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ABSTRACT

Mammalian aryl hydrocarbon receptor (AHR) is a transcription factor activated by aryl hydrocarbons including some environmental toxins. It regulates several important physiological processes. AHR target genes include members of the cytochrome P450 (CYP) protein superfamily. CYPs as a family metabolize a wide variety of substrates including drugs and toxins but also endogenous compounds such as steroids and fatty acids. The nematode *Caenorhabditis elegans* (*C. elegans*) is a simple multicellular model organism that can be used to study gene function. It has one AHR ortholog AHR-1 and 77 protein-coding *cyp* genes. While AHR responds to toxins only in vertebrates, the physiological roles of AHR and CYPs are likely evolutionarily conserved. The aim of the study was to investigate the basic physiological roles of AHR and CYPs in *C. elegans*. Loss-of-function mutants were studied using a combination of phenotyping, fatty acid profiling and transcriptomics. Another aim was to follow the development of transcriptomics methods and their usage.

The *ahr-1* mutant grew slower than wild-type and had defects in movement, egg laying and defecation. Its fatty acid compositions, as well as gene expression levels of the enzymes that control them, were altered. Gene expression profiles in the mutant supported the role for AHR in development and metabolism during the first larval stage and in regulation of neuronal function during the last larval stage. Loss of *cyp-35A* subfamily genes resulted in reduction of fat and altered expression of genes encoding enzymes that control fatty acid diversity. Fatty acid compositions showed small but statistically significant changes. The endocannabinoid N-arachidonoylethanolamine was increased in the *cyp-35A5* mutant. Feeding with the fatty acids 18:n9*cis* and -*trans* lowered the expression of *cyp-35A* subfamily genes. The use of microarrays for gene expression profiling in the neurosciences has grown until recently whereas RNA-seq is a new emerging method for this purpose.

In conclusion, the results indicate a role for AHR in neuronal development and function as well as in growth and metabolism. Both CYPs and AHR appear to be involved in the regulation of fatty acid diversity and possibly lipid signaling molecules. Many of the roles of AHR and CYPs are conserved between *C. elegans* and mammals. These physiological roles may help to understand the mechanisms of action of toxins and should be taken into consideration when assessing chemicals or before developing treatments against the adverse effects.

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Medical Subject Headings: Caenorhabditis elegans/genetics; Gene Expression Profiling; Transcription Factors; Aryl Hydrocarbon; Cytochrome P-450 Enzyme System; Fatty Acids; Microarray analysis

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TIIVISTELMÄ

Nisäkkäiden aryylihiilivetyreseptori (AHR) on transkriptiotekijä, joka aktivoituu aryylihiilivetyjen kuten eräiden ympäristömykkyjen vaikutuksesta. Se säätelee useita tärkeitä fysiologisia prosesseja. AHR:n kohdegeenejä ovat mm. sytokromi P450 (CYP) -yläperheen jäsenet. CYP:t metaboloivat perheenä monenlaisia substraatteja kuten lääkkeitä ja toksiineja mutta myös endogeenisiä yhdisteitä kuten steroideja ja rasvahappoja. Nematodi *Caenorhabditis elegans* (*C. elegans*) on yksinkertainen monisoluinen malliorganismi, jota voi käyttää geenien toiminnan tutkimiseen. Sillä on yksi AHR-ortologi AHR-1 ja 77 proteiinia koodaavaa *cyp*-geeniä. Vaikka AHR reagoi toksiineihin vain selkärankaisissa, AHR:n ja CYP:ien fysiologiset roolit ovat todennäköisesti evoluutiossa säilyneitä. Tutkimuksen tavoite oli tutkia AHR:n ja CYP:ien fysiologisia perustehtäviä *C. elegansissa*. Mutantteja, joilta puuttui toimiva geeni, tutkittiin yhdistämällä fenotyyppianalyysiä, rasvahappoprofilointia ja transkriptomiikkaa. Toinen tavoite oli seurata transkriptomiikan menetelmien kehitystä ja käyttöä.

ahr-1-mutantti kasvoi hitaammin kuin villityyppi, ja sillä oli häiriöitä liikkumisessa, munimisessa ja ulostamisessa. Sen rasvahappokoostumukset ja niitä säätelevien entsyymien geenien ilmenemistasot olivat muuttuneet. Mutantin geenien ilmenemisprofiilit tukivat AHR:n roolia kehityksessä ja metaboliassa ensimmäisen toukkavaiheen aikana ja neuronien toiminnan säätelyssä viimeisen toukkavaiheen aikana. *cyp-35A*-aliperheen geenien puuttuminen aiheutti rasvan vähentymistä sekä ilmenemismuutoksia geeneissä, jotka koodaavat rasvahappokoostumuksia säätteleviä entsyymejä. Rasvahappokoostumuksissa näkyi pieniä mutta tilastollisesti merkitseviä muutoksia. Endokannabinoidi N-arakidonoyylietanolamiinin määrä oli kasvanut *cyp-35A5*-mutantissa. Rasvahappojen 18:n9*cis* ja *-trans* syöttäminen vähensi c*yp-35A*-aliperheen geenien ilmenemistasoja. Mikrosirujen käyttö geenien ilmenemisen profilointiin neurotieteissä on kasvanut viime aikoihin saakka, kun taas RNA-seq on uusi kasvava menetelmä tähän tarkoitukseen.

Loppupäätelmänä tulokset osoittavat, että AHR:llä on rooli hermosolujen kehityksessä ja toiminnassa sekä kasvussa ja metaboliassa. Sekä AHR että CYP:t näyttävät liittyvän rasvahappojen moninaisuuden ja mahdollisesti rasvasignaalimolekyylien säätelyyn. Monet AHR:n ja CYP:ien rooleista ovat säilyneet *C. elegansin* ja nisäkkäiden välillä. Nämä fysiologiset roolit voivat auttaa ymmärtämään toksiinien toimintamekanismeja, ja ne pitäisi ottaa huomioon arvioitaessa kemikaaleja tai ennen kuin kehitetään hoitoja haitallisia vaikutuksia vastaan.

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This has been a long trek for me. The publication number I was my very first own science project, before I had even started my Master's thesis and long before I knew I would become a Ph.D. student. Officially I started in the beginning of 2007 at the Finnish Graduate School in Toxicology that funded me during 2007-2010 enabling the work for publications II and III (including many unpublished trials and errors, of course). After that, publication IV was supposed to be an easy piece but ended up taking the following three years. During this time I was lucky to receive grants from Finnish Cultural Foundation / North Savo Regional Fund, Emil Aaltonen Foundation and Faculty of Health Sciences, University of Eastern Finland all of which helped me to finish the last publication and finally write the thesis.

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Kuopio, July 2014

Vuokko

List of the original publications

This dissertation is based on the following original publications:

I Aarnio V, Paananen J and Wong G. Analysis of microarray studies performed in the neurosciences. *Journal of Molecular Neuroscience 27(3): 261-268, 2005.*

II Aarnio V, Storvik M, Lehtonen M, Asikainen S, Reisner K, Callaway J C, Rudgalvyte M, Lakso M and Wong G. Fatty acid composition and gene expression profiles are altered in aryl hydrocarbon receptor-1 mutant *Caenorhabditis elegans*. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology 151(3): 318-324, 2010.*

III Aarnio V, Lehtonen M, Storvik M, Callaway J C, Lakso M and Wong G. *Caenorhabditis elegans* mutants predict regulation of fatty acids and endocannabinoids by the CYP-35A gene family. *Frontiers in Pharmacology: Predictive Toxicology 2: 12, 2011.*

IV Aarnio V, Heikkinen L, Peltonen J, Goldsteins G, Lakso M and Wong G. Transcriptional profiling reveals differential expression of a neuropeptide-like protein and pseudogenes in aryl hydrocarbon receptor-1 mutant *Caenorhabditis elegans*. *Comparative Biochemistry and Physiology, Part D: Genomics and Proteomics 9: 40-48, 2014.*

Contents

Abbreviations

acetyl-CoA	acetyl coenzyme A
acs-2	fatty acid CoA synthetase-2
AEA	N-arachidonoylethanolamine
2-AG	2-arachidonoylglycerol
AHA-1	aryl hydrocarbon receptor-associated protein-1
AHR	Aryl hydrocarbon receptor
ARNT	AHR nuclear translocator
ATP	adenosine triphosphate
avr-15	altered avermectin sensitivity-15
bHLH	basic Helix-Loop-Helix
BODIPY	boron-dipyrromethene
CAGE	cap analysis of gene expression
cAMP	cyclic adenosine monophosphate
CAR	constitutive androstane receptor
CARS	coherent anti-Stokes Raman scattering
CB1/CB2	cannabinoid receptor type 1/2
C. briggsae	*Caenorhabditis briggsae*
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
C. elegans	*Caenorhabditis elegans*
cGMP	cyclic guanosine monophosphate
ChIP-seq	chromatin immunoprecipitation followed by high-throughput DNA sequencing
CIBEX	Center for Information Biology gene EXpression
COX	cyclo-oxygenase
C. remanei	*Caenorhabditis remanei*
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ctl-1	catalase-1
CYP	cytochrome P450
daf	abnormal dauer formation
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EET	epoxyeicosatrienoic acid
EET-EA	epoxyeicosatetraenoic ethanolamide
2-EG	2-epoxyeicosatrienoyl glycerol
egg-1	egg sterile (unfertilizable) -1
elo-1	fatty acid elongase-1
FAAH	fatty acid amide hydrolase
FACS	fluorescence-activated cell sorting

fat-1	fatty acid desaturase-1
FBXA	F-box A protein
FXR	farnesoid X receptor
FGED	Functional Genomics Data Society
FICZ	6-formylindolo[3,2-*b*]carbazole
FLP	FMRFamide-related neuropeptide
FMRF	(Phe-Met-Arg-Phe)
GABA	γ-aminobutyric acid
GCY	soluble guanylate cyclase
GEET	glycerated epoxyeicosatrienoic acid
GEO	Gene Expression Omnibus
GFP	green fluorescent protein
GO BP	Gene Ontology Biological Process
GP CC	Gene Ontology Cellular Compartment
GO MF	Gene Ontology Molecular Function
GOA-1	Go*α* receptor subunit-1
GPR	G protein-coupled receptor
GR	glucocorticoid receptor
grl-1	GRound-Like-1
HNF	hepatic nuclear factor
hpo-30	Hypersensitive to POre-forming toxin-30
HAH	halogenated aromatic hydrocarbon
HETE	hydroxyeicosatetraenoic acid
HETE-EA	hydroxyeicosatetraenoic acid ethanolamide
him	high incidence of males
HSP90	heat schock protein 90
ICZ	indolo[3,2-*b*]carbazole
L1 / L4	larval stage 1 / larval stage 4
LDL	low-density lipoprotein
let-767	lethal-767
lim-6	LIM domain family (LIN-11, ISL-1 and MEC-3) -6
LXR	liver X receptor
LOX	lipoxygenase
LRO	lysosome-related organelle
MAGE-TAB	Microarray Gene Expression Tabular format
MDT-15	Mediator subunit-15
MGED	Microarray Gene Expression Database Society
MIAME	Minimum Information About a Microarray Experiment
MINSEQE	Minimum Information About a high-throughput SEQuencing Experiment
MosDEL	Mos1-mediated Deletion
MosTIC	Mos1 excision-induced transgene-instructed gene conversion
MPSS	massively parallel signature sequencing
mRNA	messenger-RNA

N2	*C. elegans* wild-type reference strain
NAE	N-acylethanolamine
NADPH	nicotinamide adenine dinucleotide phosphate
NAPE-PLD	N-acylphosphatidyl-ethanolamine phospholipase D
NF-κB	nuclear factor κ-B
NHR	nuclear hormone receptor
NLP	neuropeptide-like protein
NPR	neuropeptide receptor
OP50	a strain of *E. coli*
OSM	OSMotic avoidance abnormal
PAH	polyaromatic hydrocarbon
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PAS	Per-Arnt-Sim
PPAR	peroxisome proliferator-activated receptor
PCB	polychlorinated biphenyl
PTENpg1	phosphatase and tensin homolog pseudogene 1
PUFA	polyunsaturated fatty acid
qRT-PCR	quantitative real-time PCR
PXR	pregnane X receptor
Rb	retinoblastoma protein
RIP-140	receptor-interacting protein-140
RNAi	RNA interference
RNA-seq	high throughput RNA sequencing
RXR	retinoid X receptor
SAGE	serial analysis of gene expression
SBP-1	Sterol regulatory element Binding Protein-1
SCD1	stearoyl-CoA desaturase 1
TBP	TATA box binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TFIIA/B/…	transcription factor II A/B/…
TGF-β	transforming growth factor β
TRPV	transient receptor potential vanilloid
VDR	vitamin D receptor
XAP2	immunophilin-like protein hepatitis B virus X-associated protein 2
XRE	xenobiotic responsive element

1 Introduction

The field of molecular toxicology studies the effects of chemicals on living systems with the ultimate aim to develop toxicity tests and treatments against the toxic effects of chemicals. In this process it is essential to understand the physiological functions of the molecules that interact with xenobiotics. Aryl hydrocarbon receptor (AHR) is a transcription factor that regulates the expression of several genes by binding to specific DNA sequences in their regulatory areas. Human AHR is inappropriately activated by environmental toxic aryl hydrocarbons. Animal knockout studies show that AHR regulates the development and function of many systems in the body (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). Genes regulated by AHR include some cytochrome P450 (CYP) genes. CYPs are a superfamily of enzymes that metabolize various exogenous and endogenous compounds and synthesize important signaling molecules. Chemicals from the environment or food can affect gene expression or enzymatic activity of CYPs and thereby interfere with their physiological functions (Amacher, 2010).

Caenorhabditis elegans (*C. elegans*) is a good model organism to study functions of genes. It is a ~1 mm long nematode with 1031 cells and a lifespan of three weeks. It is a multicellular, yet simple organism that is easy to grow, handle and store. Functions of genes can be studied in *C. elegans* using e.g. loss-of-function mutants that lack the functional protein of interest. *C. elegans* was the first multicellular organism with its entire genome sequenced and genomic microarrays are available for it. *C. elegans* has about 20500 protein-coding genes, and about 38 % of them have been shown to have orthologs in human (Shaye and Greenwald, 2011). The AHR ortholog in *C. elegans* is AHR-1 (Powell-Coffman et al., 1998). It is required for normal neuronal development (Huang et al., 2004; Qin and Powell-Coffman, 2004) but the molecular mechanisms of its function are not well understood. The nematode has 77 functional CYPs but a lot remains to be known about their function. According to earlier RNA interference (RNAi) studies, some CYPs are essential for survival and development in *C. elegans* (Piano et al., 2002; Kamath et al., 2003; Rual et al., 2004; Sönnichsen et al., 2005). *C. elegans* can provide a model system also in molecular toxicology.

As the genomic sequence is known for an ever increasing number of organisms the current challenge is to elucidate the flow of information from sequence to phenotype. DNA is transcribed to RNA which is then translated to protein. These processes and their regulation are often studied using high throughput methods. One such approach is gene expression profiling which measures the levels of different RNA species in a tissue at a given time on the level of the whole transcriptome. The most used gene expression profiling method so far is microarray technology, whereas high throughput RNA sequencing (RNA-seq) is an upcoming method. Some proteins produce metabolites such as lipids that are also important to the function of the cell. There is a growing interest to study these metabolites on a global level, and methods such as mass spectrometry have facilitated the study of cellular lipids.

In this thesis, the functions of AHR-1 and CYP-35A subfamily members were investigated in *C. elegans*. Phenotypes were characterized in mutants lacking functional *ahr-1* or *cyp-35A* subfamily genes and the role of AHR-1 and CYP-35A subfamily members in fatty acid synthesis was demonstrated by measuring overall fatty acid compositions and gene expression alterations in enzymes that control them in the mutants (**II** and **III**). The effect of the *ahr-1* mutation on overall gene expression was studied by identifying differentially expressed genes using microarrays (**II**) and RNA-seq (**IV**). In addition, the state of the microarray field and availability of microarray data were investigated in a systematic review (**I**).

2 Review of the literature

2.1 ARYL HYDROCARBON RECEPTOR (AHR)

Mammalian aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic Helix-Loop-Helix/Per-Arnt-Sim (bHLH/PAS) family. It was first found as the molecule that responds to treatment with toxic aryl hydrocarbons by inducing enzymes that metabolize them (reviewed by Nebert et al., 1993). AHR is also generally accepted as the main mediator of the toxic effects of these compounds through aberrant changes in the expression of not only the well known enzymes but also several other genes under its regulatory control. Knockout studies have shown that AHR clearly has physiological roles beyond the toxin response, and its original ligands are thought to be endogenous. Current research focuses on the physiological functions of AHR. The following subsections will first review the molecular mechanisms of gene expression regulation by AHR, ligands and other possible ways to activate AHR, physiological roles of AHR with the main focus on studies in knockout mice or cell lines, and finally the AHR ortholog in the nematode *C. elegans*.

2.1.1 Molecular mechanisms of action

The classical function of AHR is to facilitate transcription initiation of its target genes by binding to their regulatory sequences in DNA. Unactivated AHR is located in the cytosol in a protein complex that contains a dimer of the heat shock protein HSP90 (Denis et al., 1988; Perdew, 1988), the co-chaperone p23 (Kazlauskas et al., 1999), and immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2, Meyer et al., 1998; Ma and Whitlock, 1997; Carver and Bradfield, 1997). AHR is typically activated by a ligand that binds to its PAS-B domain (Fukunaga et al., 1995). The ligand binding leads to nuclear translocation and dissociation of the complex (reviewed by Beischlag et al., 2008). In the nucleus, AHR forms a heterodimer with the AHR nuclear translocator (ARNT, Reyes et al., 1992) through its PAS-A, PAS-B and HLH domains and the heterodimer binds to specific DNA sequences named xenobiotic responsive elements (XREs) through the "basic" (b) region of the bHLH domain (Fukunaga et al., 1995). AHR is exported from the nucleus and degraded via the 26S proteasome pathway (reviewed by Pollenz, 2002).

The XRE contains a core sequence to which the AHR:ARNT heterodimer binds. Denison et al. (1988) defined the core sequence as 5′-T[A/T]GCGTG-3′ while Lusska et al. (1993) observed the sequence 5′-CGTGT-3′ to be essential for AHR:ARNT binding and transcription enhancement. ARNT binds to the GTG nucleotides whereas AHR binds to the 5′ portion of the XRE core (Bacsi et al., 1995). However, the core sequence, or the binding of the AHR:ARNT heterodimer to it, is not sufficient for the transcription enhancement function that also depends on the DNA sequence surrounding the core (Denison et al., 1988; Lusska et al., 1993). The most studied AHR target gene is CYP1A1 that contains multiple upstream XRE sites required for its transcription (reviewed by Whitlock, 1999).

The binding of AHR to the XRE in the CYP1A1 enhancer leads to nucleosome disruption that makes the promoter accessible for general transcription factors (Morgan and Whitlock, 1992). AHR and ARNT interact with general transcription factors such as TATA box binding protein (TBP) and TFIIF (Rowlands et al., 1996), subunits of the Mediator complex (Wang et al., 2004) and several coactivators that can be histone acetyltransferases (Kumar and Perdew, 1999; Beischlag et al., 2002), ATP-dependent histone modifiers (Wang and Hankinson, 2002) or other types of coactivators such as the receptor-interacting protein RIP-140 (Kumar et al., 1999; reviewed by Hankinson, 2005). Transcriptional activation by AHR requires a transactivation domain that is a large glutamine-rich area in the C terminal

region of AHR and contains several regions that function synergistically (Fukunaga et al., 1995; Rowlands et al., 1996; Ko et al., 1997).

AHR can also interact directly with other transcription regulator proteins to enhance or repress their target gene expression via binding to DNA. For example, AHR forms a complex with the retinoblastoma protein Rb (Ge and Elferink, 1998) and the complex represses transcription by another transcription factor E2F by binding to its promoters and displacing the coactivator p300 (Marlowe et al., 2004). AHR can also form a complex with E2F that induces E2F target gene expression (Watanabe et al., 2010). In addition, AHR can recruit unliganded estrogen and androgen receptors to their DNA binding sites and activate their target gene expression (Ohtake et al., 2003, 2008) while it has also been shown to repress the target genes of ligand-bound estrogen receptor (Klinge et al., 1999; Ohtake et al., 2003). Ligand-bound AHR forms a complex with the nuclear factor κB (NF-κB). This complex represses the target genes of both AHR and NF-κB (Tian et al., 1999) whereas unliganded AHR can form a complex with NF-κB that induces NF-κB target gene expression (Chen et al., 2012). In addition, AHR in a complex with ARNT acts as a ligand-activated coregulator with an unknown protein that binds to a specific DNA sequence termed XREII (Boutros et al., 2004; Sogawa et al., 2004). Similarly, other transcription factors can modulate the expression of AHR target genes.

AHR can also modulate intracellular signaling pathways without binding to DNA (reviewed by Marlowe and Puga, 2005 and Haarmann-Stemmann et al., 2009). Treatment with the environmental model toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) leads initially to activation of cytosolic kinases before induction of AHR target genes but also these initial effects are AHR-dependent (Enan and Matsumura, 1995; Dong and Matsumura, 2008, 2009). Moreover, AHR without an exogenous ligand forms a complex with the D-cyclin-dependent kinase 4 that phosphorylates the retinoblastoma protein (Rb) preventing it from repressing E2F target genes (Barhoover et al., 2010).

Another function of AHR is that it promotes proteasomal degradation of specific target proteins such as estrogen and androgen receptors. Ligand-bound AHR recognizes the proteins to be degraded and functions in an ubiquitin ligase E3 complex. The complex recruits E2 ubiquitin-conjugating enzymes that add ubiquitin molecules to the target proteins. The added ubiquitins are recognized by the 26S proteasome leading to protein degradation (Ohtake et al., 2007, 2008, 2009).

2.1.2 Activation of AHR

AHR has several ligands, some of which are synthetic, some endogenous and some dietary. The most classical ligands for AHR are the synthetic and exogenous planary compounds that contain benzene rings (reviewed by Denison and Nagy, 2003 and Nguyen and Bradfield, 2008). They include halogenated aromatic hydrocarbons (HAHs) such as TCDD, 2,3,7,8′-tetrachlorodibenzofuran, 3,3′,4,4′-tetrachloroazoxybenzene or 2,3,6,7-tetrachloronaphtalene; polyaromatic hydrocarbons (PAHs) such as, 3-methylcholantrene, benzo[*a*]pyrene or β-naphthoflavone; and polychlorinated biphenyls (PCBs) such as 3,4,3′,4′,5-pentachlorobiphenyl. There are also "nonclassical" synthetic AHR ligands that typically also contain benzene rings but are somewhat different in structure (reviewed by Denison and Nagy, 2003). Among the known xenobiotic ligands, the HAHs are the most potent (pM to nM affinities) and TCDD is the most potent of them.

For a compound to be an endogenous AHR ligand it has to bind AHR at a relatively high affinity and also be present in high enough concentrations, at least locally, to activate the receptor. The local concentrations are not easy to assess and therefore most of the endogenous AHR ligands are still putative. The endogenous ligand with the highest affinity known is the tryptophan ultraviolet photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ) that binds AHR as well or even better than the prototypical xenobiotic AHR ligand TCDD (Rannug, et al., 1987, 1995). FICZ synthesis can be induced by UV-B light in a cell culture (Fritsche et al., 2007) and FICZ metabolites have been detected in human urine

(Wincent et al., 2009) suggesting that FICZ is present in the human body. FICZ is also a substrate of CYP1A1, an enzyme whose expression it induces through AHR, resulting in an autoregulatory loop (Wei et al., 2000). This resembles the "detoxification response" function of AHR in which xenobiotic AHR ligands induce genes that metabolize them. Other endogenous ligands include other tryptophan metabolites, indigoids, heme metabolites, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, equilenin (horse estrogen), and the arachidonic acid metabolites lipoxin A4 and prostaglandin G (reviewed by Denison and Nagy, 2003 and Nguyen and Bradfield, 2008).

In addition, many of the naturally occurring AHR ligands are obtained from diet. These include carotinoids, flavonoids, 7,8-dihydrocutacarbine, dibenzoylmethanes, curcumin and indolo-3-carbinol that is metabolized to e.g. indolo[3,2-*b*]carbazole (ICZ) that resembles FICZ and binds AHR almost as well as TCDD (reviewed by Denison and Nagy, 2003 and Nguyen and Bradfield, 2008). Also the naturally occurring AHR ligands typically contain benzene rings but they can vary in their molecular structure (reviewed by Denison and Nagy, 2003). Some natural compounds such as certain flavonoids, resveratrol (Casper et al., 1999) or 7-ketocholesterol (Savouret et al., 2001) bind to AHR but are antagonists rather than agonists.

There is discussion whether AHR can also be activated without binding to any ligand. Suspension of cell cultures that reduces cell adhesion, alters cell shape and influences differentiation (Sadek and Allen-Hoffmann, 1994a and b; Monk et al., 2001), sparse cell density (Ikuta et al., 2004) or disruption of cell-cell contact (Cho et al., 2004) can activate AHR and its target gene expression. In addition, shear stress-modified low-density lipoprotein (LDL) can activate AHR (McMillan and Bradfield, 2004) although it is very different in structure and many times larger than all of the known AHR ligands. AHR is also activated by high glucose in a culture of endothelial cells and forms a complex with other glucose-activated transcription factors that have putative binding sites near the binding sites of AHR (Dabir et al., 2008). The activation of the complex involves glycosylation of at least one of the proteins. Moreover, cAMP has been shown to induce nuclear translocation of AHR and formation of a complex somewhat different than when AHR is activated by TCDD (Oesch-Bartlomowicz et al., 2005). However, involvement of an unidentified endogenous ligand cannot be completely ruled out in any of these cases. Phosphorylation of AHR by kinases such as protein kinase C (Carrier et al., 1992) or mitogen-activated protein kinases (Tan et al., 2002) has been shown to be necessary for AHR activation although it may not be sufficient (reviewed by Puga et al., 2009).

2.1.3 Physiological roles of AHR

Studies in knock-out animals have demonstrated that AHR has many important physiological roles. The earliest studies reported slow growth, liver defects such as smaller size, fibrosis and fatty metamorphosis, increased formation of blood cells and lower basal expression or uninducibility of enzymes (Fernandez-Salguero et al., 1995; Gonzalez et al., 1995; Schmidt et al., 1996). The liver defects may be due to accumulated retinoid levels caused by downregulation of CYP enzymes (Andreola et al., 1997, 2004a and b), increased levels of transforming growth factor β (TGF-β, Zaher et al., 1998) or abnormal liver vasculature development (Lahvis et al., 2000, 2005; Walisser et al., 2004; Harstad et al., 2006).

Abnormal angiogenesis has been reported in AHR knockouts also in ischemia (Ichihara et al., 2007) or tumorigenesis models (Fritz et al., 2007, 2008; Roman et al., 2009). In addition, AHR knockouts have cardiac hypertrophy and fibrosis (Fernandez-Salguero et al., 1997, Thackaberry et al., 2002; Vasquez et al., 2003). It may be due to increased levels of hypoxia-inducible factor-1α, (Thackaberry et al., 2002) or endothelin-1 (Lund et al., 2006) that increases reactive oxygen species in the heart (Lund et al., 2005) or decrease in the proto-oncogene Vav3 (Sauzeau et al., 2011). AHR knockout also affects blood pressure. Studies report both hypertension (Lund et al., 2003, 2008; Villalobos-Molina et al., 2008) and

hypotension (Zhang et al., 2010; Agbor et al., 2011) depending on e.g. altitude (Lund et al., 2008) as well as on the levels and responsiveness to angiotensin II.

One of the two knockout strains also had neonatal lethality and immune system defects (Fernandez-Salguero et al., 1995; Gonzalez et al., 1995). Several studies show that AHR knockouts have reduced numbers of some types of immune system cells due to impaired formation or increased apoptosis (Gasiewicz et al., 2000; Veldhoen et al., 2009; Jux et al., 2009; Elizondo et al., 2011; Kadow et al., 2011; Singh et al., 2011, 2014; Qiu et al., 2012; Zhou et al., 2013) whereas other types of immune system cells are formed in excess in the knockouts (Singh et al., 2011; Liu et al., 2013; Qiu et al., 2013; Gasiewicz et al., 2014). Certain types of immune system cells secrete interleukins that promote the development of other cells of the immune system. In AHR knockouts, some interleukins are increased (Rodríguez-Sosa et al. 2005; Shi et al., 2007; Sekine et al., 2009; Elizondo et al., 2011) and some decreased (Veldhoen et al., 2009; Elizondo et al., 2011) and some interleukin genes are direct targets of AHR (Jeon and Esser, 2000). AHR knockouts are usually more susceptible to infections but in some cells the lack of AHR can protect from autoimmunity (Nakahama et al., 2011).

AHR knockout mice also have several reproductive defects. AHR knockout females have lesions in the uterus (Fernandez-Salguero et al., 1997) and decreased survival during pregnancy or lactation. Many the knockout pups also die during pregnancy or lactation (Abbott et al., 1999). AHR knockout females have a larger number of primordial follicles after birth (Benedict et al., 2000; Robles, et al., 2000) but a smaller number of antral follicles later in life (Benedict et al., 2000, 2003). The AHR defieciency decreases follicular growth and ovulation (Benedict et al., 2003; Barnett et al., 2007a), levels of estrogen and its receptors and follicular expression of genes promoting cell cycle (Barnett et al., 2007a; Hernández-Ochoa et al., 2010). Ovaries of AHR knockouts are also less responsive to pituitary gonadotropins (Barnett et al., 2007b; Hernández-Ochoa et al., 2013). Male AHR knockout mice have impaired prostate and seminal vesicle development (Lin et al., 2002). AHR is also required in normal sperm development (Baba et al., 2008; Hansen et al., 2014). In addition, AHR knockouts exhibit age-dependent regression of seminal vesicles, low testosterone levels, abnormal vaginal plugs and low fertility (Baba et al., 2008).

AHR knockout also causes some defects in the development and function of the nervous system. The knockout has reduced GABA receptor subtype Aα6 expression in the cerebellum (Collins et al., 2008) and decreased GABAergic neuron differentiation in the ventral telencephalon (Gohlke et al., 2009). AHR knockout mice display impaired hippocampal-dependent memory as well as decreased formation, survival and differentiation of neurons in the dentate gyrus (Latchney et al., 2013). AHR knockout also causes involuntary eye movements due to visual or visuo-motor defects (Chevallier et al., 2013).

The endogenous AHR ligand FICZ is formed by UV-B irradiation in skin cells. UV-B causes a stress response in keratinocytes (Fritsche et al., 2007), a tanning response in melanocytes (Jux et al., 2011) and local immunosuppression (Navid et al., 2013) through activation of AHR. AHR knockout keratinocytes are also more susceptible to UVB-induced apoptosis than wild-type cells (Frauenstein et al., 2013). In addition, activation of AHR by FICZ and other tryptophan photoproduct ligands may play a role in the circadian timing system in the brain, possibly by mediating a response to light. FICZ alters the circadian expression of clock genes in a suprachiasmatic nucleus cell line and inhibits glucose-induced shift in electrical activity rhythm in brain slices (Mukai and Tischkau, 2007). AHR knockout mice have an increased response to light (Xu et al., 2013) whereas xenobiotic AHR agonists decrease light-induced phase shifts in wild-type (Mukai et al., 2008; Xu et al., 2013).

Activation of AHR affects body weight and fat mass. HAHs such as TCDD typically cause a wasting syndrome with decreased weight and fat mass (reviewed by Poland and Knutson, 1982) whereas PCBs have been obesogenic in some studies (Arsenescu et al., 2008;

Rashid et al., 2013; Wahlang et al., 2013). AHR activation inhibits adipocyte differentiation and fat storage in mouse-derived cell culture systems (Phillips et al., 1995; Brodie et al., 1996; Alexander et al., 1998; Shimada et al., 2009). Another toxic effect of AHR activation is hepatic steatosis (reviewed by Poland and Knutson, 1982) through increased fatty acid uptake in the liver (Lee et al., 2010; Kawano et al., 2010, He et al., 2011; Angrish et al., 2012). AHR agonist treatment also alters lipid compositions in the liver, serum and adipose tissue (Angrish et al., 2011, 2013; Forgacs et al., 2012) as well as the expression of fatty acid transport, metabolism and synthesis genes (Boverhof et al., 2005; Sato et al., 2008; Forgacs et al., 2012; Wahlang et al., 2013). Moreover, mice with low-affinity AHR are less susceptible to diet-induced obesity than mice with a high-affinity AHR (Kerley-Hamilton et al., 2012).

AHR knockouts are often resistant to the toxic effects caused by treatment with xenobiotic AHR agonists (Fernandez-Salguero et al., 1996; Mimura et al., 1997; Dertinger et al., 1998; Peters et al., 1999; Shimizu et al., 2000; Vorderstrasse et al., 2001; Nakatsuru et al., 2004; Mulero-Navarro et al., 2005; Sagredo et al., 2006; Talaska et al., 2006; Curran et al., 2011, 2012; Lee and Riddick, 2012; Mejia-Garcia et al., 2013). However, AHR knockout affects many of the same biological processes as the xenobiotic ligands (reviewed by Poland and Knutson, 1982 and Bock and Köhle, 2006).

As a summary, the lack of functional AHR affects many systems in the body. This can be due to defects in other systems on the organ level, defects in cell cycle, differentiation or apoptosis, differential expression of AHR target genes, lack of metabolism of specific compounds, or interference with hormonal systems.

2.1.4 *C. elegans* AHR

The ortholog of AHR in *C. elegans* is AHR called AHR-1 (aryl hydrocarbon receptor-related, Powell-Coffman et al., 1998). The AHR-1 peptide is ~600 amino acids long and shares approximately 38 % identity with human AHR over the first 400 amino acids including the bHLH, PAS-A and PAS-B domains while the carboxyterminals are less similar. *C. elegans* AHR-1 does not have a similar glutamine-rich transcriptional activation domain as in mammalian AHR. However, carboxyterminal regions are likely required for transcriptional activation as suggested by experiments in a yeast expression system.

Orthologs for ARNT and HSP90 are also found in *C. elegans*, called AHA-1 (Powell-Coffman et al., 1998) and DAF-21 (Birnby et al., 2000), respectively. AHR-1 and AHA-1 bind as a dimer to the XRE core sequence TGCGTG *in vitro.* The binding is sequence-specific as the AHR-1-AHA-1 dimer does not bind to a modified XRE core sequence with one G nucleotide changed to A. Inter-species complex formation is also possible *in vitro*: *C. elegans* AHA-1 can form a complex with murine AHR and bind to DNA. Similarly, *C. elegans* AHR-1 can form a heterodimer with human ARNT that binds to the XRE. In addition, *C. elegans* AHR-1 binds to rabbit HSP90 (Powell-Coffman et al., 1998) but not to mouse XAP2 (Bell and Poland, 2000).

The main difference between the nematode and human AHR proteins is that *C. elegans* AHR-1 does not bind a TCDD analog $^{125}IN_3Br_2DD$ and is not activated by β-naphthoflavone in a yeast expression system (Powell-Coffman et al., 1998). In fact, none of the known invertebrate AHR orthologs in *Drosophila melanogaster* and *Mya arenaria* (soft cell clam), appear to bind TCDD or β-naphthoflavone (Butler et al., 2001; Crews and Brenman, 2006). Moreover, a metabolic response to xenobiotics in the study by Jones et al. (2013) was not dependent on AHR-1. This suggests that the ancestral role of AHR is not in adaptive toxin response. Neither exogenous nor endogenous ligands are currently known for invertebrate AHR. *C. elegans* AHR-1 is not constitutively active in a yeast expression system unless the PAS domain is removed (Powell-Coffman et al., 1998). This suggests that AHR-1 requires some kind of post-translational activation. However, it is not known whether the activation is achieved through a ligand or another mechanism. If there are ligands for *C. elegans* AHR-1 they are likely different from those in mammals.

The *ahr-1* gene is expressed in various neurons, blast cells and phasmid socket cells during embryogenesis and larval development (Qin and Powell-Coffman, 2004). The neurons are of various types and utilize different neurotransmitters (**Table 1**). Blast cells are precursor cells destined to divide some time after hatching. Phasmid socket cells envelop sensory neurons in a posterior sensory structure called phasmid.

Table 1. C. elegans neurons that express *ahr-1*. A question mark indicates that the neurotransmitter has not been confirmed.

Neuron	**Location**	**Neurotransmitter**	**Type**
ALNR/ALNL	tail	acetylcholine	sensory (oxygen)
AQR/PQR	pharynx and tail	unknown	sensory (oxygen)
AVM/PVM	anterior body	glutamate	sensory (touch)
BDUR/BDUL	anterior body	neuropeptide-like proteins	interneurons
PLMR/PLML	tail	glutamate	sensory (touch)
PLNR/PLNL	tail	acetylcholine	interneurons
PHCL/PHCR	tail	dopamine	sensory (temperature)
PVWL/PVWR	tail	unknown	interneurons
RMEL/RMER	nerve ring	GABA	motor
SDQR	anterior body	unknown	interneuron
SDQL	posterior body	unknown	interneuron
URXR/URXL	head	neuropeptide-like proteins	sensory (oxygen)
M1	pharynx	acetylcholine?	motor
I3	pharynx	neuropeptide-like protein?	interneuron
ASKL/ASKR	head	glutamate	sensory (chemo)
RIPL/RIPR	ring/pharynx	unknown	interneurons

Mutations of AHR-1 affect several aspects of neuronal development (Huang et al., 2004; Qin and Powell-Coffman, 2004; Smith et al., 2013). For example, AHR-1 is required for the cell fate of GABAergic neurons in the L1 larval stage (Huang et al., 2004). The four GABAergic RME neurons in the nerve ring control head muscle movements in foraging behavior. AHR-1 is expressed in two of them, namely RMEL and RMER, but not in the other two, i.e. RMED and RMEV. These neurons can be distinguished based on their morphology and expression of marker genes: RMED and RMEV have posteriorly extending neuronal processes that RMEL and RMER do not have. Moreover, RMEL and RMER express *lim-6* and *glr-1* whereas RMED and RMEV express *avr-15*. In the *ahr-1(ju145)* mutant, RMEL and RMER have the neuronal processes and marker gene expression typical of RMED and RMEV. Ectopic expression of *ahr-1* in all RME neurons rescues the abnormalities seen in RMEL and RMER but in addition it causes RMED and RMEV to develop into cells with typical RMEL and RMER morphology and marker gene expression. The presence of AHA-1 is required for the AHR-1 function in specifying RME cell fate whereas DAF-12 is not required.

AHR also specifies the fate of AVM light touch sensory neuron (Smith et al., 2013). In *ahr-1* mutants the AVM neuron that normally has one simple unbranched process develops into a PVD-like nociceptor neuron with highly branched dendrites. AHR elevates the

expression of another transcription factor MEC-3 while simultaneously repressing MEC-3 target genes such as *hpo-30* that promotes denritic branching in PVD and in the abnormal AVM neuron. The *ahr-1* mutant has a weakened response to light touch of the anterior body. Instead, the abnormal AVM neuron responds to similar nociceptive stimuli as the PVD neuron and also evokes an escape response although it forms synapses with different neurons than PVD.

AHR-1 also appears to play a role in the regulation of cell and axon migration of certain neuron subtypes (Qin and Powell-Coffman, 2004). In the *ahr-1(ia03)* mutant, AVM, SDQR and PLM neurons or their precursors fail to migrate to their correct position during the L1 larval stage more often than in wild-type. Qin and Powell-Coffman (2004) observed SDQR and AVM neurons to extend incorrectly projected axons and AVM to grow branched or too many axons in the *ahr-1(ia03)* mutant. This may be at least partially due to differentiation of these cells into PVD-like neurons as reported by Smith et al. (2013). In rare cases the *ahr-1(ia03)* mutant also has an extra neuron that expresses the PLM neuron marker *mec-18*:GFP, probably due to lineage defects or disrupted apoptosis in the mutant (Qin and Powell-Coffman, 2004). Also AHA-1 function is required at least in SDQR migration. In addition, AHR-1 and AHA-1 are required for gap junction contact formation between BDU and PLM neurons (Zhang et al., 2013).

Qin et al. (2006) demonstrated that AHR-1 is involved in social feeding behavior in which nematodes form groups on the border of the bacterial lawn (de Bono and Bargmann, 1998). The *ahr-1* gene is expressed in four neurons that play a role in the regulation of social feeding behavior, namely URXR, URXL, AQR and PQR. Social feeding is regulated by nutrition and oxygen availability and inhibited by the neuropeptide receptor NPR-1 that suppresses the function of these neurons. Unlike wild-type N2 animals, *npr-1* loss-of-function mutants are social feeders in normal laboratory conditions. Loss-of-function of *ahr-1* or *aha-1* dramatically decreases social feeding of the *npr-1* mutants (Qin and Powell-Coffman, 2004, Qin et al., 2006). Transgenic expression of the *ahr-1* gene in only URX and two other neurons greatly increases social feeding in the *ahr-1*; *npr-1* double mutant suggesting that *ahr-1* is required mainly in the URX neurons to regulate social feeding but may also function in other neurons to modulate the behavior. Induction of the *ahr-1* transgene after the development of the URX neurons partially restores social feeding in the *ahr-1; npr-1* double mutant. This suggests that the suppression of social feeding behavior in the double mutant is not due to irreversible developmental defects in URX neurons.

The URX neurons in the *ahr-1* mutant develop normal morphologies but express decreased levels of their specific marker genes *npr-1* and *gcy-32* as well as other *gcy* family genes *gcy-34*, *gcy-35*, *gcy-36* and *gcy-37* as seen with *gcy*::GFP reporter genes. The *gcy* family genes encode soluble guanylate cyclases that synthesize 3′,5′-cyclic guanosine monophosphate (cGMP). cGMP activates TAX-2/TAX-4 cGMP-gated sensory channels that promote social feeding in URX neurons (Coates and de Bono, 2002). Especially GCY-35 and GCY-36 may be key regulators of social feeding as GCY-35 likely acts as an oxygen sensor and functions as a heterodimer with GCY-36. However, transgenic expression of *gcy-35* and *gcy-36* in URX neurons is not sufficient to increase social feeding in the *ahr-1*; *npr-1* double mutant. Thus, the regulation of these two genes is not the only mechanism through which AHR-1 regulates social feeding but AHR-1 may regulate other genes important for the behavior. Also the mechanism through which AHR-1 regulates the expression of *gcy-35* and *gcy-36* genes is unclear. The 5′ flanking sequences of the *gcy* family genes contain putative XRE elements. However, even when mutations are induced in the XRE elements of *gcy-35*::GFP or *gcy-36*::GFP reporter constructs GFP is still expressed in wild-type background and under-expressed in *ahr-1* mutant background suggesting that the regulation of *gcy-35* or *gcy-36* by AHR-1 does not occur through the putative XRE sites.

In conclusion: AHR is an evolutionarily old protein that has been present before the divergence of vertebrates and invertebrates but toxin response is a relatively new function only seen in vertebrates. The old functions of AHR may include development but also

regulation of behavior in response to environmental cues as the study concerning social feeding suggests. The activation of AHR in invertebrates is still a mystery. *C. elegans* provides a simple model system for studying the evolutionarily old and possibly conserved functions of AHR.

2.2 CYTOCHROMES P450 (CYPS)

Cytochromes P450 (CYPs) are a superfamily of mono-oxygenase enzymes that are found in virtually all organisms including animals, plants, fungi, protists, bacteria, archaea and viruses (Gotoh, 2012). In eukaryotes they are expressed in mitochondrial membranes and endoplasmic reticulum whereas in prokaryotes they are soluble proteins. CYPs are known to oxidize a wide variety of both endogenous and exogenous compounds. From a toxicological perspective, understanding of the endogenous functions of CYPs is important as the induction of these enzymes with xenobiotics is likely to affect the physiological processes involving CYPs (Amacher, 2010). The following subsections will provide an overview of general CYP structure and function, evolutionarily prospects, role of CYPs in metabolizing endogenous compounds and *C. elegans* CYPs.

2.2.1 Structure and general function

The CYPs consist of an apoprotein and an iron protoporphyrin IX as a prosthetic group (reviewed by Werck-Reichhart and Feyereisen, 2000). The protoporphyrin has an iron ion in the middle whose oxidation-reduction cycle is involved in the catalysis of reactions. Cytochrome P450s were named after their absorbance maximum at 450 nm when the iron ion is in the Fe^{2+} form and the heme is in a complex with carbon monoxide. The core domain binding the protoporphyrin group located near the C terminus of the protein is highly conserved whereas other parts can vary greatly between different CYP proteins. A CYP protein contains several recognition sites for substrates. Even small mutations in these domains can result in the recognition of different substrates and the production of different metabolites. In addition, CYP proteins contain binding sites for the cytochrome b5 and NADPH-P450 reductase that provide electrons for the reactions catalyzed by CYPs, sites that can be phosphorylated by cAMP and a membrane insertion sequence through which the protein is attached to the endoplasmic reticulum or mitochondrial membrane.

General types of CYP-mediated reactions have been identified and include hydroxylations, epoxidations, demethylation, oxidative deamination, oxidation, oxidative dehalogenation and many others (reviewed by Guengerich, 2001). Generally, one atom of oxygen is incorporated into the substrate. CYPs perform phase I metabolism of drugs and toxins. In these reactions the substrate is prepared for Phase II metabolism enzymes. Sometimes the effect is detoxifying, sometimes bioactivating.

CYPs are regulated at the gene expression level by their substrates that activate transcription factors. Transcription factors known to activate CYPs include at least AHR, pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptors (PPAR), farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear factors (HNF), glucocorticoid receptor (GR) and CCAAT/enhancer binding proteins (C/EBPs, reviewed by Tompkins and Wallace, 2007). The main xenobiotic-activated transcription factors that induce CYPs are AHR, CAR and PXR (Pelkonen et al., 2008; Amacher, 2010). Also endogenous substrates induce CYP gene expression through transcription factors. For example, steroid and growth hormones have been shown to regulate CYP gene expression (Waxman, 1988; Waxman et al., 1991) and retinoids activate retinoid X / vitamin D (RXR/VDR) receptors that induce CYP3A4 (Wang et al., 2008).

2.2.2 Evolution of CYPs

Currently more than 16000 CYP proteins are known (Gotoh, 2012). They are found in a wide variety of organisms including animals, plants, fungi, protists, bacteria, archaea and

viruses, although some species such as *Escherichia coli* bacteria lack them. The diversity of CYPs has arisen through gene duplications and probably gene amplifications, conversions, genome duplications, gene loss and lateral transfer (Werck-Reichhart and Feyereisen, 2000).

In CYP nomenclature, the first number denotes the CYP family and the following letter denotes the subfamily (Nelson, 2006). CYPs within the same family share 40 % identity in peptide sequence whereas CYPs within a subfamily have at least 55 % similarity (Gotoh, 2012). The last number identifies the CYP protein. CYP proteins may share as little as 16 % similarity according to an early review (Werck-Reichhart and Feyereisen, 2000). Humans have 57 functional CYPs and more than 59 CYP pseudogenes classified in 18 families and 43 subfamilies (Gotoh, 2012). The CYP battery in human is highly conserved among mammals and even among vertebrates. The largest CYP families in human are 2, 3 and 4, and some of these are even larger in other mammals such as mouse.

CYP families in different phyla, e.g. vertebrates, nematodes and insects, can be grouped into clans based on similarity (Nelson, 1998). CYPs within a clan may be derived from a single ancestor. Eleven animal CYP clans have been identified in total (Nelson et al., 2013). Humans likely have CYPs of the clans 2, 3, 4, 7, 19, 20, 26, 46, 51 and mitochondrial whereas nematodes and insects have CYPs belonging to clans 2, 3, 4 and mitochondrial (Baldwin et al., 2009). In human and nematodes, the largest CYP clan is 2, whereas in insects clan 3 is the largest. CYPs can also be classified on an even higher level into groups that contain similar CYP clans across e.g. animals, plants and bacteria (Gotoh, 2012). In addition, all CYPs belong to one of the two major classes, B and E. The class B is specific to prokaryotes and fungi, whereas CYPs of the class E are found in all organisms.

CYP families have emerged and been lost during evolution. One interesting CYP family is CYP51 required for one step of cholesterol synthesis. This family is evolutionarily old and found e.g. in bacteria, plants and human. However, it is not found in nematodes or insects that do not synthesize cholesterol *de novo*.

Some CYPs and transcription factors that control their expression or bind ligands synthesized by them may have evolved together. This has been shown for steroid hormone receptors and the CYP19A1 that synthesizes estrogen (reviewed by Gotoh, 2012). Whether it is true in the case of AHR and CYP1A1 is currently not known.

Some CYP families or subfamilies are phylogenetically stable typically having only one or a few members conserved across many species, whereas unstable CYP families have variable numbers of members in different species. It is believed that the stable CYP families are essential in the synthesis or degradation of endogenous substrates while the highly diverse CYP families metabolize xenobiotics or secondary metabolites (Thomas, 2007; Gotoh, 2012). CYPs that have endogenous substrates typically do not have many point mutations leading to amino acid alterations in their substrate recognition sites, whereas CYP families involved in xenobiotic metabolism such as CYP2 and CYP3 accumulate such mutations. There is evidence that CYPs originally metabolizing endogenous compounds have developed into CYPs that metabolize xenobiotics but Gotoh (2012) suggests that the opposite may be even more likely.

2.2.3 Physiological roles of CYPs

All mammalian CYP families metabolize endogenous substrates while xenobiotic metabolism is mainly carried out by families 1 to 4 (reviewed by Nebert et al., 2013). Generally, the substrates that CYPs biosynthesize, bioactivate, modify or catabolize are lipophilic. Some CYPs are essential for the production of endogenous compounds. For example, CYP51A1 is a lanosterol 14α-demethylase required in cholesterol biosynthesis (Strömstedt et al., 1996). Also several other steps in the production of different steroid hormones are dependent on CYPs (reviewed by Hu et al., 2010). CYP11A1 converts cholesterol to pregnenolone by side-chain cleavage and CYP17A1 catalyzes subsequent intermediate steps. CYP19A1 (aromatase) converts androgens to estrogens whereas CYP21A2 and CYP11B1 catalyze reactions in a different pathway leading to glucocorticoids

that CYP11B2 further converts to aldosterone. Sex steroids are also catabolized by members of the CYP3A subfamily (Waxman et al., 1988; Domanski et al., 2001). In addition, cholesterol is also a precursor of bile acids, and CYPs 7A1, 8B1, 27A1, 7B1, 46A1 and 39A1 are involved in the pathways of their synthesis (Lorbek et al., 2012). CYPs 27A1, 27B1 and 2R1 are required for the bioactivation of vitamin D (Wikvall, 2001) whereas CYP24A1 degrades it (reviewed by Jones et al., 2012). Some CYPs synthesize retinoic acid (Zhang et al., 2000) and others such as CYP26A1, CYP26B1 and CYP26C1 inactivate it by hydroxylation (Ross and Zolfaghari, 2011).

CYPs also metabolize fatty acids and lipid signaling molecules. Fatty acids released from membrane lipids by e.g. phopholipases can be converted into different classes of signaling molecules by e.g. cyclo-oxygenases (COXs), lipoxygenases (LOXs) and CYPs. CYPs typically metabolize fatty acids into hydroxyl and epoxy products that are called hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs), respectively**.** CYPs have been shown to metabolize several fatty acids including at least arachidonic acid (20:4n6), docosahexaenoic acid (22:6n3), linoleic acid (18:2n6), α-linoleic acid (18:3n3), eicosapentaenoic acid (20:5n3) and adrenic acid (22:4n6, Konkel and Schunck, 2011). CYPs can also further metabolize the fatty acid-derived signaling molecules made by CYPs or other enzymes (reviewed by Nebert and Karp, 2008). CYP families responsible for the metabolism of fatty acids and their derivatives are CYP1, CYP2, CYP3 and CYP4, of which especially the subfamilies CYPs 1A1 and 1B1 are induced by AHR (reviewed by Nebert and Karp, 2008). The HETEs and EETs are potent signaling mediators and have a major role in cardiovascular and nervous systems (Iliff et al., 2010). They can activate ion channels, G protein-coupled receptors and PPAR nuclear receptors, some of which belong to the endocannabinoid system. For example, arachidonic acid can be epoxidized by CYPs into EETs that relax vascular smooth muscles through the activation of transient receptor potential vanilloid type 4 (TRPV4) receptors (Watanabe et al., 2003) or into 2-epoxyeicosatrienoylglycerols (2-EG) that activate CB2 and CB2 cannabinoid receptors (Chen et al., 2008).

CYPs can also metabolize the endocannabinoid N-arachidonoylethanolamine (AEA) that is a fatty acid-containing signaling molecule derived from membrane phopholipids. Bornheim et al. (1995) found that liver and brain microsomes metabolize AEA into several unidentified products and the metabolism is carried out by at least CYPs from subfamilies 3A, 1A and 2C. Snider et al., (2007) demonstrated that CYP4F2 hydroxylates AEA into 20-HETE-ethanolamide (20-HETE-EA) in human liver and kidney microsomes, whereas CYP3A4 epoxidizes it into four different epoxyeicosatetraenoic ethanolamides (EET-EAs). Also the brain mitochondrial CYP2D6 metabolizes AEA into the same products (Snider et al., 2008). One of the products, 5,6-EET-EA is a potent agonist of CB2 cannabinoid receptors and more stable than AEA (Snider et al., 2009). Individuals have different alleles of the CYP3A4 gene, and proteins from the different alleles have been shown to oxidize AEA into different products (Pratt-Hyatt et al., 2010). In addition, CYP4X1 converts AEA into 14,15-EET ethanolamide (14,15-EET-EA, Stark et al., 2008). CYPs also metabolize another endocannabinoid, 2-arachidonoylglycerol (2-AG), into glycerated epoxyeicosatrienoic acids (GEETs) that have vasodilatory functions (Awumey et al., 2008).

2.2.4 *C. elegans* CYPs

C. elegans has 83 *cyp* genes of which 77 are protein-coding and 5 are pseudogenes. The CYPs can be classified into 16 families and 26 subfamilies. Almost all of the *C. elegans* CYP families appear to be nematode-specific (Nelson, 1998) but they correspond to the clans 2, 3, 4 and mitochondrial found also in human (Baldwin et al, 2009). The clan 2 is highly expanded in *C. elegans* (Gotoh, 1998; Nelson, 1998) whereas only one *C. elegans cyp, cyp-44A1,* belongs to the mitochondrial clan. The same CYP clans are found also in other protostomes including insects (Feyereisen, 2006) supporting the theory that these CYP clans

diverged before the lines leading to nematodes and human (protostomes and deuterostomes) diverged about 670 million years ago (Ayala et al., 1998).

CYPs are required in several important physiological processes. The silencing of single *cyp* genes with RNAi causes alterations in survival, morphology, embryonic development, growth, larval development, dauer formation, life span, reproduction, movement, pharyngeal pumping, lawn leaving behavior, fat content, lipid composition and metabolism (Albert and Riddle, 1988; Jia et al., 2002; Piano et al., 2002; Ashrafi et al., 2003; Kamath et al., 2003; Murphy et al., 2003; Simmer et al., 2003; Rual et al., 2004; Sönnichsen et al., 2005; Kraemer et al., 2006; Ruaud and Bessereau, 2006; Ceron et al., 2007; Cui et al., 2007; Kim and Sun, 2007; Samuelson et al., 2007; Kinchen et al., 2008; Kleemann et al., 2008; Benenati et al., 2009; Coolon et al., 2009; Hannich et al., 2009; Schäfer et al., 2009; Sharma et al., 2009; Jensen et al, 2010; Jeong et al., 2010; Karp and Ambros, 2011; Kashyap et al., 2012). For example, the silencing of the *cyp* genes 31A2 and 31A3 leads to failures in meiosis and embryonic development and they synthesize lipids that are essential for correct formation of eggshell (Benenati et al., 2009). In addition, CYP-31A2 is required in sperm motility (Kubagawa et al., 2006) and it negatively regulates the synthesis of prostaglandins (Hoang et al., 2013). CYPs are also involved in aging as several *cyp* genes are upregulated in dauer larvae and in long-lived *daf-2* mutants (McElwee et al., 2004).

One *C. elegans* CYP is known to be steroidogenic: CYP-22A1 a.k.a. DAF-9. It makes cholesterol-derived compounds such as dafachronic acids (Motola et al., 2006) and cholestenoic acids (Held et al., 2006) that act as ligands of the the nuclear hormone receptor DAF-12. CYP-22A1/DAF-9 plays in several pathways controlling e.g. dauer formation (Albert and Riddle, 1988), life span and gonadal migration (Gerisch et al., 2001; Jia et al., 2002).

Some *C. elegans* CYPs also synthesize fatty acid-derived signaling molecules (Kulas et al., 2008; Kosel et al., 2011). Especially CYP-29A3 and CYP-33E2 related to mammalian CYP2 and CYP2 families, respectively, metabolize the fatty acids eicosapentaenoic acid and arachidonic acid into hydroxyl and epoxy derivatives.

Various xenobiotics induce the expression of different *C. elegans cyp* genes (Menzel et al., 2001, 2005, 2007; Reichert and Menzel, 2005; Roh et al., 2006, 2007, 2010; Chakrapani et al., 2008; Hasegawa et al., 2008; Roh and Choi, 2011; Wren et al., 2011; Laing et al., 2012; Anbalagan et al., 2013; Peltonen et al., 2013) and the induction pattern is sometimes similar between the closest CYP homologs in *C. elegans* and human (Chakrapani et al., 2008). *C. elegans* CYPs have also been shown to metabolize xenobiotics such as PCB52 (Schäfer et al., 2009). It appears that CYPs can both eliminate toxins and mediate their toxic effects, as the silencing of *cyp* genes sometimes decreases and sometimes increases the sensitivity to toxic compounds (Menzel et al., 2005, 2007; Ruaud and Bessereau, 2006; Cui et al, 2007; Kim and Sun, 2007; Ayyadevara et al., 2009; Park et al., 2009; Leung et al., 2010; Roh et al., 2010; Roh and Choi, 2011).

2.2.4.1 *C. elegans* CYP-35A subfamily

Members of the *C. elegans cyp-35A* subfamily are induced by a wide range of xenobiotics (Menzel et al., 2001, 2005, 2007; Kwon et al., 2004; Reichert and Menzel, 2005; Roh et al. 2006, 2007, 2010; Chakrapani et al., 2008; Roh and Choi, 2011; Peltonen et al., 2013) suggesting that they are involved in xenobiotic response. The CYP-35A family members may also be targets for the toxicity of PCB52 that induces them as the RNAi knockdown of *cyp-35A1*, *cyp-35A3*, *cyp-35A5*, and *cyp-35C1* in a *cyp-35A2*/*cyp-35A4* double mutant background makes the nematodes more resistant to its reproductive toxicity (Menzel et al., 2005). Also the silencing of *cyp-35A2* makes the nematodes more resistant to the toxicity of fenitrothion (Roh and Choi, 2011) or nanoparticles (Roh et al., 2010). Although members of the *cyp-35A* subfamily have putative XRE sites in their 5′ flanking sequences they are are not regulated by AHR-1 as they are expressed and induced by xenobiotics in *ahr-1(ju145)* mutants in similar levels as in wild-type nematodes (Menzel et al., 2005).

RNAi studies indicate that *cyp-35A2*, *3*, *4* and *5* are required for normal fat accumulation (Ashrafi et al., 2003; Menzel et al., 2007). Moreover, PCB increases fat accumulation in wild-type but not in *cyp-35A* subfamily mutants and this may be one of the mechanisms of its toxicity (Menzel et al., 2007). The *cyp-35A2* and *cyp-35A3* genes are expressed in the intestine which is a major location of fat stores. *cyp-35A2*, *cyp-35A3* and *cyp-35A4* may also play a part in dauer formation pathways as their silencing with RNAi leads to increased dauer formation (Jensen et al., 2010) and they are under-expressed in dauer-constitutive *daf-2* mutants (McElwee et al., 2003; Murphy et al., 2003).

2.3 *CAENORHABDITIS ELEGANS* (*C. ELEGANS*)

Caenorhabditis elegans (*C. elegans*) is an about 1 mm long nematode widely used in research. Genome comparisons show that molecular and cellular pathways found in *C. elegans* are generally strongly conserved in higher organisms including human (Shaye and Greenwald, 2011). As a simple multicellular organism, *C. elegans* is especially well-suited to the study of gene functions and molecular and cellular pathways involving intercellular interactions of multiple cells. The nematode is also partially transparent which makes it good for live imaging experiments. In this thesis, *C. elegans* has been used in the study of gene function in e.g. the metabolism of fatty acids. Fat metabolism in *C. elegans* resembles that in mammals with some exceptions. The following subsections will review *C. elegans* as a research model especially in genetics, as well as lipid homeostasis and a class of lipid signaling molecules called endocannabinoids in the nematode.

2.3.1 Basic biology of *C. elegans*

C. elegans consists of differentiated organs and tissues such as cuticle, hypodermis, muscles, nervous system, gonad and intestine. It is not parasitic like many other nematodes but naturally lives free in the soil in many parts of the world and feeds on micro-organisms. In the laboratory *C. elegans* is usually grown in room temperature on nematode growth medium agar plates and fed with bacteria. One commonly used food source is the OP50 strain of *Escherichia coli* (*E. coli*) that is auxotrophic for uracil and grows only in thin layers on nematode growth medium allowing the nematodes to grow, move, mate and be observed easily. Larger amounts of nematodes can be grown on plates or liquid media containing more nutrients and better growing bacteria such as the NA22 strain of *E. coli*. *C. elegans* also survives freezing at -70°C and can thus be stored for later use. As an invertebrate, it typically does not require legal permits for its use in research.

There are two sexes in *C. elegans*: hermaphrodite with sex chromosomes XX and male with one X chromosome. Hermaphrodites produce both oocytes and sperm and fertilize themselves but not each other. In self-fertilization, hermaphrodite progeny are about 500 times more frequent than males that arise spontaneously through an X-chromosome nondisjunction during meiosis. However, there are "high incidence of males" (*him*) mutant strains and the frequency of males can also be increased by incubating hermaphrodites at 30°C for 6 h and then allowing them to self-fertilize at 20°C. When males are mated with hermaphrodites the oocytes are preferentially fertilized with the male's sperm and an equal amount of hermaphrodite and male progeny are produced. *C. elegans* clones established in the laboratory are genetically uniform (Brenner, 1974). Self-fertilization also enables the reproduction of mutants with severe behavioral or morphological defects that prevent mating.

The hermaphrodite lays eggs that hatch and develop through four larval stages, L1 to L4, each of which ends with molting. Each individual goes through the same cell divisions and apoptoses during embryonic and larval development finally resulting in 959 somatic cells in the adult hermaphrodite and 1031 in the adult male (Sulston et al., 1983). In case of limited food source, high population density, or high temperature the L2 larva may develop into an additional long-living larval stage, i.e. dauer larva, in which the living

functions are slowed down. The dauer larva can again develop into an L4 larva and adult. Normally the cycle from a newly laid egg to fertile adulthood takes about 2.5 days at 20°C and the development rate is highly dependent on temperature. One hermaphrodite has about 300 progeny through self-fertilization during its fertile period that last about 4 days. If the hermaphrodite mates with males the total number of progeny may be more than 1000. The entire life span of *C. elegans* is about 18 to 20 days at 20°C. The short reproductive cycle and life span make *C. elegans* well suited for studies that require assessment over an entire life span or several generations.

2.3.2 *C. elegans* genetics, genomics and transcriptomics

The use of *C. elegans* as a research organism gained momentum in the 1960's as Sydney Brenner saw its potential as a small, simple, rapidly propagating yet multicellular model organism for studying cellular development. He described its culturing and genetics methods in his classical article (Brenner, 1974) in which also about 100 genes that affect behavior or morphology were characterized.

In the traditional genetic experiments in *C. elegans*, gene function is studied by identifying and isolating loss-of-function mutants (reviewed by Kutscher and Shamam, 2014). In a forward genetics approach, mutations are induced randomly to the genome and phenotypes are scored before detecting the mutations causing them. Random mutations can be induced using a chemical mutagen, irradiation or insertion of transposons. In reverse genetics, mutations are identified before proceeding to phenotype assays. This approach can also start with random mutagenesis followed by screening for mutations e.g. with PCR. Targeted knockout of specific genes was initially not possible in *C. elegans* because homologous recombination via DNA injection to oocyte nuclei widely used in mammals is ineffective in the nematode. However, there are recently developed techniques for targeted gene deletion such as MosDEL (Frøkjaer-Jensen et al., 2010) and MosTIC (Robert and Bessereau, 2007) that utilize transposons, as well as systems based on zinc finger- or transcription activator-like effector nucleases (Wood et al., 2011) or the bacterial CRISPR-Cas9 endonuclease (Friedland et al., 2013). Also a technique using bombardment with DNA-coated gold particles (Berezikov et al., 2004) has been developed but not widely used. Mutant strains can be purchased from the Caenorhabditis Genetics Center.

A very feasible technique to silence specific target genes in *C. elegans* is RNA interference (RNAi) in which double-stranded RNA (dsRNA) leads to degradation of a target mRNA or blocks its translation (Fire et al., 1998). RNAi is systemic in *C. elegans* and can be induced by injecting, soaking or feeding the dsRNA to the nematodes (Tabara et al., 1998; Timmons and Fire, 1998). There are dsRNA libraries that represent a large fraction of all *C. elegans* genes and RNAi screens have been performed genome-wide to detect genes involved in specific phenotypes (reviewed by Lee et al., 2004; Kaletta and Hengartner, 2006). The disadvantage of RNAi is that it does not always silence the gene completely and some cell types, especially neurons, are resistant to it (Timmons et al., 2001).

Another way to study gene function is transgenesis that is relatively easy in *C. elegans*. Transgene expression can be directed to cells of interest using cell type-specific promoters. The traditional method is to inject the transgene to the gonad in a vector such as a plasmid (Stinchcomb et al., 1985; reviewed by Kadandale et al., 2009; Rieckher et al., 2009). To easily detect transgenenic animals, the transgene is often co-injected with a marker gene such as *rol-6* that causes abnormal movement or production of green fluorescent protein (GFP). The injected DNA is taken into oocyte nuclei where it replicates independently as an extrachromosomal array that is transmitted unevenly to progeny (Mello et al., 1991). To produce stable Mendelian inheritance of the transgene it has to be integrated into the genome by e.g. gamma or X ray irradiation. There are also new techniques that produce integrated transgenic lines that utilize microparticle bombardment (Praitis et al., 2001), transposons (Frøkjaer-Jensen et al., 2008) or the CRISPR-Cas9 system (Chen et al., 2013; Dickinson et al., 2013). Transgenesis can be used e.g. to model diseases by over-expressing a

disease-related gene (Kaletta and Hengartner, 2006), to rescue the function of a mutated gene in specific cells or to visualize the anatomic expression pattern of a gene of interest by combining its promoter to GFP (Chalfie et al., 1994; Dupuy et al., 2004).

C. elegans was the first multicellular organism with its entire genome sequenced (The *C. elegans* Sequencing Consortium, 1998). The relatively small genome of about 100 Mb is organized in six chromosomes including the X chromosome. *C. elegans* is predicted to have about 20500 protein-coding genes (WormBase, 2011). For comparison, the human genome is many times larger in size (3000 Mb) but the number of protein-coding genes (20700) is close to that in *C. elegans* (The ENCODE Project Consortium, 2012). However, the human genes produce a larger variety of mRNAs and proteins. Human orthologs have been identified for ~38 % of *C. elegans* genes (Shaye and Greenwald, 2011). Estimates about the proportion of human genes that have a *C. elegans* ortholog vary from 60 to 80 % but are not up to date (reviewed by Kaletta and Hengartner, 2006). An unusual feature of nematode genomes is that they contain polycistronic clusters of genes that are expressed under the control of one regulatory signal, resembling bacterial operons (Blumenthal and Gleason, 2003). About 15 % of *C. elegans* genes are estimated to be in such polycistronic clusters but these are typically genes that do not need to be regulated individually.

C. elegans has also been used in transcriptomics (Reisner et al., 2005). The early completion of the *C. elegans* genome project allowed the production of *C. elegans* gene expression profiling microarrays. Also other methods such as serial analysis of gene expression (SAGE) and more recently RNA-seq have been used. The main limitation of *C. elegans* in transcriptomics is that because of its small size, cell- or tissue-specific gene expression is difficult to assess. Typically, RNA for the experiment is derived from populations of whole animals. Specific cell types can be harvested by expressing GFP in them and subsequently isolating them with fluorescence-activated cell sorting (Christensen et al., 2002). Another technique is to co-immunoprecipitate poly(A)-RNA with a poly(A) binding protein expressed in specific cells (Roy et al., 2002). The use of single cells requires RNA amplification for which sophisticated methods have been developed (Hashimshony et al., 2012).

2.3.3 Lipid storage and fatty acids in *C. elegans*

C. elegans can also be used as a model to study lipids. It differs from mammals on a cellular level in that it does not have adipocytes. Instead, fat is stored as triacylglyceroles in intestinal lysosome-related organelles (Hermann et al., 2005) and in lipid droplets found in intestinal and epidermal skin-like cells (O'Rourke et al., 2009). Fat stores can be visualized with different methods such as Sudan black (Kimura et al., 1997, Ogg et al., 1997), Nile Red (Ashrafi et al., 2003; McKay et al., 2003), Oil-Red-O (O'Rourke et al., 2009; Soukas et al., 2009), boron-dipyrromethene (BODIPY) fluorescently labeled fatty acids (Mak et al., 2006; Klapper et al., 2011) or coherent anti-Stokes Raman scattering (CARS) microscopy (Hellerer et al., 2007).

The fat stores are dynamically regulated and major regulatory pathways are conserved between *C. elegans* and human (reviewed by Jones and Ashrafi, 2009; Mullaney and Ashrafi, 2009). Fatty acids are taken up into the stores by diffusion and active transport, released by lipases and used for energy in a series of β-oxidation reactions (Zhang et al., 2010). An RNAi screen by Ashrafi et al. (2003) identified hundreds of genes that increase or reduce fat content in *C. elegans*. *C. elegans* responds to environmental signals such as availability of food. Neuroendocrine signaling systems regulate both behaviors and peripheral lipid metabolism in response to sensory cues. Such neuroendocrine systems include e.g. insulin (Kimura et al.; 1997, Ogg et al., 1997), serotonin (Srinivasan et al., 2008), TGF-β (Greer et al., 2008) and Tubby signaling (Mak et al., 2006). Other factors function directly at the sites of fat storage and can sometimes feed back to the nervous system. These include e.g. several transcription factors that regulate the expression of lipid metabolism genes (reviewed by Brey et al., 2009).

Several classes of lipids contain fatty acids. Fatty acids vary in length and in the number and position of double bonds. In fatty acid nomenclature (Davidson and Cantrill, 1985), the first number refers to the number of carbons and the second to the number of double bonds. Saturated fatty acids do not contain double bonds, monounsaturated fatty acids contain one and polyunsaturated fatty acids (PUFAs) more than one double bond. The denotion "n3" means that starting from the methyl end of the fatty acid chain the closest double bond is between the 3rd and 4th carbons, whereas in "n6" fatty acids it is between the 6th and 7th carbons. n3 can also be written as ω-3 (omega-3).

C. elegans takes up fatty acids from diet and synthesizes them *de novo* (Perez and Van Gilst, 2008). According to Zhang et al. (2010), the food source OP50 *E. coli* contains mainly palmitic (16:0) and stearic (18:0) acids. Palmitic acid can also be synthesized *de novo* from acetyl-CoA by acetyl-CoA carboxylase and fatty acid synthase (Rappleye et al., 2003). Unlike humans, *C. elegans* can create the long-chain PUFAs arachidonic acid (20:4n6) and eicosapentaenoic acid (20:5n3) from palmitic acid by modifying the fatty acids with desaturase and elongase enzymes that are sequentially named FAT-1 to 7 and ELO-1 to 8, respectively (Watts and Browse, 2002). **Figure 1** does not show all fatty acids in *C. elegans* but focuses on the steps required for the generation of arachidonic (20:4n6) and eicosapentaenoic (20:5n3) acids.

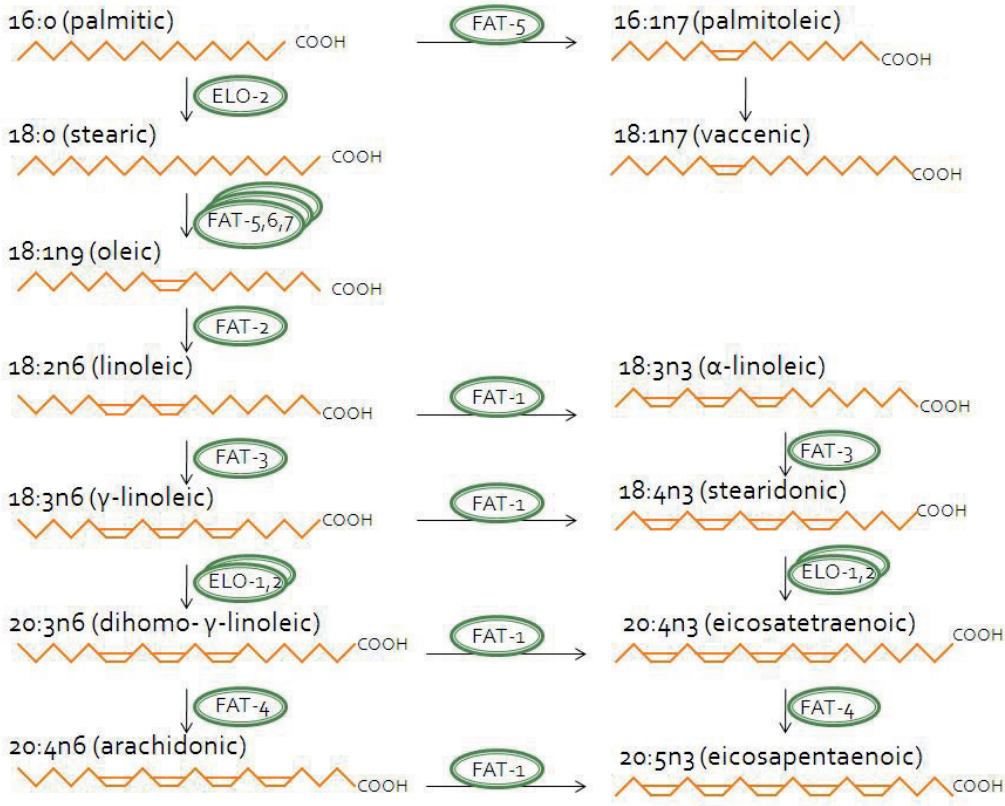

Figure 1. Synthesis of long-chain polyunsaturated fatty acids from fatty acid precursors by fatty acid desaturase and elongase enzymes in *C. elegans* (modified from Watts and Browse, 2002)

Palmitic acid (16:0) is elongated by ELO-2 to obtain stearic acid (18:0). The *elo-2* gene expression is required for e.g. normal growth, reproduction and biological rhythms (Kniazeva et al., 2003). Palmitic acid (16:0) can also be desaturated into palmitoleic acid (16:1n7) by FAT-5 (Watts and Browse, 2000). Stearic acid (18:0) is desaturated into oleic acid

(18:1n9) by the desaturases FAT-5, FAT-6 and FAT-7 that are functionally redundant. While single mutations of *fat-5*, *fat-6* and *fat-7* genes appear normal with only subtle alterations in fatty acid composition, the triple mutation is lethal (Brock et al., 2006).

Linoleic acid (18:2n6) is a precursor for n6 PUFAs and α-linoleic acid (18:3n3) for n3 PUFAs. While humans need to obtain these fatty acids from diet, *C. elegans* can synthesize them. Linoleic acid (18:2n6) is synthesized from oleic acid (18:2n6) by the desaturase FAT-2, and α-linoleic acid (18:3n6) from linoleic acid (18:2n6) by FAT-1. FAT-1 can also synthesize other n3 PUFAs from n6 percursors. *fat-2* mutants display slow growth, abnormal body shape, sluggish movement, cuticle defects and reduced brood sizes. *fat-1* mutants lack n3 PUFAs but are apparently normal in growth, morphology, reproduction and movement. The *C. elegans fat-1* gene has been transgenically expressed in mice, cattle and pig (Kang et al., 2004; Guo et al., 2011; Zhang et al., 2012). The decreased n6/n3 fatty acid ratio in the transgenic mice has been shown to e.g. have anti-inflammatory effects and protect against tumorigenesis and pathological angiogenesis (reviewed by Kang, 2007).

Linoleic (18:2n6) and α-linoleic (18:3n3) acids are further desaturated into γ-linoleic (18:3n6) and stearidonic (18:4n3) acids, respectively, by the desaturase FAT-3 (Watts and Browse, 2002). *fat-3* mutation causes embryonic lethality, abnormal morphology, small brood sizes and slow growth and movement (Watts et al., 2003). The γ-linoleic (18:3n6) and stearidonic (18:4n3) acids are elongated to dihomo-γ-linoleic (20:3n6) and eicosatetraenoic (20:4n3) acids, respectively, by the elongase ELO-1 and possibly also ELO-2. While *elo-1* mutants appear normal, suppression of *elo-2* in the *elo-1* mutants causes nearly total depletion of 20-carbon polyunsaturated fatty acids, abnormal body morphology, small brood sizes and death of some progeny in the first larval stage (Kniazeva et al., 2003). The final step of arachidonic and eicosapentaenoic acid synthesis is catalyzed by FAT-4. *fat-4* mutants appear normal despite the lack of these fatty acids. *fat-1*; *fat-4* double mutants have small abnormalities such as defects in TRPV receptor-mediated olfaction (Kahn-Kirby et al., 2004) or sensitivity to dihomo-γ-linoleic acid-induced sterility (Watts and Browse, 2006). Unlike human, *C. elegans* cannot synthesize 22-carbon or longer fatty acids from 20:4n6 and 20:5n3 (Watts and Browse, 2002).

In addition, *C. elegans* synthesizes other kinds of fatty acids such as monomethyl branched fatty acids that are important for its larval development (Kniazeva et al., 2004). The fatty acid desaturase and elongase genes are regulated by several transcription factors such as the nuclear hormone receptor NHR-49 (Van Gilst et al., 2005a, b) that functions together with the Mediator subunit MDT-15 (Taubert et al., 2006), as well as by NHR-80 (Brock et al., 2006) or the sterol regulatory element binding protein SBP-1 (Kamath et al., 2003; Kniazeva et al., 2008) some of which control also other aspects of lipid homeostasis.

Defects seen in the nematodes lacking fatty acid desaturase or elongase enzymes show the importance of the different fatty acids. Proportions of different fatty acids in membrane phospholipids affect the structure of the membranes and several cellular functions (reviewed by Spector and Yorek, 1985; Ibarguren et al., 2014). The normal appearance of *fat-1* and *fat-4* mutants at least in standard laboratory conditions is somewhat surprising considering the importance of arachidonic acid and n3 fatty acids in mammals.

2.3.4 Endocannabinoid system in *C. elegans*

The main psychoactive compound in cannabis, Δ(9)-tetrahydrocannabinol, acts via CB1-type cannabinoid receptors in the mammalian brain (Devane et al., 1988; Matsuda et al., 1990). Endogenous ligands for these receptors were named endocannabinoids. The first found endocannabinoids were N-arachidonoylethanolamine (a.k.a. anandamide or AEA, Devane et al., 1992) and 2-arachidonoylglycerol (2-AG, Mechoulam et al., 1995). The endocannabinoid system consists of the endocannabinoids themselves, their receptors and enzymes for their synthesis and degradation as well as other factors regulating their actions or transport. *C. elegans* has endocannabinoids but other components of its endocannabinoid system are not yet well defined.

Endocannabinoids have a fatty acyl group linked to another chemical group that depends on the type of the endocannabinoid. The chemical group can be an ethanolamine in N- or O-acylethanolamines (NAEs and OAEs, Hanus et al., 1993; Porter et al., 2002; Hansen and Diep, 2009), glycerol in monoacylglycerols (Hanus et al., 2001) or dopamine in N-acyldopamines (Huang et al., 2002; Chu et al., 2003). AEA is a NAE and 2-AG a monoacylglycerol, and both have their fatty acyl moieties derived from arachidonic acid. There are likely also many other types of endocannabinoids that have not yet been identified. In *C. elegans*, several endocannabinoids have been identified including AEA, 2-AG, N-palmitoleoyl ethanolamine, N-eicosapentaenoyl ethanolamine, N-lineoyl ethanolamine, N-oleoyl ethanolamine and N-palmitoyl ethanolamine (Lehtonen et al., 2008, 2011; Lucanic et al., 2011).

In mammals, endocannabinoids are derived from membrane phospholipids through several routes. The most studied NAE synthesis pathway involves a hydrolysis by N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD, Okamoto et al., 2004) but there are also other NAE synthesis pathways not dependent on NAPE-PLD (reviewed by Hansen and Diep, 2009). The *C. elegans* homolog of NAPE-PLD named NAPE-1 is not necessary for the synthesis of NAEs (Lucanic et al., 2011) but this may be due to the presence of alternative NAE synthesis routes as in mammals (Leung et al., 2006). In mammals 2-AG is made by diacylglycerol lipase (DAGL, Bisogno et al., 2003). Its homolog in *C. elegans* is F42G9.6 whose function is not known (McPartland et al., 2006).

AEA and other NAE-type endocannabinoids are catabolized mainly by fatty acid amide hydrolase (FAAH, Schmid et al., 1985; Cravatt et al., 1996). The *C. elegans* FAAH ortholog FAAH-1 appears to degrade NAEs since its silencing elevates and transgenic over-expression reduces NAE levels (Lucanic et al., 2011). In mammals, 2-AG is mainly catabolized by monoacylglycerol lipase (MAGL, Dinh et al., 2002) although other enzymes such as cyclo-oxygenase 2 (COX2, Yu et al., 1997a), lipoxygenases and CYPs have also been shown to metabolize endocannabinoids (reviewed by Ueda et al., 2010). The closest homologs for MAGL and COX2 are F01D5.7 and C46A5.4, respectively, but it is not known whether they degrade 2-AG or not (McPartland et al., 2006). It is also possible that *C. elegans* CYPs metabolize endocannabinoids.

Mammalian receptors for endocannabinoids include the CB1, CB2 (Munro et al., 1993), the transient receptor potential vanilloid type 1 (TRPV1, Zygmunt et al., 1999), the G protein-coupled receptors GPR-55 (Baker et al., 2005) and GPR-119 (Overton et al., 2006) and peroxisome proliferator-activated receptors (PPARs) of subtypes α and γ (Fu et al., 2003; Bouaboula et al., 2005; Lo Verme et al., 2005; Rockwell et al., 2006). *C. elegans* has proteins with some homology to the CB1/CB2, TRPV1 and GPR-55 receptors (NPR-19, OSM-9 and NPR-9, respectively) but they are not very likely to function as cannabinoid receptors (McPartland et al., 2006). The CB1 homolog NPR-19 lacks many of the amino acid residues required for ligand binding or signal transduction (McPartland and Glass, 2001, 2003). The *C. elegans* homolog of TRPV1, OSM-9, appears insensitive to the exogenous TRPV1 ligand capsaicin (Tobin et al., 2002). Jordt and Julius (2002) identified amino acid residues that determine the sensitivity of the TRPV1 receptor to both capsaicin and AEA. The *C. elegans* OSM-9 has a substitution at T550 amino acid residue as do some other capsaicin-insensitive TRPV receptors (McPartland et al., 2006) suggesting that OSM-9 is not likely activated by AEA.

Although endocannabinoid receptors have not been identified in *C. elegans*, endocannabinoids do have functions in the nematode. Lucanic et al. (2011) demonstrated with a series of experiments that NAE-type endocannabinoids mediate the signal of nutrient availability which prevents dauer formation and dietary restriction-related lifespan extension in *C. elegans*. First they showed that NAEs are required for normal growth rate. In the presence of abundant food NAEs reach their highest levels in L2 stage during which the nematode commits to reproductive development instead of entering into dauer stage. In dauer-constitutive *daf-2* mutants, NAE levels are lower than in wild-type after the decision

to enter into dauer stage. Treatment with the major NAE, N-eicosapentaenoylethanolamine, prevents dauer formation in *daf-2* and other dauer-constitutive mutants. Moreover, NAEs are also low in starved animals and rise after refeeding. Dietary restriction increases lifespan (Klass, 1977), and the lowering of NAE levels by transgenic over-expression of *faah-1* has a similar effect. The lowering of NAE levels does not have an additive effect on lifespan in dietary restriction conditions suggesting that NAE reduction and dietary restriction are mechanistically equivalent. Correspondingly, EPEA treatment dramatically reduces the lifespan extension in dietary restriction. Another, different function of AEA is that it inhibits axon regeneration in mature *C. elegans* neurons through the Goα receptor subunit GOA-1 (Pastuhov et al., 2012).

The endocannabinoid system is related to energy metabolism also in mammals. Over-activity of the endocannabinoid system has been associated with obesity by acting through the cannabinoid receptor CB1 in several tissues such as brain, adipose tissue, liver, pancreas and skeletal muscle (Matias and Di Marzo, 2007; Maccarrone et al., 2010). CB1 antagonists alleviate obesity and improve the plasma lipid profile and insulin sensitivity in obese mice and rats (Cota et al., 2009; Jourdan et al., 2010). In mammals the endocannabinoid system has also many other functions playing a modulatory role in e.g. immune response, cognition, emotion, perception, behavioral reinforcement, motor coordination, body temperature, wake/sleep cycle, bone formation and resorption, and various aspects of hormonal control, pain, inflammation and reproductive functions (Wang et al., 2006; Fine and Rosenfeld, 2013; Robson, 2013).

2.4 TRANSCRIPTOMICS

The central dogma of molecular biology is that DNA directs the synthesis of RNA which then directs the assembly of proteins. Proteins present in a particular cell type determine its structure and functions by serving as structural proteins or enzymes that catalyze the synthesis of other classes of biological molecules. The process of decoding the genetic information into a functional gene product is called gene expression.

Gene expression must be regulated to determine which proteins are found in each cell at a given time point. This regulation generates the variety of cell types with different appearences and functions in an individual with the same genome in all its cells and makes it adaptable to environmental signals. Gene expression is regulated in several steps, such as transcription, mRNA processing and stability, translation and protein modification.

As genome sequences are known for more and more organisms, current research focuses on understanding the dynamic functions of the genomes. One approach is transcriptomics i.e. the study of transcriptomes that can also be called gene expression profiling. Transcriptome refers to the complete set of transcripts and their quantity in a cell for a specific developmental stage and physiological condition (Wang et al., 2009). Gene expression profiling experiments typically compare trancriptomes between e.g. different cell types or developmental stages, or study the effect of a disease or treatment, while advanced methods also allow the finding of new transcripts.

The transcriptome is greatly influenced by factors that regulate transcription initiation, including also AHR. The following subsections give a quick overview on the regulation of transcription initiation, as well as on some of the methods used in transcriptomics and initiatives to facilitate the sharing of datasets generated by the methods.

2.4.1 Regulation of transcription initiation

Transcription of eukaryotic protein-coding genes is catalyzed by RNA polymerase II. Other factors necessary for transcription initiation include general transcription factors such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH some of which consist of several subunits, and the Mediator complex. These proteins form a transcription preinitiation complex and bind co-operatively to DNA regions called core promoters located near the transcription start

site (Maston et al., 2006). General transcription factors have many functions including promoter recognition, interaction with regulatory factors, DNA unwinding and RNA polymerase II regulation (Sikorski and Buratowski, 2009; Grünberg and Hahn, 2013).

The general transcription machinery drives only low levels of gene expression. Transcription is greatly activated or repressed by another class of transcription factors that bind to specific DNA sequences further away from the transcription start site (Maston et al., 2006). The human genome is predicted to have over a thousand such transcription factors and they can be classified based on the structure of their DNA binding domains (Vaquerizas et al., 2009). For example, AHR belongs to the bHLH class. Transcription factor binding sites in DNA are short (~6-12 bp) consensus sequences. Genes have regulatory elements that contain binding sites for several transcription factors acting synergistically (Maston et al., 2006). Regions up to a few hundred bp upstream of the core promoter are called proximal promoter elements, whereas enhancers and silencers can be located tens of kilobases upstream or downstream of the transcription start site and be brought close to the core promoter by DNA looping.

The specific transcription factors can recruit coactivators and corepressors that participate in the regulation of transcription without binding to DNA sequence-specifically (Maston et al., 2006). Several coactivators and corepressors can modify the structure of chromatin i.e. the complex formed of DNA and proteins, primarily histones. Some coactivators and corepressors covalently add chemical groups to histones or remove them (reviewed by Rosenfeld et al., 2006). Histone modifications include acetylations, phosphorylations, methylations, ubiquitylations, ribosylations and sumoylations. The activating or repressing function of these modifications depends on the chemical group, the amino acid residue modified and the context of other modifications (Berger, 2007). Histone modifications can directly alter chromatin structure or recruit effector proteins. Another class of coactivators are ATP-dependent chromatin remodelers that disassociate stretches of DNA from histones in various ways exposing binding sites for proteins of the transcription machinery (Clapier and Cairns, 2009; Tang et al., 2010). Activator transcription factors and coactivators can also facilitate the formation of the preinitiation complex and subsequent steps in transcription (reviewed by Lee and Young, 2000).

Repressor transcription factors can inhibit activation in many ways (Latchman, 1996; Lee and Young, 2000). They can block the binding of activator transcription factors by competing for binding sites, by binding to the activator transcription factor itself or by recruiting corepressors that modify chromatin. Repressor transcription factors may also interact with DNA-bound activator transcription factors inhibiting their function. They can also inhibit the assembly or activation of the preinitiation complex by binding directly to its components or upstream silencer elements in DNA. One transcription factor can have both activator and repressor roles depending on the presence of other factors.

While some transcription factors are constitutively active or regulated only on the level of their own expression, other transcription factors are post-translationally activated by a signal (Brivanlou and Darnell, 2002). The signal can be e.g. an extracellular ligand such as a steroid hormone or another lipid-soluble compound that enters the cell. Mammalian AHR is an example of a transcription factor activated this way. For some transcription factors, the activating ligand is intracellular. Also peptide hormones binding to cell surface receptors can activate transcription factors via intracellular signal transduction cascades. In addition, signal transduction pathways can regulate the composition and function of coactivator complexes (Rosenfeld et al., 2006).

2.4.2 Transcriptomics methods

The field of transcriptomics utilizes high-throughput methods that can measure thousands of transcripts simultaneously rather than a gene-by-gene approach. In the 1980's the first methods assaying large numbers of mRNA species included differential hybridization (reviewed by Liang and Pardee, 2003) and subtractive hybridization (Zimmermann et al.,

1980). These methods were followed by differential display PCR (Liang and Pardee, 1992) and serial analysis of gene expression (SAGE, Velculescu et al., 1995). Microarray technology started becoming popular in gene expression profiling in the late 1990's likely because it is very high-throughput, simple for the end-user and cheaper than the other methods. Newer methods based on sequencing have been developed, including massively parallel signature sequencing (MPSS, Brenner et al., 2000), cap analysis of gene expression (CAGE, Shiraki et al., 2003) and newer variants of SAGE (Saha et al., 2002; Gowda et al., 2004; Matsumura et al., 2005). However, only the rather recent method high throughput RNA sequencing (RNA-seq) that utilizes next-generation sequencing technologies is challenging microarrays (Wang et al., 2009). Microarrays and RNA-seq are overviewed in the following subsections.

2.4.2.1 Microarrays in transcriptomics

Gene expression profiling microarrays consist of a series of DNA targets immobilized onto a solid surface in an ordered fashion so that the location of each target can be referenced. Their use in transcriptomics is based on hybridization of labeled complementary DNA or RNA probes derived from a sample.

There are many kinds of microarrays depending on the manufacturer. In the early DNA microarrays (Schena et al., 1995, 1996), the targets were hundreds of nucleotides long cDNA fragments spotted onto the surface. Several research groups and microarray core facilities have made their own custom microarrays this way. Another type of microarray consists of oligonucleotides (25-60 nt) that can be synthesized directly onto the platform, allowing a higher target density (Lockhart et al., 1996). In a newer technology, oligonucleotide targets are attached to beads that then assemble into arrays (Kuhn et al., 2004). The two latter types of microarrays can only be obtained from commercial sources.

In a microarray experiment, the RNA molecules isolated from a tissue of interest are converted to fluorescence-labeled cDNA or complementary RNA probes. The probes hybridize specifically to their complementary targets on the array and are detected by laser confocal microscopy. Signal intesities on the array are assumed proportional to the abundance of the corresponding RNA species in the original sample (Mirnics and Pevsner, 2004). The signals are quantified, filtered and normalized to remove non-biological variation between and within arrays (Wolkenhauer et al., 2002; Saviozzi and Calogero, 2003). Differentially expressed genes between conditions are determined from the normalized values using relative expression levels and statistical tests.

Although DNA microarrays can assay a very large number of transcripts in a single experiment, they also have limitations. Firstly, they require knowledge on the sequence to be assayed. Obtaining genome sequences is getting easier as genome projects are being completed for more and more organisms. However, the reliance on existing sequence data makes it difficult to find new transcripts. For this kind of purposes there are tiling arrays in which areas of the genome are represented at a high density (Kapranov et al., 2002) but they are expensive and have only a limited ability to distinguish between different isoforms (Wang et al., 2009). Another limitation of DNA microarrays is that they do not measure absolute levels of expression and comparing expression levels between different experiments is problematic. In addition, there are technical issues such as high background noise and saturation of signal that prevent the detection of transcripts with very low or high abundances.

2.4.2.2 RNA-seq

RNA-seq is a trascriptomics application that uses next-generation sequencing technologies (Wang et al., 2009). Next-generation sequencing refers to recent sequencing technologies that are dramatically faster and more affordable than the traditional Sanger sequencing (Sanger et al., 1977). The techniques are variable and constantly developing. The most used technologies in RNA-seq have so far been Illumina, Roche/454 and SOLiD.

In an RNA-seq experiment, a library of cDNAs is generated from the isolated RNA and the cDNAs are then sequenced to obtain short sequence reads (Wang et al., 2009). The details of library preparation, sequencing and intermediate steps vary between the different technologies. In all current platforms, mRNA or the reverse-transcribed cDNA needs to be fragmented into smaller pieces (200-500 nt). Small non-coding RNAs do not need fragmentation. Typically adapter sequences are ligated to both ends of the cDNAs and there can also be amplification steps before sequencing. The cDNA fragments are sequenced from one or both ends and the reads obtained are 30-400 nt in length depending on the sequencing technology. In data analysis, the sequence reads are either aligned to an existing reference genome or assembled *de novo* to identify the transcripts and their abundancies.

RNA-seq has several advantages compared to microarrays (Wang et al., 2009). RNA-seq does not require prior knowledge about the transcripts to be sequenced. Even a reference genome is not necessary although it can be utilized in read assembly. RNA-seq reveals the precise boundaries of transcripts as well as single-base variations. Thus, novel transcripts such as different isoforms or entirely new RNA classes can be found. RNA-seq expresses the amounts of different transcripts as absolute values, not as relative amounts. In addition, RNA-seq can detect transcripts expressed in very low and very high levels due to low background noise and no saturation of signal. Also the amount of RNA sample required is smaller compared to microarray. The older sequencing-based methods SAGE, CAGE and MPSS differ from RNA-seq in that they analyze only a part of the transcript and cannot distinguish between all isoforms. RNA-seq does not have this limitation as the short sequence reads generated should span the whole length of the transcript. What also makes RNA-seq more popular than the older sequencing-based methods is the significantly lower price. The prices of microarrays and RNA-seq are comparable. At the moment RNA-seq is a bit cheaper than tiling arrays while more old-fashioned microarrays are still cheaper than RNA-seq.

RNA-seq also has some challenges (Wang et al., 2009). The manipulation steps during the cDNA library construction, such as fragmentation and amplification, can cause bias. The experiment produces massive amounts of data. In data analysis, reads that span exon junctions or contain polyA have to be taken into account.

2.4.3 Transcriptomics data sharing and standards

Transcriptomics experiments produce large amounts of data. Public databases were started in order to store microarray data and make it available to the whole research community in an appropriate and usable format that would allow new data comparisons and the analysis of existing results with new methods and tools. The most used gene expression databases are Gene Expression Omnibus (GEO, Edgar et al., 2002) by National Center for Biotechnology Information and ArrayExpress (Brazma et a., 2003) by the European Bioinformatics Institute. Both databases accept array- and sequencing-based data. Another major gene expression database, Center for Information Biology gene EXpression (CIBEX) by the DNA DataBank of Japan (Ikeo et al., 2003), is currently being migrated to a new system.

Since gene expression profiling data is dynamic and highly dependent on the conditions in which it was generated it is necessary to record detailed description of how the expression values were obtained. The Minimum Information About a Microarray Experiment (MIAME) document was developed by the Microarray Gene Expression Database Society (MGED, now called Functional Genomics Data Society, FGED) to provide a standard on what information to record and report (Brazma et al., 2001). Similar guidelines have been developed for high-throughput sequencing data: Minimum Information About a high-throughput SEQuencing Experiment (MINSEQE, Brazma, 2009). The essential information to be presented according to both MIAME and MINSEQE include the complete raw data, the final processed data, description of the samples and

experimental conditions, experimental design, and experimental and data processing protocols (Functional Genomics Data Society, 2014). Although the information can be in any format, standard data exchange formats have been developed to allow communication of MIAME-supportive data between different data systems. Currently FGED recommends the use of the standard format MAGE-TAB that is relatively simple and spreadsheet-based (Rayner et al., 2006). Also an MGED Ontology has been developed to provide a controlled vocabulary to describe a microarray experiment (Whetzel et al., 2006). The public repositories GEO, ArrayExpress and CIBEX are designed to accept, hold and distribute MIAME compliant data and several scientific journals (Functional Genomics Data Society, 2010) require submission of MIAME-compliant data to databases prior to publication.

3 Aims of the study

While the roles of AHR and CYPs in toxicology are well established the whole range of their physiological functions is not thoroughly understood. Most of the studies so far have been performed in mice and rats but the knowledge on the functions of AHR and CYPs in *C. elegans* is still very limited. Research in *C. elegans* can elucidate which functions of AHR and CYPs are evolutionarily conserved, or result in finding novel functions not yet studied in higher organisms. Progress in genomics has lead to the development of methods such as microarrays that enable measuring entire transcriptomes. Therefore, the general aims of the present study were to investigate the basic physiological roles of AHR and CYPs in *C. elegans* using a combination of phenotyping, fatty acid profiling and transcriptomics approaches, and follow the development of transcriptomics methods and their usage.

The more detailed aims of the study were to:

1. Derive gene expression profiling microarray usage statistics from neuroscience publications in order to determine current trends and infer future directions in the application of microarrays (**I**)

2. Study the physiological function of AHR in *C. elegans*, focusing on its possible roles in neuronally controlled behaviors, development, regulation of fatty acid diversity and control of gene expression (**II** and **IV**)

3. Study the physiological function of CYP-35A subfamily members in *C. elegans*, focusing on their possible roles in the regulation of lipid homeostasis and endocannabinoid levels (**III**)

4 Materials and methods

The methods used in the original publications are summarized in **Table 2**.

Table 2. List of methods used in the publications **I**-**IV**.

Method	**Original publication**
Systematic review on microarray studies	**I**
C. elegans strains and their growth	**II, III, IV**
Overall fatty acid compositions using Gas Chromatographic Mass Spectrometry	**II, III**
Gene expression using Quantitative Real-Time PCR (qRT-PCR)	**II, III, IV**
Phenotype assays (larval development, egg laying, embryonic lethality, movement, defecation, brood size)	**II**
Gene expression profiling using microarrays	**II**
Sequence alignments of human and *C. elegans* CYPs	**III**
Fat content using Oil-Red-O staining	**III**
Feeding of fatty acids in triglycerides to *C. elegans*	**III**
Endocannabinoid levels using Liquid Chromatographic Mass Spectrometry	**III**
Gene expression profiling using RNA-seq	**IV**
Search for putative XRE sites	**IV**

5 Results and discussion

5.1 MICROARRAY STUDIES IN THE NEUROSCIENCES

Microarrays were a relatively new and rapidly growing technology in 2004. The field of neuroscience had been thought to particularly benefit from it as nervous tissue is the most transcriptionally complex system and brain disorders involve interaction of many genetics factors (Mirnics and Pevsner, 2004). To determine overall usage statistics of microarrays in the neurosciences, data was collected on laboratories, source tissues, study designs, microarray platforms, analysis tools and availability of datasets from published literature.

5.1.1 Microarray field grew rapidly and was used in many types of studies

The literature search resulted in 2025 publications of which 502 fulfilled the criteria of being original gene expression profiling microarray studies on neural or glial material and published in peer-reviewed English language journals (**I, Table 1**). Of these, 448 could be accessed. The first five microarray studies in the neurosciences had been published in 1999 and the number of studies had increased every year being 283 in 2004 (**I, Figure 1A**). In the early years, the design and manufacture of microarray platforms for neuroscience applications had likely been limited by the lack of sequence data from higher organisms, while the completion of genome projects facilitated the growth of the field. The geographical distribution of publications was international although highly weighted towards the USA (**I, Figure 1B**).

The microarray articles represented different study types (**I, Table 2**). In many, disease had been studied using either animal models or post-mortem tissue from human patients. Fourty-four (44) different brain-related diseases had been studied using microarrays, most of them only in one or two studies (**I, Table 3**). The most studied disease was ischemic stroke (26 studies). Also age-related neurodegenerative diseases, mainly Alzheimer's disease and Parkinson's disease, had been studied in several publications. There were also many publications that could be classified as physiology studies. These had investigated the effect of e.g. genetic manipulation, endogenous compound or development. Studies on the effect of an exogenous compound, either toxic or beneficial, were also common. In addition, there were some publications that had studied the effect of mechanically or chemically induced injury, infection or non-chemical treatment, or compared brain areas or species. Some studies had to be classified in more than one category.

A quick search in PubMed using the same words as in Publication **I** indicates that the number of neuroscience studies utilizing microarrays appears to have still grown reaching the top value in 2011 after which it has slightly decreased (data not shown). The use of RNA-seq is constantly growing (data not shown). It is not straightforward to compare the numbers of publications between the two methods as the use of synonyms in the search greatly influences the results. If only the words "microarray" or "RNA-seq" are used microarray appears the more used method in neuroscience although the numbers have been getting closer every year since the first RNA-seq neuroscience publication in 2008 (data not shown). In the future there may be a shift in the field towards RNA-seq. The early neuroscience studies utilizing RNA-seq also tend to answer different scientific questions, such as finding novel genes and isoforms, rather than being "traditional" gene expression comparisons between conditions as microarray experiments typically are.

5.1.2 Source material

The coverage of different animal species in the studies was rather limited. Mouse and rat studies constituted more than two thirds of all studies (**I, Table 4**). Studies utilizing human

tissues or cell lines made up approximately one fifth of the studies. Non-human primates were used in 21 studies. There were also some rarer model organisms each used in 1 to 5 studies, including honey bee, fruit fly, Siberian hamster, cat, cattle, channel catfish, guinea pig, planaria and rabbit. The use of other model organisms has likely been limited by the lack of microarray platforms available. Microarrays for mouse, rat and human were readily available from several sources. Non-human primates were studied using human microarrays because of a high sequence similarity, but these animals are not grown specifically for laboratory use. Also the focus on neuroscience may explain why most of the species used were relatively large vertebrates.

No studies in *C. elegans* found in the literature search fulfilled the criteria of being microarray studies on neuronal material because the nematodes are usually assayed as whole animals instead of using only neurons. Microarrays have already long been available for *C. elegans*, and Reisner et al. (2005) review 26 *C. elegans* microarray studies published before 2005. A study by Link et al. (2003) was related to neuroscience as it involved an Alheimer's disease model. However, also in this publication the nematodes were profiled as whole animals. Isolation of sufficient amounts of RNA from *C. elegans* neural tissue requires isolation of cells, that is not easy, and amplification of RNA (or cDNA) that can cause bias to the results. However, cell type-specific gene expression can be measured using mRNA tagging (Roy et al., 2002) or primary cell cultures obtained from dissociated embryos with fluorescence-activated cell sorting (FACS, Christensen et al., 2002). These methods have been used after the time of the survey (**I**) to find genes whose expression is enriched in neurons or specific neuron types (Colosimo et al., 2004; Cinar et al., 2005; Fox et al., 2005; Kunitomo et al., 2005; Von Stetina et al., 2007; Takayama et al., 2009; Smith et al., 2010; Spencer et al., 2011). The literature search (**I**) also missed an earlier study that compared gene expression profiles of isolated wild-type and mutant touch receptor neurons in *C. elegans* (Zhang et al., 2002).

In more than half of the studies (305) a discrete brain area was used, as different brain areas can vary greatly in their gene expression and diseases or functions involve specific brain areas. Microarray datasets have been created from a very wide range of brain regions. There were especially many studies on cerebral cortex and brain regions affected in common neurological disorders. For example, CA1 region of the hippocampus, amygdala, cingulate cortex and superior temporal gyrus that are associated with Alzheimer's disease were well represented in the literature. Hippocampus had been used in many physiology studies because of its role in e.g. learning and memory, whereas the striatum involved e.g. in the development of drug dependence was often used in pharmacology/toxicology studies. Cell cultures were used the second most often (94 studies). Whole brain was used in 57 studies although the use of whole brains may mask regional expression changes. Single isolated cells were used in only nine studies as the isolation requires special techniques and amplification of RNA. One study used voxelation in which the brains were cut into equal-sized cubical parts to be profiled separately.

5.1.3 Experimental designs often lacked replicates

About half of the studies did not state using any biological replicates. In the studies that did use biological replicates, there were usually 2 to 10, most typically 3 replicates. Each biological replicate was often an individual animal or cell culture but in about 38 % of the studies, samples derived from several animals were pooled likely to obtain a sufficient amount of RNA. It can also be argued that a sample pooled from several individuals represents the population better than a sample from a single animal if biological replicates are not used. Technical replicates were used even more rarely. About 72 % of the studies did not report to use them at all. Sometimes the sample was hybridized onto two replicate chips, more rarely onto three or four chips. However, some microarray platforms contain targets spotted on the array in duplicate, which provides a measure of the variation in

hybridization. The number of replicates or details in description of methods did not change over the years from 1999 to 2004.

Economical reasons may limit the number of replicates used, as the commercial microarrays have been relatively expensive. In some cases scarcity of tissue can also limit the use of replicates. Experimental designs were often complex involving several treatments, genotypes, time points etc. and the use of replicates would obviously have multiplied the already large number of microarrays required. However, the lack of biological replicates is surprising considering their necessity for the calculation of appropriate statistics. Technical replicates are not as important as biological replicates in order to obtain biologically relevant information although they help to handle possible variations arising from e.g. differential labelling efficiencies, uneven hybridizations or technical differences between microarrays.

5.1.4 Platforms, imaging systems and analysis software

Microarrays were obtained from commercial sources in 76 % of the studies. Among the 15 commercial platform manufacturers reported, the most commonly used was Affymetrix (184 studies) followed by Clontech (97 studies, **I, Figure 2A**). The non-commercial microarrays were mainly custom microarrays designed by the laboratories (105 studies). The distinction was not made whether they were produced by the laboratory itself or by a commercial or academic facility. The proportion of custom microarrays remained approximately the same over the years 1999-2004 and they were made for both commonly and rarely used model organisms. Microarrays were purchased directly from microarray core facilities located in non-profit academic institutions or organizations in only three studies despite the apparent cost advantage compared to commercial microarrays. This may be due to a more limited selection of microarray types available. Some microarrays contained probes for only a few selected genes whereas some represented entire genomes containing up to 65000 probes.

Several scanning systems and image quantification software (**I, Figure 2B**) as well as data analysis software were used. The scanning systems reported were by about 20 different manufacturers. There were about 50 different image quantification software and 100 data analysis software reported. As in the case of microarray platforms, mostly commercial software were used. The use of the same manufacturer's microarray platforms, analysis instruments and software was usually preferred when possible. The two most frequently mentioned companies Affymetrix and Clontech provided integrated tools for image and data analysis, which may be one reason for their popularity. The data shows that a leader on the market can become a generally accepted "standard". However, even when an integrated platform and analysis system were used, one data set was often analyzed with more than one data analysis tool, e.g. one for calculating the fold change, one for statistical tests, one for clustering, one for visualization, and one for formatting the data to be imported from a software to another. Among the data analysis software, there were also about 20 non-commercial programs the most common of which was Michael Eisen's software "Cluster" used in 21 studies.

5.1.5 Microarray data was not effectively shared in the early years of the technology

Full data set containing information for all features measured in each condition was available for only 53 out of the 448 studies. The data was most often published on the author's or institute's website (29 studies) or as supplementary material of the journal (11 studies). In 13 studies the data was deposited in a public database that was GEO in 7 studies, Array Express in 3 studies, Neumetrix in 2 studies, or China Medical College database in one study. In two additional studies it was stated that the data was available upon request directly from the authors, which may apply to many other studies as well. In most studies, typically only the differentially expressed genes were listed although the

criteria of differential expression were not uniform making it difficult to compare the results between different studies.

Data on author's or institute's website is often available only for a limited time whereas journals and databases likely hold it longer. However, the Neumetrix and China Medical College databases are not available anymore. Full data sets were presented usually as output from software, either as raw data or processed data. The format depends on the software used. In rare cases even scanned microarray images were published.

In the beginning, MIAME referred only to the information on the microarray experiment and not to the data itself. Now also the data sets in both raw and processed forms are considered an essential part of MIAME. GEO and Array Express databases support and encourage the submission of MIAME compliant data. The ten studies in which data was deposited to either GEO or Array Express should be MIAME compliant and two of these are explicitly stated to be MIAME compliant. However, data in these studies is usually available either in raw or processed form, not both. Also the information directly accessed through the databases often lacks information such as descriptions of data analysis. Four studies contained a microarray experiment description in a MIAME-compliant format in a separate document but the whole data sets were not available. In other studies, including those with the datasets available on authors' websites or supplementary material, the only source of information on the experiment was the article itself. Public deposition of microarray data appears to have improved a great deal as the public databases GEO and Array Express currently have tens of thousands of datasets related to neuroscience.

5.2 *AHR-1* MUTANT *C. ELEGANS*

The function of AHR-1 was investigated in *C. elegans* using mutants that lack the functional AHR-1 protein. First, effects of the mutation on growth and behavior were observed. The hypothesis was that AHR-1 might play a role in the regulation of fatty acid diversity in *C. elegans* as it had earlier been shown to be involved in the transcriptional regulation of fat metabolism enzymes in mouse liver (Boverhof et al., 2005; Sato et al., 2008). The overall fatty acid compositions were measured as well as transcriptional alterations of genes that maintain fatty acid diversity. Finally, global transcriptomic alterations due to the lack of functional AHR-1 were investigated in the L1 larval stage using microarrays and in the L4 larval stage using RNA-seq.

5.2.1 Mutant alleles of *ahr-1*

Two available *C. elegans* strains carrying different mutant alleles of *ahr-1* were used, namely *ia03* and *ju145* (**IV, Figure 1**). The *ia03* allele has a 1517 bp deletion that removes exons 4 to 7 and causes a premature stop codon in exon 8, while the *ju145* allele has a C to T point mutation in exon 7 leading to a premature stop codon in place of the amino acid 302. If the mutant *ahr-1* alleles are translated into truncated proteins both of them contain the bHLH domain responsible for DNA binding and parts of the sequence that forms the PAS-B domain. The *ju145* mutant protein would contain the sequence for a full PAS-A domain whereas the *ia03* mutant protein would lack it completely. Any carboxyterminal transcriptional activation regions would be missing from both of the mutant proteins. Thus, the truncated proteins should lack the transcriptional activation function of AHR-1. However, there is still a possibility that they could bind DNA to repress transcription or function through some other mechanism resulting from protein-protein interactions. The remaining functions may differ between the two mutant proteins and explain the phenotypic and transcriptomic differences between the mutant strains.

5.2.2 Slow growth, movement, egg laying and defecation

The role of AHR-1 in neuronal development had been demonstrated earlier by Qin and Powel-Coffman (2004) and Huang et al. (2004) but little was known about other phenotypes

of the *ahr-1* mutant *C. elegans*. Development was studied in *ahr-1(ju145)* mutant (**II, Figure 3**) and behavioral phenotypes were characterized in both *ahr-1(ju145)* and *ahr-1(ia03)* mutants (**II, Table 1**). The *ahr-1(ju145)* mutant grew slower than wild-type as the time from L1 to L4 larval stage was 6 h longer. Both of the *ahr-1* mutants moved slower than wild-type. They also defecated in a slower rhythm and laid fewer eggs in 4 hours than wild-type and there was a small but significant increase in embryonic lethality. Brood sizes of the *ahr-1(ju145)* mutant were also slightly smaller than those of wild-type although the difference was not statistically significant.

Slow larval development or embryonic lethality had not been reported in earlier RNAi studies (Fraser et al., 2000; Rual et al., 2004; Sönnichsen et al., 2005). The differences may have been too subtle to be reported or detected in these genomic level screens, or RNAi may not have silenced the gene sufficiently. The involvement of AHR-1 in movement, defecation cycle or egg laying had not been studied before, except that the silencing of the *ahr-1* gene with RNAi had been shown to suppress the egg laying defect of another mutant (Gort et al., 2008).

Locomotion, egg laying and defecation behaviors are controlled by specific motor circuits in *C. elegans*. The well-characterized developmental defect in the GABAergic RME motor neurons in *ahr-1* mutant *C. elegans* (Huang et al., 2004) does not completely explain the behavioral phenotypes observed, as the affected behaviors are controlled by other classes of motor neurons. However, the *ahr-1* gene is mainly expressed in sensory neurons and interneurons (Qin and Powel-Coffman, 2004) that can modulate activities in the motor neurons as a response to environmental cues. For example, HSN motorneurons that control egg laying receive sensory input from PLM mechanosensory neurons (Schafer, 2005) that express *ahr-1*.

5.2.3 *A*lterations in fatty acid compositions and expression levels of fatty acid elongase and desaturase genes

Ligand-activation of AHR had earlier been shown to alter gene expression of fat metabolism enzymes in mouse liver (Boverhof et al., 2005; Sato et al., 2008) but a more precise role in the regulation of fatty acid composition had not been investigated. In **II**, relative amounts of different fatty acids were measured in *ahr-1(ju145)* mutant and wild-type *C. elegans* (**II, Figure 1**). The proportions of 15-methylpalmitic (17isoA), elaidic (18:1n9*trans*), dihomo-γ-linoleic (20:3n6) and arachidonic (20:4n6) acids were significantly increased, whereas the proportion of oleic acid (18:n9*cis*) was significantly decreased. The increased arachidonic acid (20:4n6) is a precursor of epoxy and hydroxyl products made by CYPs also in *C. elegans* (Kulas et al., 2008; Kosel et al., 2011).

The relative amounts of different fatty acids are controlled by desaturase and elongase enzymes that in *C. elegans* are named FAT and ELO, respectively (Watts and Browse, 2002). Gene expression of several fat metabolism-related genes is controlled e.g. by the nuclear hormone receptor NHR-49 and the Mediator subunit MDT-15 (Van Gilst et al., 2005a, b, Taubert et al. 2006). To understand the mechanisms underlying the changes in the fatty acid composition, the expression levels of the genes *fat-1*, *fat-2*, *fat-3*, *fat-4*, *fat-5*, *fat-6*, *fat-7*, *elo-1*, *elo-2*, *elo-5*, *elo-6*, *nhr-49* and *mdt-15* were measured in L4 larval stage *C. elegans* using qRT-PCR. The *elo-5*, *fat-7*, *nhr-49*, and *mdt-15* genes were over-expressed in the *ahr-1* mutant while the other genes studied were not altered significantly (**II, Figure 2A**). The increase in *elo-5* gene expression is consistent with the observed increase in 15-methylpalmitic acid (17isoA) observed in *ahr-1(ju145)* mutant (**II, Figure 1**) as ELO-5 catalyses the synthesis of 17iso from 15iso (**II, Figure 2C**). The expression of fatty acid desaturase and elongase genes was measured also in L1 stage using qRT-PCR. In this stage, the *elo-2*, *elo-5*, *fat-2*, and *fat-4* genes were over-expressed and the *fat-5* and *fat-7* genes under-expressed in the *ahr-1* mutant (**II, Figure 2B**).

Activation of AHR by TCDD has later been shown to alter fatty acid compositions and the expression levels of fatty acid-modifying genes also in mouse and rat liver (Angrish et

al., 2011; Forgacs et al., 2012). In mouse this is partially due to over-expression and increased activity of the stearoyl-CoA desaturase SCD1 (Angrish et al., 2011) that catalyzes the desaturation of e.g. 18:0 into 18:1n9. In *C. elegans* this reaction is catalyzed by FAT-7, FAT-6 and FAT-5 that are also the closest homologs of mouse SCD1 in *C. elegans*. Chromatin immunoprecipitation high-throughput DNA sequencing (ChIP-seq) data and band shift assays suggest that AHR regulates SCD1 directly in mouse (Angrish et al., 2011). In *C. elegans*, however, anatomical expression patterns of the differentially expressed genes *fat-7*, *elo-5*, *nhr-49*, *mdt-15*, *elo-2*, *fat-2* and *fat-5* do not largely overlap with that of *ahr-1* (Kniazeva et al., 2003, 2004; Huang et al., 2004; Qin and Powell-Coffman, 2004; Van Gilst et al. 2005a, b; Brock et al., 2006; Hunt-Newbury et al., 2007; McGhee et al., 2007) suggesting that the regulation of these genes in the *ahr-1* mutant is indirect. However, it should also be noted that these anatomical expression patterns obtained using promoter-GFP constructs may not be entirely accurate as the "promoter" areas in the constructs can lack regulatory elements located elsewhere in the genome (Hunt-Newbury et al., 2007).

Defects in fat metabolism affect several biological processes. Similar phenotypes as observed in the *ahr-1* mutant can be seen when the different genes required in fatty acid metabolism are silenced with RNAi. Earlier RNAi studies show that *nhr-49*, *mdt-15*, *fat-7*, *elo-5*, *elo-2*, *fat-2* and *fat-5* genes are required in normal growth rate (Gönczy et al., 2000; Kamath et al., 2003; Simmer et al., 2003; Kniazeva et al., 2003, 2004; Sönnichsen et al., 2005; Lehner et al., 2006; Horikawa et al., 2008), *nhr-49*, *fat-2* and *fat-7* genes in movement (Simmer et al., 2003; Yang et al., 2006; Horikawa et al., 2008), *nhr-49*, *mdt-15*, *fat-7*, *elo-2*, and *fat-2* genes in embryonic survival (Maeda et al., 2001; Kamath et al., 2003; Simmer et al., 2003; Rual et al., 2004; Lehner et al., 2006; Horikawa et al., 2008), *elo-2*, *fat-2* or *fat-7* genes in egg laying (Horikawa et al., 2008) and *elo-2* gene in the regulation of the defecation cycle (Kniazeva et al., 2003).

5.2.4 Gene expression profile in L1 larval stage using microarrays

Transcriptomic alterations due to the lack of functional AHR-1 protein were measured on a whole-genome level in L1 larval stage *C. elegans* using microarrays. In total, 238 genes were over-expressed and 324 genes under-expressed in the *ahr-1(ju145)* mutant compared to wild-type (**II, Supplementary Table 2**). While the *C. elegans* AHR homolog does not bind TCDD, the results from our microarray study surprisingly suggest that the *ahr-1* gene still affects the expression of genes related to xenobiotic metabolism and the direction of the expression change was also unexpected: the most over-expressed genes included the Phase I and II metabolic enzyme genes *cyp-13A4*, *ugt-19*, and *gst-21* (**II, Table 2**) and the over-expressed genes included also *cyp-35A5*, *ugt-20*, *ugt-22*, *ugt-29*, *ugt-33*, *ugt-38*, *ugt-41* and *gst-31* (**II, Supplementary Table 2**). However, a clear majority of the affected genes were not related to xenobiotic metabolism, but rather to physiological functions (**II, Supplementary Tables 2 and 3**). The most under-expressed genes included the fatty acid desaturase gene *fat-7* and the enoyl-CoA hydratase *ech-6* that is required for the catabolism of fatty acid to acetyl CoA and energy (**II, Table 2**).

Enriched Gene Ontology (GO) level 3 Biological Process (BP) and Molecular Function (MF) terms as well as PANTHER BP and MF terms were found for the over- and under-expressed genes (**II, Supplementary Table 3**). Enriched BP terms among the over-expressed genes were associated with carbohydrate metabolism or synaptic transmission. Enriched MF terms among the over-expressed genes were also mainly related to metabolism, such as transferase or oxidoreductase activity, but included also protein binding, calcium binding, mitochondrial carrier protein or cytoskeletal protein.

Among the under-expressed genes, many enriched BP terms were associated with growth and development (**II, Supplementary Table 3**), which is consistent with the slow larval development observed in the *ahr-1(ju145)* mutants (**II, Table 1**). One of the enriched GO BP terms was "locomotory behavior" consistent with the slow movement phenotype of the *ahr-1* mutant (**II, Table 1**). Also genes required in reproductive development were

under-expressed in the *ahr-1* mutant. Other enriched BP terms among the under-expressed genes were associated with cellular biosynthesis and catabolism, homeostasis, electron transport, cell surface receptor-mediated signaling, and metabolism of several types of compounds such as alcohols, organic acids, amines, amino acids, nitrogen compounds and pyrimidines. Most of the enriched MF terms were related to enzymatic activity such as peptidase, oxidoreductase, hydrolase, small GTPase, metalloprotease, transferase, and coenzyme or heme binding.

This data suggests that the loss of function of AHR affects similar processes in *C. elegans* as previously observed in mammals. Knockout of AHR has been shown to affect the expression of several genes, many of which have metabolic and enzymatic functions, in mouse liver (Tijet et al., 2006; Minami et al., 2008; Boutros et al., 2009; Reyes-Hernández et al., 2010), mouse kidney (Boutros et al., 2009), or rat liver (Boutros et al., 2011). Overall, the data reveals a widespread role for AHR-1 during early larval development.

5.2.5 Gene expression profile in L4 larval stage using RNA-seq

Gene expression profile alterations were also measured in L4 larval stage in *ahr-1(ia03)* and *ahr-1(ju145)* mutants compared to wild-type nematodes using RNA-seq. Initially, 51 to 61 million reads were obtained in each sample of which 85 to 88 % aligned directly to the *C. elegans* "virtual transcriptome" compiled based on known gene models in TopHat (Langmead et al., 2009, **IV, Table 1**). The expression levels were calculated for 45343 predicted transcripts as expected fragments per kilobase of transcript per million fragments sequenced (FPKM) using Cufflinks (Trapnell et al., 2010). Over 18000 transcripts were detected in each sample (**IV, Figure 2 A and B**).

Fourteen (14) transcripts were over-expressed and 125 transcripts under-expressed in both of the *ahr-1* mutants when compared to wild-type (**IV, Figure 2 C and D, Table 2**, **Supplementary files 1 and 2**). In total, 198 transcripts were over-expressed and 318 transcripts under-expressed in the *ahr-1(ia03)* mutant **(IV, Supplementary file 1**) while 41 transcripts were over-expressed and 487 transcripts under-expressed in the *ahr-1(ju145)* mutant compared to wild-type (**IV, Supplementary file 2**). There was only little overlap in the differentially expressed genes between the L1 and L4 stages (**II, IV**). This was expected since gene expression greatly depends on the developmental stage (Agarwal et al., 2010).

5.2.5.1 *gcy* and *fbxa* family genes and *nlp-20* were under-expressed

The most under-expressed genes included the soluble guanylate cyclase genes *gcy-32* and *gcy-36*, the neuropeptide-like protein gene *nlp-20* and the F-box A protein genes *fbxa-191*, *fbxa-192* and *fbxa-193* (**IV, Table 2**). The under-expression of *gcy-36*, *nlp-20* and *fbxa-192* was confirmed with qRT-PCR in both mutant strains and that of *fbxa-191* and *fbxa-193* in the *ahr-1(ia03)* mutant (**IV, Figure 3**). *gcy-32* was detected in only two replicates of the *ahr-1(ia03)* mutant and not at all in the *ahr-1(ju145)* mutant in qRT-PCR although it was detected in all of the four wild-type samples. This is likely due to a low level of expression in the mutant strains as observed in RNA-seq (**IV, Supplementary files 1 and 2**). In addition, *gcy-33*, *gcy-35* and *gcy-37* were significantly and over 2-fold under-expressed in the *ahr-1(ju145)* mutant which was also confirmed with qRT-PCR (**IV, Figure 3**).

The finding that several *gcy* family genes were significantly under-expressed is in agreement with previous studies performed with promoter-GFP fusion constructs showing that *gcy-32*, *gcy-34*, *gcy-35*, *gcy-36* and *gcy-37* are down-regulated in *ahr-1* mutants (Qin and Powell-Coffman, 2004; Qin et al., 2006). All of the *gcy* genes altered in the *ahr-1* mutant, except *gcy-33*, are expressed in neurons that also express *ahr-1* (Yu et al., 1997b; Cheung et al., 2004; Gray et al., 2004; Qin et al., 2006). RNA-seq was sensitive enough to detect expression differences of these genes although they have been detected in only 2-20 cells in the promoter-GFP studies and total RNA for RNA-seq was extracted from whole animals. A similar sensitivity of *C. elegans* whole-animal RNA-seq to detect differences in expression

even for genes expressed in only a few cells was seen also in another study from our laboratory (Peltonen et al., 2013).

Another dramatically under-expressed gene in *ahr-1* mutants, *nlp-20,* encodes a neuropeptide-like protein whose receptor is unknown and whose knockdown by RNAi leads to embryonic lethality. *nlp-20* is expressed in four head neurons, four tail neurons, one pharyngeal neuron, spermatheca, and intestine (Nathoo et al., 2001). Although *ahr-1* modulates social feeding behavior in *npr-1* mutants (Qin and Powell-Coffman, 2004) it is not likely that NLP-20 is a ligand of NPR-1 since the two known ligands for NPR-1, FLP-18 and FLP-21, are FMRFamide-related neuropeptides, a neuropeptide class distinct from NLPs (Li et al., 1999; Rogers et al., 2003). NLP-20 may be part of another neuronal pathway that mediates social feeding or other AHR functions such as movement or neuronal development. Interestingly, among the most under-expressed genes in L1 stage *ahr-1* mutants there was another neuropeptide gene, *nlp-30* (**II, Table 2**), suggesting a role for AHR-1 in neuropeptide regulation also in the earliest larval stage. In addition, TCDD has been shown to affect the expression of neuropeptides in rat neurons through AHR (Fetissov et al., 2004; Solak et al., 2013).

A novel role for AHR-1 transcription is the regulation of proteins that contain F-box motifs. *fbxa-191* and *fbxa-193* are pseudogenes whereas *fbxa-192* is protein-coding. Pseudogenes, mutated genes that no longer encode a functional protein, were originally considered to be junk DNA and to eventually degenerate due to accumulating mutations in the absence of selection pressure. More recent studies suggest that transcribed pseudogenes can regulate RNAs from the corresponding functional genes by binding to them as antisense RNAs, producing small interfering RNAs or competing for factors that regulate mRNA stability such as microRNAs (reviewed by Pink et al., 2011). The coordinate regulation of three similar *fbxa* genes of which two are pseudogenes and one is protein-coding may be due to a conserved regulatory sequence. Alternatively, the transcripts may affect the levels of each other. One such example is the phosphatase and tensin homolog (PTEN) tumor suppressor gene that is regulated negatively and positively by different transcripts from its pseudogene PTENpg1 (Poliseno et al., 2010; Johnsson et al., 2013). The extent to which AHR-1 controls non-coding RNA expression remains to be determined. However, about 12 % of the under-expressed transcripts were pseudogenes or other non-coding RNAs (data not shown).

5.2.5.2 Putative XRE sites upstream of the under-expressed genes

The XRE core sequence GCGTG was searched in 2000 bp 5′ flanking sequences of the *gcy*, *nlp-20* and *fbxa* genes. Putative XREs were found in front of *nlp-20*, *gcy-32*, *gcy-33*, *gcy-35*, *fbxa-191* and *fbxa-192* (**IV, Figure 4**) supporting the idea that AHR-1 induces these genes via binding to the conserved XRE sequence. The different known isoforms for the *nlp-20*, *gcy-33* and *gcy-35* genes have different transcription start sites and therefore the putative XREs are in different locations for them, sometimes in introns of the other isoforms (**IV, Supplementary file 3**). The closest putative XRE site for *fbxa-193* is 2164 bp upstream of the transcription start site (data not shown). *gcy-36* does not have a GCGTG pattern in its 2000 bp flanking sequence but has one in its 5′UTR (data not shown) which likely is the one found earlier by Qin et al. (2006).

In the study by Qin et al. (2006), a *gcy-35* 5′ flanking sequence of 1250 bp as well as a *gcy-36* 5′ flanking sequence of 1172 bp were sufficient to drive GFP transgene expression in wild-type background and the expression was significantly decreased in *ahr-1(ia03)* mutants. Similarly in **IV**, *nlp-20* 5′ flanking sequence of ~1800 bp was sufficient for GFP transgene expression in the presence of wild-type *ahr-1*, and the expression was significantly reduced in *ahr-1(ia03)* mutant background. This suggests that AHR-1 regulates *nlp-20*, *gcy-35* and *gcy-36* genes through these flanking sequences. However, a point mutation of the XRE in the *gcy-35*::GFP or *gcy-36*::GFP transgenic constructs did not diminish the GFP transgene expression in wild-type, suggesting that the predicted XRE

sites are not necessary for transcriptional activation of *gcy-35* or *gcy-36* by AHR-1 (Qin et al., 2006). The point mutation used by Qin et al. (2006) may not be sufficient to abolish the binding of AHR-1:AHA-1 heterodimer *in vivo* although it does so *in vitro* (Powell-Coffman et al., 1998), or the regulation of *gcy-35* and *gcy-36* by AHR-1 may be indirect or involve another AHR-1 binding sequence.

5.2.5.3 Two catalase genes were modestly over-expressed

There were far fewer over-expressed than under-expressed genes in the *ahr-1* mutants (**IV, Figure 2 C and D**). The over-expressed genes in both *ahr-1* mutant strains included two catalase genes *ctl-1* and *ctl-3*. Over-represented GO level 5 terms among the 14 over-expressed genes were "cellular response to reactive oxygen species", "hydrogen peroxide catabolic process", "response to hydrogen peroxide" and "catalase activity", and each term involved only the two catalase genes (**IV, Supplementary file 4**). AHR has been previously associated with the induction of genes in the oxidative stress pathway in higher organisms (Nebert et al., 2000; Dalton et al., 2002). However, in **IV** only two genes in the pathway were differentially expressed, and their over-expression in the mutants suggests that AHR-1 represses rather than induces these genes. The over-expression of *ctl-1* and *ctl-3* was modest: 3.0 to 4.5-fold in RNA-seq and 1.3 to 1.8-fold in qRT-PCR (data not shown). Catalase activity was not significantly increased in either *ahr-1(ia03)* or *ahr-1(ju145)* mutants when compared to N2 (data not shown). The data does not strongly support activation of oxidative stress pathways via AHR-1 in *C. elegans* and it is therefore possible that the role of AHR in oxidative stress observed in higher organisms has evolved separately from *C. elegans*.

5.3 *C. ELEGANS* CYP-35A SUBFAMILY

Earlier studies have indicated a role of *cyp-35A* subfamily members 2, 3, 4 and 5 in the regulation of *C. elegans* fat storage (Ashrafi et al. 2003, Menzel et al. 2007). In **III**, the effect of the *cyp-35A* subfamily genes 1, 2, 4 and 5 on fatty acid regulation was studied at multiple levels using mutants lacking the functional CYP-35A proteins. First, fat content was visualized in the mutants and wild-type. To investigate the role of the *cyp-35A* subfamily genes on the regulation of the diversity of fatty acids molecules, relative amounts of several fatty acids as well as expression level alterations of genes that control their proportions were measured. Also the levels of the endocannabinoids AEA and 2-AG in the *cyp-35A* subfamily mutants and the effect of dietary fatty acids on the gene expression of *cyp-35A* subfamily members in wild-type *C. elegans* were measured. In addition, protein sequences of human and *C. elegans* CYPs were compared.

5.3.1 Sequence alignments of human and *C. elegans* CYPs

Peptide sequences of human and *C. elegans* CYPs were compared to construct a phylogenetic tree showing the relations of the different CYPs (**III, Figure 1**). The *C. elegans* CYPs clustered in four major sets that corresponded to the currently accepted clans found in nematodes, i.e. 2, 3, 4 and mitochondrial (Baldwin et al., 2009). As most *C. elegans* CYP families, the CYP-35 family is found in other nematode species such as *C. briggsae* and *C. remanei* but not in vertebrate-, insect-, plant-, fungi-, or bacterial species investigated to date (Nelson, 2009).

The *C. elegans* CYP-35 family belongs to clan 2 together with the human CYP1 and CYP2 families. This clan is diverse in both human and in *C. elegans*. The CYP-35 family and the CYP-35A subfamily are relatively large (ten and five members, respectively) and the *cyp-35* genes are located in a cluster in the genome. This suggests that the *cyp-35* genes have undergone frequent duplications and losses during evolution likely in response to changing xenobiotic exposure (Thomas, 2007). The role of CYP-35A members in xenobiotic metabolism is also supported by studies showing that the *cyp-35A* family genes are induced

by a number of xenobiotics (Menzel et al., 2001, 2005, 2007; Kwon et al., 2004; Roh et al., 2006, 2007, 2010; Chakrapani et al., 2008; Roh and Choi, 2011; Peltonen et al., 2013,). The human CYP1 and CYP2 families metabolize xenobiotics and also a wide variety of endogenous compounds including fatty acids and their derivatives (Nebert et al., 2013). The CYP-35 family is also somewhat related to the *C. elegans* CYP-33E2 that metabolizes fatty acids into signaling molecules (Kulas et al., 2008; Kosel et al., 2011).

The CYP-35A peptides are very similar to each other especially at the first 100 amino acids from the N terminus and from amino acid 300 to 400 (**III, Supplementary Figure 1**). Within the family, CYP-35A2 and CYP-35A3 are the most similar and CYP-35A1 the most different in sequence. CYP-35A1 is missing a 38 amino acid sequence that the other four CYPs have in the middle of the peptide chain (amino acids 254 to 291) while it has additional amino acids closer to the C terminus.

5.3.2 Fat was reduced in *cyp-35A1* and *cyp-35A5* mutant *C. elegans*

To investigate the role of *cyp-35A* subfamily members 2, 3, 4 and 5 in the regulation of *C. elegans* fat storage, fat content was visualized in *cyp-35A1*, *cyp-35A2*, *cyp-35A4*, and *cyp-35A5* mutants and wild-type using Oil-Red-O (Soukas et al., 2009; Horikawa and Sakamoto, 2010) instead of Nile Red used in earlier studies (Ashrafi et al., 2003; Menzel et al., 2007). Fat content was reduced in *cyp-35A1* and *cyp-35A5* mutants (**III, Figure 2**), which supports the role of the CYP-35A subfamily in fat storage. The reduced fat in the lack of CYP-35A1 had not been reported before, likely due to allelic differences. Fat content was reduced in the *cyp-35A1(ok2306)* mutant (**III**) but not in the *cyp-35A1(ok1414)* mutant (data not shown) that was also used by Menzel et al. (2007).

Unlike in the study by Menzel et al. (2007), any marked reduction in fat stores could not be seen in *cyp-35A2(gk317)* or *cyp-35A4(ok1393)* mutants. The difference is most likely due to the dyes that stain distinct, mostly nonoverlapping fat compartments. Nile Red stains the intestinal acidic lysosome-related organelles (LROs) whereas Oil-Red-O stains neutral lipid vesicles in the intestine, hypodermis, gonad, and eggs (O'Rourke et al., 2009). In fasting conditions and several mutants Oil-Red-O signal correlates with triglyceride mass measured with biochemical assays whereas Nile Red signal does not (O'Rourke et al., 2009). The *cyp-35A2* and *cyp-35A4* mutants may be depleted in Nile Red-positive LRO fat but not in Oil-Red-O-positive lipid droplet fat. More sensitive methods such as solid-phase chromatography followed by gas chromatography/mass spectrometry (O'Rourke et al., 2009) could detect possible alterations in the total triglyceride content of the *cyp-35A2* and *cyp-35A4* mutants.

Our results suggest that CYP-35A1 and CYP-35A5 are necessary, but not sufficient to maintain fat storage. The molecular mechanism by which these CYPs act may be complex as fat storage is influenced by several processes such as food sensation, feeding, neuroendocrine control of metabolism, nutrient absorption and transport, *de novo* synthesis of fatty acids, triglyceride production, lipid droplet/LRO formation, lipolysis, and energy production from fats (Ashrafi, 2007). Genome-wide RNAi studies in *C. elegans* have revealed 305 gene inactivations that reduce Nile-Red-positive body fat (Ashrafi et al., 2003). These genes include metabolic enzymes, lipid interacting proteins, transcription factors, signal transduction molecules, receptors, channels and transporters, energy metabolism enzymes, cell surface molecules, vesicular transport proteins, protein degradation enzymes, cytoskeletal proteins, and general cellular machinery proteins.

5.3.3 Fatty acid compositions and gene expression levels of enzymes that regulate them were altered in the *cyp-35A* subfamily mutants

To investigate the possible role of the *cyp-35A* subfamily genes on the regulation of fatty acid diversity, relative amounts of several different fatty acids were measured in the *cyp-35A* subfamily mutants. Also gene expression levels of the fatty acid desaturase genes *fat-1*,

fat-2, *fat-3*, *fat-4*, *fat-5*, *fat-6* and *fat-7* and the elongase genes *elo-1*, *elo-2*, *elo-5*, and *elo-6* that control the proportions of different fatty acids were measured in the mutants.

The fatty acid compositions showed modest but statistically significant changes (**III, Figure 3**). The most affected fatty acid profiles were seen in the *cyp-35A1* and *cyp-35A5* mutants in which also fat content was reduced. Also gene expressions were mostly altered in these two mutants (**III, Figure 5A**). The *cyp-35A5* mutant differed from the other mutants in the directions of the changes: its fatty acids were slightly shifted towards longer chain fatty acids whereas in the other mutants the trend was opposite. In addition, all of the genes measured appeared under-expressed in the *cyp-35A5* mutant (decreases in *fat-1* and *fat-3* being statistically significant) whereas in the other mutants, particularly *cyp-35A1*, the same genes were over-expressed.

The differentially expressed *fat* and *elo* genes are regulated by transcription factors such as NHR-49, SBP-1 or DAF-16 (Kamath et al., 2003; Murphy et al., 2003; Van Gilst et al., 2005a, b; Kniazeva et al., 2008). One possible mechanism for the altered gene expression is that the CYP-35A members synthesize or degrade ligands for such transcription factors.

Some of the gene expression changes of the enzymes appear to explain the alterations in the levels their fatty acid substrates or metabolites (**III, Figure 5B**). For example, γ-linoleic acid (18:3n6) is increased in the *cyp-35A1* mutant and the *fat-3* gene encoding the enzyme that synthesizes it is over-expressed. However, not all alterations appear concordant in the same sense. For example, the over-expression of the *fat* and *elo* genes in the *cyp-35A1* mutant would suggest a shift towards longer and more unsaturated fatty acids but this was not seen in its fatty acid composition. Alterations in gene expression levels of enzymes precede (and not always predict) alterations in their protein levels or enzyme activities. The fatty acids might also form feedback loops to control gene expression of the enzymes responsible for their synthesis or metabolism. Thus, the alterations seen in the assays may depend on timing issues.

Overall, the alterations in the fatty acid compositions were small considering the obvious reduction of fat in *cyp-35A1* and *cyp-35A5* mutants. Possible local alterations may be diluted, as whole animals were used and different tissues or subcellular fractions were not separated. Alternatively, the small changes might reflect a strong metabolic drive to maintain the diversity of fatty acids. It is possible that other factors, such as those that cause the reduced fat phenotype, also affect amounts of specific fatty acids and the gene expression alterations of the *fat* and *elo* enzymes are a homeostatic response to compensate these changes. Moreover, major changes in fatty acid composition profiles are typically seen only in *C. elegans* mutants lacking enzymes required for fatty acid desaturation or elongation (Watts and Browse, 2002; Kniazeva et al., 2004) while changes are more moderate in e.g. mutants lacking transcription factors that regulate genes in the fatty acid metabolism pathways (Van Gilst et al., 2005a, b; Taubert et al., 2006; **II**).

The *acs-2* gene that encodes the fatty acid CoA synthetase 2 enzyme required in the first step of fatty acid β-oxidation was only slightly over-expressed in the *cyp-35A2* mutant (fold change 1.7) and under-expressed in the *cyp-35A5* mutant (fold change 0.8), and unaffected in *cyp-35A1* and *cyp-35A4* mutants. The results do not indicate increased usage of fatty acids for energy in β-oxidation in the *cyp-35A* mutants that would explain the depletion of fat stores.

5.3.4 The endocannabinoid AEA was increased in the *cyp-35A5* mutant

The endocannabinoids AEA and 2-AG are arachidonic acid-containing signaling molecules that control e.g. food intake and fat synthesis in mammals. Levels of AEA and 2-AG were measured in the *cyp-35A* subfamily mutants. AEA was increased 4.6-fold in the *cyp-35A5* mutant when compared to wild-type, but unaltered in the other *cyp-35A* mutants (**III, Figure 4**). 2-AG was not significantly altered in any of the *cyp-35A* mutants tested.

One hypothesis is that CYP-35A5 could metabolize AEA as some mammalian CYPs have been shown to do in earlier studies (Bornheim et al., 1995; Snider et al., 2007, 2008; Stark et

al., 2008; Pratt-Hyatt et al., 2010). In *C. elegans*, the degradation of AEA depends on the FAAH ortholog FAAH-1 (Lucanic et al., 2011). Therefore it seems unlikely that AEA catabolism would also require CYP-35A5 as an AEA-metabolizing enzyme. Rather, enzymes of AEA synthesis and/or degradation could be regulated in the *cyp-35A5* mutant.

AEA signaling is also connected to the regulation of energy homeostasis. It increases feeding and fat content in mammals (Matias and Di Marzo, 2007; Maccarrone et al., 2010). In *C. elegans*, AEA and other NAEs mediate the signal of nutrient availability: Dietary restriction leads to a decrease in NAE levels that is necessary for metabolic alterations involved in dauer formation and longevity (Lucanic et al., 2011). Metabolic alterations during dauer formation include also fat accumulation (Watts, 2008). Whether the increased AEA could be a homeostatic response or part of an underlying mechanism causing the reduced internal fat stores observed in the *cyp-35A5* mutant remains to be investigated.

5.3.5 Feeding with oleic and elaidic acids decreased the expression of *cyp-35A* subfamily genes in wild-type *C. elegans*

Gene expression of the CYP35A subfamily members was investigated in response to feeding of fatty acids. Diet affects CYP activities in mammals (Murray, 2006) and for example high-fat diet has been shown to lower the gene expression of a CYP in mouse liver (Yoshinari et al., 2006). Wild-type *C. elegans* were fed with oleic (18:n9*cis*) and elaidic (18:1n9*trans*) acids that are not present in the standard OP50 *E. coli* diet but have been detected in *C. elegans* (**II**, **Figure 1**). Feeding of oleic or elaidic acid in triglycerides lowered the expression levels of the *cyp-35A2*, *cyp-35A4*, and *cyp-35A5* genes whereas the *cyp-35A1* gene was not differentially expressed (**III, Table 1**). There was no difference between the effect of the *cis* and *trans* forms on *cyp-35A* subfamily gene expression.

Similar feeding of oleic and elaidic acids in triglycerides has been shown to result in an altered expression of some fat metabolism genes in *C. elegans*, including the fatty acid desaturase *fat-7*, the fatty acid β-oxidation gene *acs-2*, the transcriptional regulator genes *mdt-15* and *daf-16* and a steroid dehydrogenase gene *let-767* that is also involved in the production of fatty acids (Reisner et al., 2011). Our result supports the hypothesis that the CYP-35A subfamily members function in the same or parallel pathways with these genes to regulate fat metabolism in response to dietary fatty acids.

6 Summary and conclusions

The aims of the present study were to investigate the basic physiological roles of AHR and CYPs in *C. elegans* and follow the development of gene expression profiling methods and their usage. Loss-of-function mutants were studied using a combination of phenotyping, fatty acid profiling and transcriptomics approaches.

To summarize the main findings:

1. Systematic analysis of neuroscience publications between years 1999 and 2004 revealed a growing trend in the application of microarrays in gene expression profiling experiments that has continued until recent years (**I**). The use of RNA-seq is growing although microarrays may still be a more used method in neuroscience. Most of the animal species used were relatively large vertebrates. Neuron-specific gene expression can be profiled also in *C. elegans* with new techniques but it is far more challenging than the profiling of whole animals. Microarray data in the neurosciences was not shared effectively during 1999-2004. To date, the situation has improved as public databases contain tens of thousands of datasets related to neuroscience. However, the reporting of the experiments and especially data analysis steps as well as the inclusion of both raw and processed data could still be better.

2. The lack of functional AHR-1 affected neuromuscular behaviors including movement, egg laying and defecation (**II**), which is consistent with the role of AHR-1 in neuronal development. Fatty acid compositions suggest a role for AHR in regulating the synthesis of long-chain, unsaturated fatty acids, that eventually produce lipid signaling molecules. AHR was also required for normal larval development rate. This is consistent with the gene expression profile of L1 stage *ahr-1* mutants that supports a role of AHR in growth, and also in metabolism, homeostasis and signal transduction. In the L4 stage, AHR-1 regulates the expression of genes involved in neuronal function as well as transcribed pseudogenes or other noncoding RNAs (**IV**). Overall, the results suggest that many of the physiological roles of AHR-1 are conserved despite the apparently different activation of AHR in vertebrates and invertebrates.

3. Members of the highly xenobiotic-inducible CYP-35A subfamily were required for the normal accumulation of fat in *C. elegans* (**III**). They were demonstrated to have a role in the dynamic regulation of the variety of different fatty acid molecules in the nematode. In addition, the results suggest that CYP-35A5 plays a part in the regulation of the endocannabinoid AEA levels in *C. elegans*. These results provide a framework for future mechanistic studies to investigate fatty acid signaling using *C. elegans*. These findings also predict an elaborate role for CYPs overall in regulating signaling molecules in higher organisms.

Taken together, many of the roles of AHR and CYPs are conserved between *C. elegans* and mammals and *C. elegans* can be used as a model in molecular toxicology. This information on physiological roles of AHR and CYPs may help to understand the mechanisms of action of their altered activity caused by mutations or toxins and should be taken into consideration when assessing chemicals humans are exposed to or before developing treatments against the undesired effects.

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Vuokko Aarnio

Functions of AHR-1 and CYP-35A Subfamily Genes in Caenorhabditis elegans

Aryl hydrocarbon receptor (AHR) is a transcription factor aberrantly activated by some toxins. Its target genes include cytochrome P450 (CYP) enzymes. In this thesis, *Caenorhabditis elegans* is used to demonstrate a role for the AHR ortholog AHR-1 in neuronal function, growth and metabolism. Moreover, AHR-1 and *Caenorhabditis elegans* CYP-35A subfamily members are shown to take part in the regulation of fatty acid diversity and possibly lipid signaling. Many of the roles presented here appear conserved in mammals and may help to understand mechanisms of action of toxins.

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