], as well as higher plasma glucose concentrations [409]. In addition, our previous unpublished findings, recently summarised by Lensu et al. [204], support the idea of differences in glucose metabolism between L–E and H/W rats: In contrast to the H/W strain, it has proved impossible to induce diabetes by streptozotocin (a pancreatic B cell-specific toxicant [356]) administration to L–E rats, and they were also unresponsive to 2DG-elicited eating. Finally, the strains respond differently to taste and other "distal cues" [416] in regulating meal size: The H/W rats prefer saccharin and drink it more avidly than L-E rats [276] and, contrary to the L–E strain, increase their total energy intake when offered palatable food in addition to normal feed [377].

As a more general question, the importance of the hypothalamic neuropeptides in relation to other systems (inside and outside brain) in eating regulation has not been definitively resolved. Thus, the role of the alterations in the adiposity and satiety signals (see 2.4.4) and neurotransmitters other than neuropeptides (see 2.4.8), as well as that of the brainstem, reward circuitry and food aversion (2.4.7), require further study in relation to TCDD-induced energy balance derailment. Finally, the extent to which changes in mRNA expression affect cellular protein abundances is a moot point. Recent studies employing techniques that provide the ability to directly survey mRNA and protein abundances on a large scale, suggest that only about 40% of the variation in protein concentrations can be explained by knowing the mRNA levels [411], and alterations in translation (not transcription) appeared to be the dominant mediator of changes in gene expression during adaptation to minor stress in yeast cells [121]. On the other hand, higher correlations between protein and mRNA abundances have also been observed [63], and little is known about the effects of perturbations *in vivo*, let alone about the regulation of highly specialised factors such as neuropeptides, receptors or bHLH/PAS proteins. The activated AHR seems to be controlled by protein degradation [143, 144, 219, 274, 288], while *Ahrr* appears to be activated at the transcription level [94, 143, 144]. Studies at the protein expression level would have been a logical step to verify the mRNA alterations detected in studies I and II. However, the anatomical "robustness" of the sampling (whole hypothalami) and the minor alterations detected prompted us not to undertake these analyses, but to try to ascertain the reproducibility of the RT-qPCR data.

6.3 RT-qPCR ROBUSTNESS IN TCDD STUDIES

The most common way to perform the critical normalisation in RT-qPCR is to relate the measured mRNA concentration for the gene of interest to the concentration of the

reference gene, or preferably to a set of genes. However, no such set, let alone any single gene, is universally suitable for all purposes and must be validated for each experimental setting, a task attempted in studies III and IV: First, the most stable reference genes in response to TCDD or feed restriction were sought, and their performance in RT-qPCR was then scrutinised. These technical aspects are both of particular importance in bHLH/PAS, neuropeptide and receptor measurements. The extremely heterologous organisation and diversity of function in hypothalamic blocks may well lead to generally subdued alterations of gene expression in the absence of a direct cellular insult (see discussion in previous paragraphs), calling for the best attainable accuracy and precision in mRNA abundance measurements.

The *Actb* mRNA is perhaps the most widely employed gene for normalisation in gene expression experiments, and it was also the only reference gene resorted to in studies I and II. The use of a single gene is generally not regarded to be sufficient for normalisation (see 2.5), but the inertness of hypothalamic *Actb* expression after TCDD treatment and feed restriction was ascertained in both studies, and later confirmed in study III, where its mRNA levels remained unchanged in the hypothalamus (and spleen) of fatally intoxicated L–E rats at an advanced stage of acute toxicity. All in all, the use of only one reference gene has most likely not therefore induced marked bias in the results attained in studies I and II, while it may have inadvertently increased variation due to the lack of RT stability and poor correlation of RT efficiency with the genes of interest, as observed in study IV.

Taken together, the findings of study III substantiate the need for a meticulous pre-validation of reference genes, especially with dioxins, whose mechanism of toxicity inherently involves the readjustment of gene activities via the AHR. However, even for TCDD, it is still possible to find normalisation genes that do not alter their expression levels during acute intoxication, and for the hypothalamus, *Actb*, *Sdha*, *Gapdh* and *Pgk1* all appear to be acceptable. On the other hand, RT stability has a strong influence on the usability of the potential reference genes. The intrinsic RT efficiencies of the "stable" *Gapdh* and *Pgk1* transcripts closely followed the general transcription efficiency in each RT reaction and had little variation besides the general RT (tube-specific) variability. In contrast, the "unstable" *Sdha* and *Actb* transcripts had their own independent intrinsic RT efficiencies that did not correlate with the general RT-specific transcription efficiency and exhibited somewhat less stability from one RT reaction to another.

Generally, the covariances of RT efficiency and RT reproducibility appear to be mainly gene or RNA related, while the RT enzymes behaved as expected based on their known origins and biochemical modifications. Importantly, the marked lack of covariation of *Actb* and *Sdha* transcription efficiencies with the general RT-specific transcription efficiency and differences in gene-specific intrinsic RT reproducibility extend the previous findings of between-sample gene expression instability [268, 354, 363, 395] to RT reaction effects, which bears on the selection and use of reference genes to reduce RT reaction variation in gene expression measurements by RT-qPCR; any statistical method relying in RT normalisation without observing the possible lack of RT efficiency covariance between the reference genes and genes of interest, will lead to increased confounding variation [361]. The hypothetical correlation between gene-specific transcription efficiency and "general" RT reaction efficiency appeared to be poor with both a low and high (apparent) RNA transcript abundance. However, we did not assess the secondary structures (e.g.

stem and loop conformation) of the RNA transcripts, although these have a substantial impact on the efficiency of reverse transcription and PCR [44].

The RT-to-RT CV was estimated to be approximately 3 times greater than that of qPCR, which is in agreement with previous results estimating the reproducibility of the RT step to be worse than that of qPCR [349, 361]. qPCR consistency remained remarkably similar across the four transcripts studied, and the overall precision of qPCR was in excellent agreement with previous data [196]. There were appreciable differences in the reproducibility of the individual qPCR enzymes, but these appear to be of little significance in a practical analysis setting. On the contrary, the salient differences between replicate runs in the qPCR experiment attest to an unexpectedly large run-to-run variation, and should be taken into account while analysing RT-qPCR data. This variation appears to be unrelated to the genes or qPCR enzymes and is most probably induced by the use of external standard curves and/or differences in pipetting and the reaction setup between runs.

The complications arising from the lack of technical reproducibility in an RT-qPCR experiment may (at least partly) be circumvented if the least precise part of the study, generally the biological units, can be pinpointed and replication in this step increased [176]. When an increase in the number of replicates and/or reference genes is not feasible, a linear hierarchical model and Bayesian inference offers the possibility to build a coherent statistical model of the whole experiment with normalisation and error reduction over all expression measurements, thereby maximising the use of the data. The hindrance, whilst also an asset, of the Bayesian approach is the necessity to develop a unique model for each experimental setting, or at least a careful modification of a pre-developed model structure, and meticulous consideration of the prior probability distributions of the parameters.

7 CONCLUSIONS AND FUTURE PROSPECTS

1. The small constitutive differences in the hypothalamic expression of *Ahr*, *Arnt* and *Arnt2* between L–E and H/W rats are most likely not causally related to the development of the wasting syndrome. The induction of *Ahrr* expression and the sharp increase in the *Cyp1a1* and *Cyp1a2* mRNA induced by TCDD attests to the presence of a functional AHR signalling cascade in the hypothalamus, but warrants anatomically more detailed sampling of neural, supportive and vasculature-related tissue in order to establish the magnitude and importance of cytochrome induction in neurons.

2. The lack of any drastic up- or downward changes in hypothalamic neuropeptide or receptor mRNA following TCDD treatment and the stability of hypothalamic reference gene expression in studies I–III speak against a severe cytotoxic effect on, or permanent hyperexcitation of the cells taking part in eating regulation. The late orexigenic elevation brought about by TCDD seems to be a compensatory reaction to body weight loss, and an increase in hypothalamic *Agrp* expression appears to be a very sensitive response to lowered energy availability.

3. The methods employed in studies I and II may cause expression changes confined to a localised point and/or circadian time to elude detection, and the complex neurophysiology and anatomy of the hypothalamus may lead to reciprocal cancellation or dwindling of alterations. As a more general question, the importance of the hypothalamus in eating regulation in comparison to other systems or centres, especially reward circuitry, food aversion and the hindbrain, has not been definitively resolved. In addition, the role of alterations in adiposity, energy and satiety signals or their CNS "interpretation", as well as non-peptidergic neurotransmission, requires further study in relation to TCDD-induced eating derailment.

4. In the wider context of energy balance regulation, the "AHR-unrelated" genetic and physiological differences between L–E and H/W strains warrant further research. The extent to which changes in mRNA expression affect cellular protein abundances is questionable, and more study is needed regarding the level of regulation, especially *in vivo*, and concerning highly specialised tissues and proteins. However, an unfocused protein or neurotransmitter analysis without definite targets, anatomical direction or exact timing of sampling is of little value.

5. The number of genes displaying acceptable expression steadiness in the face of lethal TCDD toxicity is small (for the hypothalamus, *Actb*, *Sdha*, *Gapdh* and *Pgk1* are acceptable), and the RT stability has a strong influence on the usability of the potential reference genes, since the covariances of RT efficiency and RT reproducibility appear to be mainly gene or RNA related. The "unstable" transcripts have their own independent intrinsic RT efficiencies that do not correlate with the general RT-specific transcription efficiency and exhibit somewhat less stability from one RT reaction to another than the "stable" transcripts. Hence, the use of only one

steadily expressed but unstably reverse transcribing reference gene (*Actb*) has most likely not induced marked bias in the results attained in studies I and II, but it may have inadvertently increased variation.

6. RT variance markedly exceeded qPCR variance, stressing the importance of replication at the RT, or preferably the biological level, while the consequences of appreciable differences in the reproducibility of the individual qPCR enzymes seem to be of little practical significance. On the contrary, the salient differences between replicate PCR runs should be taken into account in the design and data analysis of RT-qPCR experiments.

7. The use of linear hierarchical models and Bayesian inference in RT-qPCR data analysis offers the possibility to build a coherent statistical model of the whole experiment with normalisation over all expression measurements, thereby maximising the use of the data.

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