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**CYP2C19 AND BRAIN
DEVELOPMENT: IMPLICATIONS
FOR SUSCEPTIBILITY
TO ANXIETY IN A TRANSGENIC
MOUSE MODEL**

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The cover image displays a coronal section of the mouse hippocampus with cells expressing calbindin in red and cells expressing calretinin in green. Cell nuclei are blue from DAPI counterstain. Photo: A. Persson

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Abstract

The cytochrome P450-2C19 enzyme is involved in the metabolism of about 10 % of all drugs used today and displays high genetic polymorphism, causing absent, decreased or elevated enzyme activity that divides the population into different metabolic phenotypes. CYP2C19 enzymatic activity is also highly influenced by different substances including drugs and *in vivo* studies have shown that estradiol and 17 α -ethinylestradiol, commonly used in hormone replacement therapy and oral contraceptives, decreased CYP2C19 mediated metabolism *in vivo* in humans. We investigated by which mechanisms this inhibition is mediated and found that the estrogens at rather high concentrations competitively inhibit CYP2C19 activity, but more importantly, at low clinically relevant concentrations caused a decreased gene transcription through a novel estrogen responsive element half-site in the *CYP2C19* promoter region. Such estrogen CYP2C19 interactions are important to consider during drug development.

Recently it was described by our laboratory that subjects lacking functional CYP2C19 enzyme had lower depressive symptoms based on analyses of a large twin cohort. To investigate CYP2C19's potential effect on behavior and brain function a transgenic mouse model expressing the human *CYP2C19* gene was characterized. We found that CYP2C19 is expressed in the developing fetal but not in adult brain. Newborn pups homozygous for the *CYP2C19* gene insert display high neonatal lethality and severe brain malformations with complete commissural agenesis and a severely reduced hippocampus. Hemizygous mice (CYP2C19Tg-Hem) showed less extensive phenotypes, thus survived and were characterized at 7 (adolescent) and 15 weeks (young adult) of age. CYP2C19Tg-Hem mice display increased stress sensitivity and anxiety-like behavior, which was more pronounced in young adult mice. Furthermore, a smaller hippocampal formation was seen at both ages as measured by manual outlining of brain sections and confirmed in adult mice by magnetic resonance imaging. The CYP2C19Tg-Hem mice hippocampal formation furthermore displayed an increased neuronal activation, or *c-fos* expression, after acute stress. This might be explained by the drastic reduction of immature neurons and the reduced number of GABAergic interneurons observed in the dentate gyrus of the hippocampus in the *CYP2C19* transgenic mice.

The results indicate that CYP2C19 expression during brain development increases the susceptibility to develop anxiety-related disorders later in life. This is interesting since, as mentioned above, absence of CYP2C19 enzyme is protective against depressive symptoms in humans, a phenotype displaying high comorbidity with anxiety disorders. Since the pathophysiology behind major depressive disorder and anxiety disorders is still mostly unknown, the model presented could be used for the investigation of factors important in the pathogenesis of these disorders and might also be used in the development of novel anxiolytic drugs.

List of publications

- I. Mwinyi J, Cavaco I, Pedersen RS, **Persson A**, Burkhardt S, Mkrtchian S and Ingelman-Sundberg M.
Regulation of CYP2C19 by estrogen receptor α . Implications for estrogen dependent inhibition of drug metabolism.
Molecular Pharmacology 2010; 78:886-894
- II. **Persson A**, Sim SC, Virding S, Onishchenko N, Schulte G and Ingelman-Sundberg M.
Decreased hippocampal volume and increased anxiety in a transgenic mouse model expressing the human CYP2C19 gene.
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List of abbreviations

AC	Anterior commissure
ADHD	Attention deficit hyperactivity disorder
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
CC	Corpus callosum
CES-D	Center for epidemiologic studies-depression
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CORT	Corticosterone
CYP	Cytochrome P450
CYP2C19Tg-Hem	Hemizygous <i>CYP2C19</i> transgenic mice
CYP2C19Tg-Hom	Homozygous <i>CYP2C19</i> transgenic mice
DCX	Double-cortin
DG	Dentate gyrus
E	Embryonic day
E1	Estrone
EE	17 β -estradiol/estradiol
EM	Extensive metabolizer
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERE	Estrogen responsive element
ETE	17 α -ethinylestradiol
FST	Forced-swim test
GCL	Granular cell layer
GAD	Generalized anxiety disorder
HA	Harm avoidance
HC	Hippocampus
HCC	Hippocampal commissure
HEK	Human embryonic kidney
HPA	Hypothalamic-pituitary-adrenal
HRT	Hormone replacement therapy
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LDB	Light-dark box
MAOI	Monoamine oxidase inhibitor
MDD	Major depressive disorder
MRI	Magnetic resonance imaging
MWM	Morris water maze
OC	Oral contraceptive

OF	Open-field
PA	Parvalbumin
PM	Poor metabolizer
PND	Postnatal day
PTSD	Post-traumatic stress disorder
RM	Rapid metabolizer
SGZ	Subgranular zone
SIH	Stress-induced hyperthermia
SSRI	Selective serotonin reuptake inhibitor
SVZ	Subventricular zone
TCA	Tricyclic antidepressant
TCI	Temperament and character inventory
TST	Tail-suspension test
WB	Western blot
Wt	Wildtype
UM	Ultra-rapid metabolizer

1 | Introduction

1.1 | Cytochrome P450 enzymes

Cytochrome P450s (CYPs) constitute a large family of important phase I enzymes involved in the oxidative activation or deactivation of both endogenous and exogenous substances.^{1,2} Besides their important functions in the metabolism of e.g. toxins and pharmaceuticals, CYPs have an essential part in endogenous metabolic functions such as cholesterol, fatty acid, and vitamin metabolism.³ CYP enzymes are most abundant in the liver, where they are membrane-bound and localized mainly in the endoplasmic reticulum whereas some forms are found in the mitochondria. CYPs are heme-proteins and function mainly as monooxygenases, transferring one oxygen atom from molecular oxygen to a substrate.⁴ Apart from being highly expressed in the liver, significant expression can also be found in extrahepatic tissues such as lung, gastrointestinal tract, and adrenal gland.^{2,5}

CYP nomenclature is based on amino acid homology with enzymes being divided into different families (>40 % homology, e.g. CYP2), subfamilies (>55 %, e.g. CYP2C), and individual enzymes (e.g. CYP2C19).^{2,4} For further reading see the CYP allele nomenclature database (<http://www.cypalleles.ki.se>).⁶ In humans, CYP family 1-3 are responsible for approximately 80 % of all phase I drug metabolism.^{1,7,8}

CYPs are, as the majority of both phase I and phase II drug metabolizing enzymes, highly polymorphic. This polymorphism has in many cases been connected to high interindividual differences in drug response and can lead to adverse drug reactions and loss of efficacy of drugs.^{1,9} Furthermore, the functional consequences of the different alleles differ and there are large differences in allele frequencies between ethnic groups.¹⁰ Based on this polymorphism the population can be divided into different metabolic phenotypes. Poor metabolizers (PMs) are defined by a complete lack of enzyme function, whereas intermediate metabolizers (IMs) are carriers of one functional and one nonfunctional allele. The wildtype allele is usually denominated *1 in CYP nomenclature and extensive metabolizers (EMs) are homozygous for this allelic variant. Some CYP alleles display increased transcription¹¹ or multiple copies¹² thus generating a ultrarapid metabolizer (UM) phenotype.⁸ Besides the genetic variants other factors such as age,^{13,14} gender,¹⁵ environmental factors, and drug interactions highly affect CYP expression and activity.¹

1.1.2 | CYPs in the brain

Besides being mainly expressed in the liver, and to a lesser extent in the gastrointestinal tract and other extrahepatic tissues, a limited number of CYPs are also found within the central nervous system (CNS). Many drugs with effects in the CNS are metabolized by CYPs^{16,17} and there is a pronounced interindividual variation in the response to these substances that is not always related to the drug plasma levels. Numerous studies have investigated CYP expression in the rodent brain, however only a limited number of CYPs expressed in brain have been detected in humans.^{17,18} It is suggested that most of

the brain-expressed CYPs predominantly have endogenous effects in e.g. steroid metabolism; nonetheless locally expressed CYPs could be crucial for the individual response to centrally acting drugs due to polymorphism and induction. Although the overall brain content of cytochrome P450 enzymes is relatively low, these enzymes display great regional and cell specificity that can lead to rather high levels in specific cells.¹⁹

1.1.3 | Human CYP2D6 and brain function

Some CYPs traditionally categorized as drug metabolizing enzymes have during the last decade been implicated also to contribute in the biotransformation of endogenous compounds. One enzyme extensively studied with regards to this is CYP2D6.²⁰ CYP2D6 is an important drug metabolizing enzyme, involved in the metabolism of 20 % of all drugs⁸ and around 50 % of all centrally acting drugs on the market¹⁷ such as antidepressants, antipsychotics and opioids.²¹ CYP2D6 also displays genetic polymorphism creating metabolic phenotypes ranging from PM to UM with multiple gene copies.¹²

CYP2D6 mRNA and protein has been detected in several human brain areas²² and it was recently revealed that brain CYP2D6-mediated metabolism alter codeine-induced analgesia in rats.²³ CYP2D6 is suggested to be involved in the endogenous metabolism of transmitter precursors into serotonin and dopamine.^{24,25} Besides the production of neurotransmitters CYP2D6 is possibly also involved in the 21-hydroxylation of progesterone²⁶ and in the metabolism of the endogenous cannabinoid anandamide.²⁷ These endogenous substrates for CYP2D6 might be the reasons for the many associations of polymorphism in the *CYP2D6* gene with several personality traits and neurological conditions. As reviewed by Cheng et al. (2013)²⁸ PMs are in some cohorts significantly associated with an anxious personality trait and are less successful in socialization than EMs.^{28,29} However, not all studies find consistent correlations between *CYP2D6* polymorphism and personality traits.³⁰ The UM phenotype has on the other hand been suggested to be associated with higher suicidal risk^{31,32} and increased suicidal behavior.³³ CYP2D6 PMs have additionally been shown to exhibit higher brain perfusion rates in the thalamus and the right hippocampus in healthy subjects, further suggesting an endogenous function of CYP2D6 in the human brain.²¹ CYP2D6 function in the brain is still not well understood but the hypothesis regarding a possible endogenous role for this enzyme and its effects on brain function has served as inspiration for this thesis work.

1.2 | CYP2C19

The human CYP2C subfamily contains 4 highly homologous genes, *CYP2C8*, *-2C9*, *-2C18*, and *-2C19*, clustered together on chromosome 10 (10q24). In this thesis the focus has been on CYP2C19, one of the major drug metabolizing enzymes in humans responsible for approximately 7-10 % of all hepatic phase I drug metabolism.^{8,34} The *CYP2C19* gene contains nine exons encoding a 490 amino-acid protein. CYP2C19 is

mainly expressed in the liver but some expression has also been found in the small intestine.³⁵⁻³⁷

1.2.1 | CYP2C19 and drug metabolism

The importance of CYP2C19 in drug metabolism is widely known and intensively studied, especially with regards to the polymorphic nature of the gene. CYP2C19 is involved in the metabolism of approximately 7-10 % of all clinically used drugs on the market today displaying broad substrate specificity.^{8,34} Substrates for CYP2C19 include several proton-pump inhibitors with omeprazole being the most well-known and studied substrate. The formation of the metabolite 5-hydroxyomeprazole from the R-enantiomer (R-omeprazole) is highly specific and extensively used for measuring CYP2C19 enzyme activity.³⁸⁻⁴⁰ CYP2C19 is furthermore involved in the metabolism of several different psychotropic drugs including selective serotonin reuptake inhibitors (SSRIs) e.g. sertraline^{41,42} and citalopram,^{43,44} tricyclic antidepressants (TCAs) like amitriptyline⁴⁵ and clomipramine,⁴⁶ and the monoamine oxidase (MAO) inhibitor moclobemide.⁴⁷ Other psychotropic substrates include benzodiazepines e.g. diazepam⁴⁸ and the anticonvulsant drug mephenytoin. CYP2C19 metabolic phenotypes (see 1.1.3) can be characterized by using racemic mephenytoin and measuring the urine R/S ratio since CYP2C19 specifically metabolizes S-mephenytoin.^{39,49,50} CYP2C19 also participates in the activation of the antimalarial drug proguanil⁵¹ and the antiplatelet drug clopidogrel.⁵² CYP2C19 is involved in the metabolism of many additional drugs, however with a minor role due to the main contribution of other drug metabolizing enzymes.

1.2.2 | Polymorphism in the CYP2C19 gene

Like many other CYPs CYP2C19 is highly polymorphic with both common and rare allelic variants leading to everything from absent to high enzyme activity with great differences in allelic frequencies between populations. There are more than 30 different allelic variants of CYP2C19 characterized today and depending on the allelic variants individuals can be classified into metabolic phenotypes.^{1,10}

Eight different allelic variants (*CYP2C19*2* to *CYP2C19*8*) encode a nonfunctional CYP2C19 enzyme, with the *CYP2C19*2* and the *CYP2C19*3* null alleles displaying the highest frequencies. The allele frequency of the null alleles varies between 12 and 23 % in Asians compared to 1-6 % in Caucasian populations. In contrast, the PM phenotype does not seem to exist in the Cuna Indians of Panama whereas 79 % of the population on the island of Vanuatu in the Pacific Ocean displays this phenotype (reviewed by Desta et al.).⁵³

Table 1 | Overview of selected CYP2C19 substrates

Substrate	Function	Reference
Omeprazol	Proton-pump inhibitor	38-40
Sertraline	Selective serotonin reuptake inhibitor	41,42
Citalopram	Selective serotonin reuptake inhibitor	43,44
Amitriptyline	Tricyclic antidepressant	45
Clomipramine	Tricyclic antidepressant	46
Moclobemide	Monoamine oxidase inhibitor	47
Diazepam	Anxiolytic, anticonvulsant	48
Mephenytoin	Anticonvulsant	39,49,50
Proguanil	Antimalarian	51
Clopidogrel	Anticoagulant	52
Estradiol	Endogenous*	75
Estrone	Endogenous	76
Progesterone	Endogenous	78
Testosterone	Endogenous	78
Polyunsaturated fatty acids	Endogenous	3,81

*Also used in hormone replacement therapy.

*CYP2C19*2* is the most common defective allele and is defined by a point mutation (G681A) leading to a premature termination of protein synthesis. The allele frequencies of the *CYP2C19*2* allele ranges from 15 % in Caucasians to 17 % and 30 % in African-Americans and Chinese, respectively.⁵³ The *CYP2C19*3* variant is defined by a single base transition (G636A) that results in a truncated protein and contributes to the PM phenotype mainly in Asian populations with the allelic frequency of around 10-12 % in Japanese and Korean subjects,^{54,55} compared to being almost nonexistent in Caucasians.⁵⁶

The *CYP2C19*17* allele represents a rapid metabolizer (RM) phenotype and is defined by two linked single nucleotide polymorphisms (SNPs) in the *CYP2C19* gene promoter region. The SNPs are located in the 5'-flanking region at -806(C>T) and -3402(C>T) relative to translation start and the -806 SNP introduces a novel transcription factor

binding site that causes increased gene transcription.¹¹ The allele frequency of *CYP2C19*17* is around 18 % in Swedes but ranges between 18 and 27 % in different European populations.^{11,57,58} The variant is rarer in Asian populations with an allele frequency of e.g. 4 % in Chinese¹¹ and 1.3 % in Japanese subjects.⁵⁴

Polymorphism in the *CYP2C19* gene is important in respect to drug metabolism as well as therapeutic outcome after treatment.¹⁷ The effect of *CYP2C19* genetic polymorphism on drug metabolism is mostly studied and has the greatest clinical impact in activating the antiplatelet drug clopidogrel. Defective *CYP2C19* alleles have been associated to increased risk of cardiovascular events and reduced bleeding risk in patients having undergone percutaneous intervention. Additionally, the RM phenotype is associated with increased risk of bleeding with clopidogrel treatment. Thus, dose adjustment based on *CYP2C19* genotypes could be beneficial but is still debated.⁹ Furthermore, psychiatric patients being RMs (*CYP2C19*17/*17*) display lower plasma levels of escitalopram^{59,60} and imipramine,⁶¹ and the genotype furthermore predicts remission in patients taking citalopram, with RMs displaying lower remission rates compared with PMs.⁶² Escitalopram serum concentrations were found to be 42 % lower in patients homozygous for the *CYP2C19*17* allele and 5.7-fold higher in PMs, compared to EMs.⁵⁹ This correlates with previous predictions of 35-40 % lower and a 2.1-fold decrease in omeprazole plasma concentrations in *CYP2C19*17/*17* subjects.^{11,63}

1.2.3 | *CYP2C19* gene regulation

Besides the described genetic polymorphism, *CYP2C19* enzyme activity is also affected by a variety of substances, including different drugs. *CYP2C19* is inducible by the antibiotic rifampicin and the corticosteroid dexamethasone and, as will be described in more detail below, inhibited by estrogens. The *CYP2C19* promoter region contains many putative transcription factor sites but the transcriptional regulation of *CYP2C19* has not been completely elucidated.^{64,65} It has however been shown that, like for many other CYPs, gene expression is up-regulated by the nuclear receptors: constitutive androstane receptor (CAR), pregnane X receptor (PXR), and the growth hormone receptor.⁶⁶ Other suggested transcription factors potentially involved in the regulation of *CYP2C19* include hepatocyte nuclear factor 3 γ (HNF3 γ)⁶⁷ and GATA-4.⁶⁸

As described above, the -806 SNP in the *CYP2C19* promoter region introduces a novel transcription factor binding site that leads to increased gene transcription of the *CYP2C19*17* allele. This SNP was suggested to create a consensus binding site for the transcription family GATA.¹¹ It was however recently discovered by our laboratory that the heterogeneous nuclear ribonucleoprotein L (hnRNP L) binds to this site and might therefore be the protein responsible for the increased gene transcription (Isa Cavaco *et al.*, unpublished).

1.3 | CYP2C19 activity and oral contraceptives

Induction or inhibition of CYP2C19 mediated drug metabolism is an important aspect of drug assessment since it could lead to unwanted drug-drug interactions associated with increased risks of side-effects or therapeutic failure.¹ Several studies have shown that exogenous estrogens affects CYP activity and most studies propose enzyme inhibition by these hormones.⁶⁹

17 β -estradiol or estradiol (EE), the major endogenous estrogen in humans, and 17 α -ethinylestradiol (ETE) are the most commonly used estrogens in hormone replacement therapy (HRT) and oral contraceptives (OCs), respectively. OCs are among the most commonly prescribed drugs for women in childbearing ages with more than 60 million users world-wide. HRT is also commonly used for women in menopausal ages and therefore possible drug interactions with both OCs and HRT are important to investigate.^{70,71} Since many studies have shown drug interactions with OCs, most pharmaceutical companies screen for possible interactions with ETE-containing OCs during drug development.⁷²

Regarding CYP2C19, both *in vitro* and *in vivo* studies have shown significant inhibition of enzyme activity by ETE. *In vitro* studies in liver microsomes have shown that a high concentration (100 μ M) of ETE strongly inhibits CYP2C19 enzyme function, as shown by CYP2C19-specific R-omeprazole hydroxylation.⁷⁰ This supports previous *in vivo* findings in healthy Swedish subjects where OCs including ETE increased the S/R-ratio of mephenytoin 2.5-fold and doubled the omeprazole/hydroxyomeprazole-ratio, both highly specific ratios for CYP2C19 activity.³⁹ Other *in vivo* studies have found similar interactions between CYP2C19 activity and OCs.⁷²⁻⁷⁴

Most oral contraceptives are usually combined with progestins to obtain a normal hormone cycle.⁷⁰ The effects seen on CYP2C19 activity using combination OCs is most definitely due to the effect of ETE since progestins by themselves do not seem to cause any CYP2C19 enzyme inhibition *in vivo*.^{72,73} ETE can inhibit CYP enzymes by both reversible and irreversible mechanisms and it is still largely unknown and difficult to predict how ETE inhibit CYP2C19 enzyme function. It is however suggested that ETE might be a weak reversible inhibitor of CYP2C19 enzyme activity.^{16,22}

1.4 | Potential endogenous substrates for CYP2C19

CYP2C19 has broad substrate specificity as described above and despite its important role in drug metabolism relatively few studies have investigated potential endogenous substrates. CYPs involved in the metabolism of endogenous substrates are most commonly involved in cholesterol, vitamin A, steroid or arachidonic acid turnover, as described previously. CYP2C19 is still regarded as a drug metabolizing enzyme but some studies suggest other functions of this enzyme in the human body that have not been completely elucidated yet. Most studies regarding a possible endogenous function of CYP2C19 have covered its role in steroid hormone metabolism. *In vitro* studies in human liver microsomes have shown that CYP2C19, together with CYP2C8 and

CYP2C9, effectively catalyzes the 17 β -hydroxy dehydrogenation of estradiol (EE) into estrone (E1). EE has many different metabolites with E1 and 2-hydroxy-estradiol being the most important and found in higher abundance than the other metabolites. E1 was found to be the most abundant metabolite at lower substrate concentrations suggesting that CYP2C19-mediated metabolism might be the most important pathway *in vivo*.⁷⁵ Concentrations of EE in plasma are usually very low, ranging from around 70 pmol/L (postmenopausal) to 2 nmol/L, but can however be significantly higher in specific tissues due to local synthesis.^{76,77} CYP2C19 has also been shown to contribute to the formation of the E1 metabolite 16 α -OH-estrone in liver microsomes.⁷⁶

Another suggested substrate for CYP2C19 is progesterone. In a study by Yamazaki et al. (1997) CYP2C19 mediated the formation of 21-OH-progesterone, and to some extent 16 α -OH-progesterone, in human liver microsomes.⁷⁸ CYP2C19 has furthermore been shown to oxidize testosterone to form androstenedione as a major metabolite, but also low levels of the metabolites: 6 β -, 16 β -, and 2 β -OH-testosterone.⁷⁸ Taken together, it can be hypothesized that CYP2C19 is involved in the metabolism and biotransformation of steroid hormones in humans. It has furthermore also been suggested that the *CYP2C19*17* variant, leading to increased gene expression, decreases breast cancer risk in women using hormone replacement therapy for more than 10 years. This also emphasizes the possible involvement of CYP2C19 in steroid hormone metabolism.^{79,80}

Apart from steroid hormones, other endogenous substrates have been proposed for CYP2C19. These include several different polyunsaturated fatty acids (PUFAs) e.g. arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid.^{3,81} Other members of the CYP2C family also contribute to this metabolism, e.g. CYP2C9 that moreover displays higher hepatic expression than CYP2C19.

CYP2C19 is also suggested to be important in the metabolism of the exogenous cannabinoid cannabidiol and related substances thus suggesting that it could be involved in the metabolism of endogenous cannabinoids as well.^{82,83} However this remains to be further investigated.

1.5 | CYP2C19, personality traits, and depressive symptoms

For many drug metabolizing enzymes, genetic polymorphism does not render any obvious phenotypes without a drug challenge. This is probably due to the fact that they do not have a critical function in endogenous metabolism.⁸⁴ From what is known today, CYP2C19 polymorphism does not have a clear impact on endogenous phenotypes in humans. Nevertheless, two studies have reported associations between *CYP2C19* polymorphism and personality traits in healthy Japanese subjects using the Japanese version of the Temperament and Character Inventory (TCI).^{85,86} The TCI investigates the intensity and relationship between seven personality dimensions divided into the temperament dimensions: harm avoidance (HA), novelty seeking, reward dependence, and persistence, and the character dimensions: self-directedness, cooperativeness, and self-transcendence.⁸⁷ The first study to investigate personality dimensions and

CYP2C19 polymorphism found significantly lower TCI-score in HA of homozygous EMs compared to heterozygous EMs and PMs.⁸⁶ Low scores in HA are associated with a carefree, courageous, outgoing, and optimistic personality and studies have previously found that high scores in HA is highly associated with depression and furthermore highly predicts MDD.⁸⁸⁻⁹⁰

In the other study by Ishii *et al.*,⁸⁵ other associations were found with female CYP2C19 PMs scoring significantly lower on the dimensions reward dependence, cooperativeness, and self-transcendence, compared to EMs. People with low scores in reward dependence are more practical, cold, and withdrawn and low scores in cooperativeness are associated with a more socially intolerant, critical, unhelpful, and opportunistic personality.⁸⁵ Low cooperativeness has also been correlated to a current state of depression.⁸⁸ Furthermore, low scores in self-transcendence correlates with an impatient, unimaginative, and proud character. There was however no differences found between male subjects.⁸⁵

Some aspects of these studies are interesting since high scores in HA and low scores in cooperativeness are associated to depression, something that seems to correlate with a PM phenotype. However, the results from these two studies are rather inconclusive, with major gender differences, and should be reproduced in a larger cohort and possibly also other ethnic groups before any conclusion can be made regarding personality traits and *CYP2C19* genetic variants.

CYP2C19 genetic polymorphism has also been associated with depressive symptoms as measured by the center for epidemiologic studies depression scale (CES-D). The CES-D scale measures depressive symptoms during the last week and consists of four subscales that together form the total score (T1): depressed mood, psychomotor retardation and somatic complaints, wellbeing, and interpersonal differences.⁹¹ Higher scores in all subscales indicate higher levels of depressive symptoms. In the study by Sim *et al.*,⁹² T1, depressed mood, and psychomotor retardation and somatic complaints were assessed in 1,472 subjects from the Swedish twin registry. *CYP2C19**2/*2 subjects, i.e. PMs had significantly lower T1, depressed mood, and psychomotor retardation and somatic complaints scores compared to EMs (*CYP2C19**1/*1), indicative of lower depressive symptoms.⁹²

It is difficult to draw any conclusion regarding *CYP2C19* genetic polymorphism and its effect on personality traits and depressive mood with regards to previous published results. Firstly, two different tests were used and TCI and CES-D scores do not measure the same parameters. However, previous studies have found significant association between high HA and high T1 scores.⁹⁰ PMs score high on HA but low on T1, depending on the study, thus making the results contradictory. The results from the twin study, using CES-D scores, are however from a much larger population and can therefore be considered more reliable. It seems that *CYP2C19* genetic polymorphism influence personality traits and depressive state but this remains to be further elucidated.

1.6 | Major depressive disorder

Major depressive disorder (MDD) is a common, heterogeneous affective disorder with a life-time prevalence of approximately 17 %, ⁹³ being twice as common in females as in males. MDD can be limited to a single episode but is frequently reoccurring or chronic. ⁹⁴ MDD is associated with high mortality due to the increased risk of suicide, and is one of the major causes of morbidity world-wide. ⁹⁵ The symptoms of MDD include persistent low mood and/or the inability to experience reward and pleasure i.e. anhedonia and associated symptoms. Furthermore, cognitive deficits such as poor concentration and impaired working memory are often a part of the symptomatology. ⁹⁶

Although being intensively researched, the pathophysiology and neurobiology of MDD are still largely unknown. The etiology of MDD is partly genetic, displaying 40-50 % heritability as shown by large family and twin cohorts. ⁹⁷ However, no major risk alleles have been identified, supporting the hypothesis that MDD is under polygenic influence and that the etiology is largely influenced by environmental factors. ⁹⁴ MDD is mostly associated with polymorphism in e.g. the serotonin transporter (*SLC6A4*) and brain-derived neurotrophic factor (*BDNF*) genes. ⁹⁷ These associations are logical since most antidepressants affect the serotonergic system ⁹⁷ and low BDNF levels are suggested to correlate with depression severity and to increase with recovery. ⁹⁸ Furthermore, many studies are investigating how environmental factors can influence the risk of MDD, both alone but also in combination with genetic risk. Environmental factors known to influence MDD are e.g. childhood adversities ⁹⁹ and stressful life events. ¹⁰⁰

The major brain systems involved in MDD are suggested to be subcortical areas involved in emotion and reward processing e.g. amygdala, hippocampus, and the ventral striatum and cortical areas such as the (medial and lateral) prefrontal cortex and anterior cingulate cortical regions, highly implicated in emotion processing and cognitive control. ⁹⁴ Monoaminergic signaling is thought to be of major importance within these structures, both in the pathophysiology and treatment of depression. The monoamine deficiency theory, reduced neuroplasticity, dysregulation of the hypothalamic-pituitary-adrenal gland (HPA) axis, and immune abnormalities are all considered important factors in the pathogenesis of MDD. ⁹⁵

1.6.1 | Pharmacotherapy of major depressive disorder

Current antidepressant treatment is directed against the monoaminergic systems and is designed to enhance its transmission. The most commonly prescribed drugs are selective serotonin re-uptake inhibitors (SSRIs) such as citalopram but other treatment strategies also include serotonin and noradrenaline re-uptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs) and furthermore noradrenaline and dopamine reuptake inhibitors like bupropion, and noradrenergic and specific serotonergic antidepressants like mirtazapine, mainly acting as α_2 -receptor antagonists. ⁹⁵ Around 30 % of depressed patients do not respond to the treatments available, ^{95,101,102} thus making the identification of new targets and new

treatment strategies extremely important for the seriously disabling and increasing health problem that MDD is today.

1.7 | Generalized anxiety disorder

Generalized anxiety disorder (GAD) has a life-time prevalence of around 4-7 %^{93,103} with females displaying an almost twice as high risk of developing the disorder.¹⁰⁴ The life-time prevalence of anxiety disorders is around 30 %⁹³ which beside GAD also include panic disorder, phobias, post-traumatic stress disorder (PTSD), and obsessive-compulsive disorder (OCD). The symptomatology of GAD includes uncontrollable worry, anxiety, and physical symptoms like disturbed sleep, restlessness, and muscle tension.¹⁰³ The disorder furthermore displays high co-morbidity with other psychiatric disorders,¹⁰⁴ including MDD as reviewed by Kessler *et al.* (2008).¹⁰⁵ It has been proposed by population twin studies that genetic effects are the most important common causes of MDD and GAD.¹⁰⁶

Compared to e.g. MDD relatively little is known regarding risk factors, genetics and neurobiology of generalized anxiety disorder but a heritability of approximately 15-20 % has however been proposed.¹⁰⁴ Neuroimaging studies suggest several brain areas involved in the pathophysiology of generalized anxiety disorder, most being a part of the so-called fear network including the amygdala, anterior cingulate cortex, and insula cortex. These structures seem important in both the pathogenesis and the neurobiology of the disorder.^{107,108}

1.7.1 | Pharmacotherapy of generalized anxiety disorder

For anxiety disorders the remission rate is poor, with between 30-50 % of patients not reaching full remission.¹⁰⁹ Even without a comorbid depression antidepressants are the first choice in the pharmacotherapy of GAD. This includes both SSRIs and SNRIs. Also MAOIs are sometimes used in treatment-resistant anxiety disorders. Other treatment choices include the acute and often short-term use of benzodiazepines, buspiron, and the anticonvulsant pregabalin.¹¹⁰ Pregabalin resembles benzodiazepines in its mechanism of action, including a rapid onset of action, and does also improve depressive symptoms when co-morbid with GAD. Unlike benzodiazepines, no issues regarding abuse, tolerance and withdrawal symptoms can be seen with pregabalin. Atypical antipsychotics might furthermore be used in treatment-resistant anxiety disorders either as monotherapies or in combination with other treatments.¹⁰⁹

1.8 | The hippocampal formation and its role in psychiatric disorders

Evidence is emerging of the involvement of the hippocampal formation in a wide range of psychiatric disorders including Alzheimer's disease, schizophrenia, anxiety disorders and MDD.¹¹¹ The hippocampus is considered a part of the limbic system with humans and other mammals having two hippocampi, one in each hemisphere. The hippocampal formation is a bilaminar grey-matter structure that consists of the dentate gyrus (DG) and the hippocampus proper, the cornu ammonis (CA). The CA cell layer contains mainly glutamatergic pyramidal neurons and based on their different properties the CA can be divided into the CA1, CA2, and CA3 regions, as seen in Figure 1.¹¹² These

pyramidal cells together with the glutamatergic granule cells of the DG constitute around 90 % of the hippocampal neurons, with the remaining 10 % being mainly γ -aminobutyric acid (GABA) producing interneurons.¹¹³

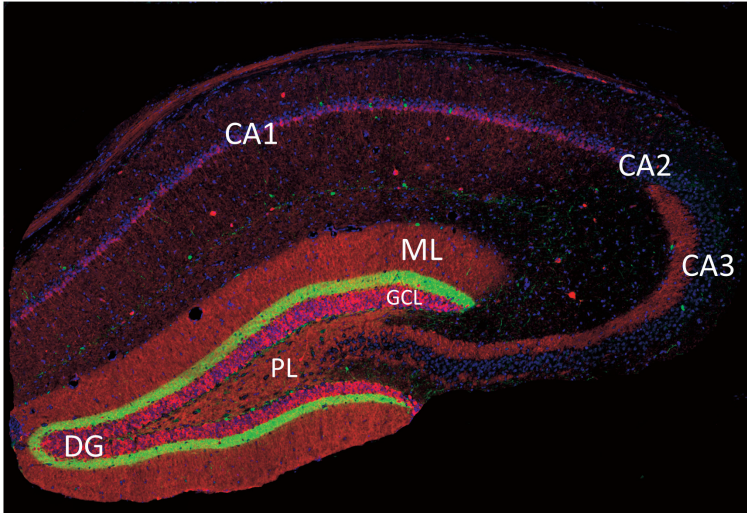


Figure 1 | The mouse hippocampus. Coronal section of the hippocampus in an adult mouse. The dentate gyrus (DG) contains the granule cell layer (GCL), the polymorphic layer (PL) or the hilus, and the molecular layer (ML). The hippocampus proper, the cornu ammonis (CA) is divided into different areas depending on their pyramidal neuron properties. Calbindin positive cells are seen in red and calretinin positive cells in green. DAPI was used as a nuclear counter stain. Photo: A Persson.

The HC is one of the most connected areas in the brain, receiving its major input from the entorhinal cortex through the perforant pathway. The entorhinal cortex serves as the major connector between the hippocampus and several different cortical areas including the auditory and olfactory cortices, but also the amygdala.¹¹¹ Despite intensive research on the function of the hippocampus there is still some controversy about the basic functions of this structure. However, its important role in the formation of episodic and spatial memory is widely known and generally accepted.^{112,114} Furthermore, pattern separation is thought to be essential for creating a specific memory when exposed to similar sensory inputs or experiences. It is believed that the DG is responsible for the process of separating memories that are formed in the hippocampus.¹¹⁵

As described above, involvement of the hippocampus in psychiatric disorders implicate that there are other possible functions of the hippocampus not involving memory. Even though traditionally considered a memory structure the hippocampal formation seems critically important in regulation of emotions as well.¹¹⁶ It furthermore seems important in regulating the stress response, a major risk factor for psychiatric disease.^{110,117}

Functions along the dorso-ventral axis

The hippocampus is involved in many different tasks including both cognitive and emotional processes. So how are all of these functions connected and regulated within the hippocampus? The hippocampal formation seems to exhibit significant differences in these functions along its dorso-ventral axis.^{116,118,119} Gene expression and differential projection patterns, mostly studied in rodents, suggest that the hippocampus can be divided into two separate structures; the rostral/dorsal part (posterior in primates), mostly involved in cognition and memory formation and the caudal/ventral part (anterior in primates), more implicated in emotion and stress regulation. The dorsal part of the hippocampus projects mostly to associational cortical areas whereas the caudal/ventral hippocampus on the other hand connects with the pre-frontal cortex, amygdala, and the hypothalamus. This theory is supported by the fact that ventral but not dorsal inactivation/lesions of the rodent hippocampus leads to anxiolytic behavior but does not seem to affect memory task performances.^{116,118,120} Also human studies indicate that the anterior part of the hippocampus is more activated when exposed to an emotional stimuli or face.¹²¹

Hippocampal size

The hippocampal formation has mostly been associated to psychiatric disorders by observations using magnetic resonance imaging (MRI). Many studies have demonstrated a small reduction of hippocampal volumes, between 4-10 %, in patients suffering from MDD.¹²²⁻¹²⁵ The reduction in hippocampal volume is furthermore suggested to correlate with severeness and duration of the disorder.^{125,126} Although it has been questioned whether reduced hippocampal volume is merely a symptom, volume reductions have also been observed in for example first episode depression,¹²⁷ and in subjects with familiar high risk for MDD¹²⁸ thus suggesting that reduced hippocampal size might also be predisposing for the disorder. Besides the effects seen on the hippocampal formation in MDD, reduced hippocampal volumes have also been associated with several anxiety disorders.¹²⁹ Reduced hippocampal volumes are found in adult patients with chronic PTSD, when compared to healthy or trauma-exposed controls using MRI.^{130,131} Also patients with social anxiety disorder display reduced hippocampal volumes.¹³² In the same way as for MDD, reduced hippocampal volumes could potentially also be a risk factor for PTSD.¹³³ The causality for the morphological changes seen in the hippocampus is still not known. One hypothesis suggests that the elevated glucocorticoid levels commonly observed in MDD patients can cause e.g. retraction of dendrites, decreased neurogenesis in the dentate gyrus, and loss of glial cells, all of which potentially could cause a smaller hippocampus.¹³⁴

Cognitive impairment

Also neuropsychological studies support the involvement of the hippocampus in the pathophysiology of MDD, with changes in hippocampal-related tasks being connected to cognitive impairment in patients.¹³⁴ Spatial navigation depends on hippocampal function and is severely impaired after hippocampal damage. In virtual reality tasks, patients with MDD display impaired spatial memory. These impairments have also been

functionally connected to abnormal activity in the hippocampus and the parahippocampal cortices.¹³⁴ Also recollection memory is highly dependent on hippocampal integrity and MDD patients display impairments in recollection memory tasks.¹²³

Stress and the hypothalamic-pituitary-adrenal axis

Dys-regulation of the hypothalamic-pituitary-adrenal (HPA) axis probably plays a central role in MDD since patients display increased cortisol levels and reduced negative feedback of the HPA-axis.¹³⁵ The hippocampus is highly responsive to glucocorticoids and plays an important part in the regulation of the stress response through a relatively high expression of glucocorticoid receptors.^{110,134} Chronic stress and sustained levels of glucocorticoids have negative effects on learning and on the survival of hippocampal neurons.^{113,134}

The hippocampus is of course not by itself creating the symptoms of depression and other related disorders. However, the plasticity of the structure, including the stress-sensitivity reported, this formation is most certainly playing an essential role in the pathobiology of several psychiatric disorders.

1.8.2 | Hippocampal plasticity

Adult neurogenesis is today generally accepted to occur in two discrete regions of the adult brain, the subventricular zone (SVZ) of the lateral ventricles and in the DG of the hippocampal formation.^{136,137} These areas are referred to as neurogenic niches and neurogenesis continues throughout life for many species,¹³⁸ including humans.^{139,140} Neurogenesis in the DG is restricted to the subgranular zone (SGZ) and new neurons are integrated in the granular cell layer (GCL) as mature granule cells. The stem cells of the SGZ are suggested to be radial glia-like stem cells,¹³⁸ also referred to as type 1 cells. As seen in figure X, these cells furthermore express glial fibrillary acidic protein (GFAP). Type 1 cells are characterized by an apical process that reaches into the molecular layer of the DG, suggested to be in contact with blood vessels.¹⁴¹ The maturation process of new neurons is suggested to be relatively linear with type 1 cells giving rise to fast-proliferating intermediate progenitor cells, type 2 cells. This cell population is characterized by a small soma and an irregularly shaped nucleus and is responsible for the large expansion of new cells seen in the DG, i.e. the expansion phase.

Early type 2 cells, type 2a, express the stem cell marker and transcription factor Sox2. At this stage cell fate is determined and newborn progenitor cells either become apoptotic or start to differentiate into type 2b cells, expressing the immature neuronal marker double-cortin (DCX).^{141,142} The expression of DCX in these cells is linked to specific properties such as structural plasticity, cell migration, axonal guidance, and dendrite sprouting.¹⁴³ DCX positive cells are frequently used as a substitute marker of neurogenesis, however the function of these cells during neurogenesis is still not known.¹⁴³ DCX positive cells do however have distinctive electrophysiological

properties, being highly excitable, and have been suggested important roles in hippocampal signal processing. Furthermore they display greater synaptic plasticity than mature neurons and long-term potentiation is more easily induced in DCX positive cells compared to more mature neurons in the DG. The pool of DCX cells in the hippocampus is highly dependent on proliferation rates but also the degree of apoptosis in the maturation process.^{144,145}

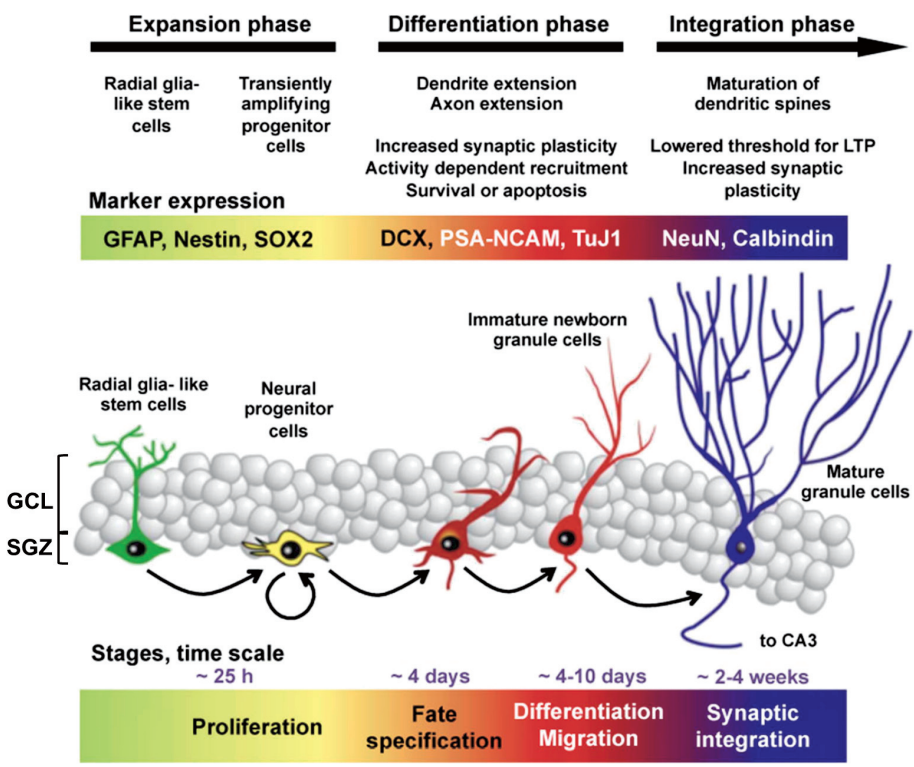


Figure 2 | Overview of adult hippocampal neurogenesis in the dentate gyrus.
The neurogenesis process in the adult hippocampus is thought to be rather linear with an overall time frame of approximately 4-6 weeks from neural progenitor to mature granule cell. The radial glia-like stem cells resides in the subgranular zone (SGZ) of the granular cell layer (GCL) and give rise to fast-proliferating progenitor cells, thus referred to as the expansion phase. The neurogenesis process seems critical for hippocampal function and is suggested to be involved in the pathobiology of psychiatric disorders. The process can be divided into different stages and by utilizing the different cell properties and specific markers expressed in the different phases the process can be studied in great detail revealing potential factors involved in its regulation. Adapted from: Schouten *et al.*, 2012, with permission from the publisher.¹⁴⁶

The majority of newborn cells undergo apoptosis during the first 1-4 days of the differentiation process.¹⁴⁷ During the differentiation phase cells are usually referred to

as type 3 cells. For the duration of this phase cells differentiate into immature neurons and migrate into the granule cell layer. At the end of the differentiation process cells develop and elongate their dendritic trees toward the molecular layer of the DG and their axons towards the CA3 area.^{114,141} The whole process from stem cells to mature granule cells takes approximately 4-6 weeks during normal conditions.¹⁴² The neurogenesis process is usually divided into different phases, with several specific cell markers for each phase, as visualized in Figure 2. This gives the opportunity for a more in detail study of the different cells involved and factors that affect hippocampal neurogenesis.¹⁴¹ Numerous studies have investigated the effects of e.g. antidepressants, stress, and exercise on neurogenesis in the DG and the neurogenesis process is shown to be extremely dynamic in rodents.^{114,142,148}

BrdU and Ki-67

The lack of knowledge regarding neuronal stem cells has made it difficult to detect and study this specific cell population. However, the discovery of the exogenous thymidine analog 5-bromo-2'-deoxyuridine (BrdU) made it easier to study proliferating cells and their survival rates in the neurogenic niches of the brain. BrdU is injected in the rodents and is incorporated in the DNA during the S-phase of the cell cycle.¹⁴⁹ BrdU is however toxic and Ki-67, another proliferation marker, has gained increased popularity for studying proliferation rates. Ki-67 is endogenously expressed during the whole mitotic period and requires less preparation compared to BrdU, both with regards to pretreatment of the animals but also the tissue, since the immuno-labeling requires denatured DNA for proper visualization of BrdU.¹⁵⁰ However, one of the major advantages of BrdU compared to Ki-67 is that after BrdU incorporation it can be visualized for a long time thus enabling studies of cell survival and cell fate in the neurogenesis process.¹⁴⁹ BrdU and Ki-67 labeled cells usually display rather similar numbers when examined acutely after BrdU injection.¹⁵⁰

Adult neurogenesis and disease

Adult neurogenesis is important for hippocampal function with a critical role in hippocampal-dependent learning and memory formation.^{114,151} The large pool of DCX positive cells in the DG is thought to be available for encoding new experiences and DCX cell numbers have been shown to adapt in rodents depending on how often the hippocampus is challenged with novel stimuli.^{152,153} Furthermore, recent studies suggest that hippocampal neurogenesis is not only regulated by stress but can in fact also buffer or regulate the stress response.^{110,117} Another important and interesting aspect is if hippocampal neurogenesis can affect other hippocampal-related behaviors such as depressive mood. The neurogenesis hypothesis postulated for both affective and anxiety disorders is based on the hypothesis that reduced neurogenesis in the DG is causative for the psychopathology seen and that treatments available are dependent on restored neurogenesis levels for a successful response.¹⁴² It has furthermore been suggested that the volumetric changes in HC volume seen in MDD and anxiety disorders are due to reduced neurogenesis in the DG. These hypotheses have been the topic for many

studies, mostly in rodents, but there are still no conclusive data supporting these theories and the subject is still under intense discussion and investigation.¹⁴²

1.9 | Modeling human psychiatric disorders in mice

For investigating gene function and for modeling human psychiatric disorders transgenic expression and genetic manipulation of target genes in animals is an important and valuable tool.^{154,155} These techniques are mostly developed in mice and due to these advantages the mouse is still the most commonly used species for transgenic expression and gene manipulation.¹⁵⁴ However, new transgenic rat models are developed and this would provide a better model in neuroscience and behavioral pharmacology where the rat in many aspects is the preferred animal model.¹⁵⁶

The usefulness and validity of animals as human disease models must be evaluated carefully and three aspects are generally considered. Construct validity refers to the etiology of the disease, i.e. the effect of a human gene causes similar conditions in the animal model. Face validity incorporates the symptoms of the disease into the validation of the model, i.e. the behavioral symptoms display common features. Many psychiatric disorders are rather complex and therefore also endophenotypes including neuroanatomical pathology and neurophysiological responses can be regarded as face validity. Predictive validity on the other hand takes in to account the treatment aspect of the modeled disease. Classes of drugs that reverse the human symptoms must similarly be effective in the model.¹⁵⁷

It is rather obvious that mice are not ideal models of human psychiatric disorders, mainly due to the great differences in brain anatomy, and we can never truly know whether a mouse is anxious or feeling depressed. However, the validity aspects described above are based on similarities in etiology, symptomatology, and treatment aspects and makes it possible to objectively investigate genetic variants or new treatments strategies for anxiety and depression in mouse but also rat models.

1.9.1 | Rodent models of mood disorders

The pathophysiology of mood disorders is still largely unknown and this is partly due to the lack of valid animal models. The main reason for the difficulties in finding good models for elucidating the pathophysiology is probably due to the fact that e.g. MDD and other mood disorders are so heterogeneous. Furthermore, many of the core symptoms of human depression are not possible to study in animals. These include e.g. depressed mood and suicidality. Due to these major issues most animal models of depression today are based on two major principles: predicting antidepressant effects or response to stressors.¹⁵⁸

Two of the most commonly used behavioral tests for assessing antidepressants are the forced-swim test (FST or Porsolt's test) and the tail-suspension test (TST).^{159,160} Both tests present inescapable environments that initially engage in intensive escape-oriented movements that eventually proceed into immobility or despair behavior.

Antidepressants give an acute increase in escape-oriented behavior and these tests are widely used as rapid screening tests for novel antidepressants due to their predictive validity. This does however raise some concerns regarding which systems that are involved since the FST and TST are sensitive to acute administration of antidepressants whereas a more chronic treatment is required for clinical efficacy in humans. These tests are also frequently used as phenotypic screens of rodent models to assess depressive-like behaviors. Increased basal immobility can in this respect be interpreted as increased depressive-like behavior and decreased immobility as a sign of an antidepressive phenotype.¹⁶¹ However, since the basis of the behavior in these tests is rather unknown it is also likely that the response seen, without pharmacological manipulation, might have more to do with stress coping than anything else.¹⁶²

The learned-helplessness model is another test that can be used to study active versus passive stress coping strategies in rodents. Rodents frequently exposed to inescapable foot shocks are subsequently incapable of fleeing even when offered a possibility. Not all rodents develop helplessness and it is by no means a chronic state since it usually only persists for 2-3 days, but antidepressants do however reverse this despair behavior giving predictive validity to this behavioral model.¹⁵⁸

Inability to cope with stressors is one of the major known risk factors of mood disorders as described above and the previously described models have stress coping as an important aspect of the behavioral response. However, the most commonly used stress-related rodent models are chronic mild stress and early life stress paradigms such as maternal separation.¹⁶¹ Chronic mild stress paradigms include a variety of mild unpredictable stressors, are more validated than early life stress models, and do in many cases display construct, predictive, and face validity of specific depression endophenotypes.¹⁶³ Anhedonia is defined as a reduced interest in normally pleasurable things. It is a core symptom of human depression and an endophenotype that can be modeled in rodents and is usually an acquired phenotype after chronic mild stress. Anhedonia in rodent models is usually assessed by investigating the preference for a highly palatable solution, e.g. sucrose over water.¹⁶¹

There are a few validated genetic rodent models of human depression with one example being the Flinders sensitive line (FSL) rat. This strain was initially developed through selective breeding for increased cholinergic sensitivity but was shown to display several important features of human depression.¹⁶⁴ One interesting and important aspect is that the FSL rat displays antidepressant effects after chronic treatment with a wide variety of antidepressants, thus displaying high construct validity.¹⁶⁵

1.9.2 | Rodent models of anxiety disorders

Anxiety is usually defined as a pathological response to fear, with the fear response being stronger than the situation requires and usually persisting for longer periods of time. Animal models of anxiety were initially based on the anxiolytic effects of benzodiazepines. The models are therefore not always so suitable for assessing new

anxiolytic agents. Animal models of anxiety can be classified into conditioned and unconditioned tests. Both groups of assessments measure the response to stressful stimuli but conditioned models often include painful events e.g. electric foot shocks. In this thesis only unconditioned models will be addressed since the other models usually involves memory and nociception influences and unconditioned responses are furthermore the most commonly used anxiety models for mice.^{166,167} However, none of the tests described here are thought to display pathological anxiety-related behaviors but are most commonly referred to as models of state anxiety. State anxiety refers to the response to the level, or type of stress, at a specific moment whereas trait anxiety does not vary from moment to moment but is a persistent feature of animal behavior. Trait anxiety models are most commonly specific strains displaying high anxiety behavior or knock-out mice,^{167,168} with one example being the 5-hydroxytryptamine_{1A} receptor knock-out mouse model.^{169,170}

Most unconditioned tests are based on the fact that small rodents have an innate aversion for open and brightly lit spaces and the conflicting nature of being exploratory animals.^{161,166} These test include e.g. the open-field, elevated plus maze, elevated zero maze, light-dark box etc. Avoidance of the aversive environment in these tests displays some face validity since avoidance of fearful situations or objects is a common feature of human anxiety disorders. However, it is important to remember like for most depression models, anxiety tests mostly display predictive validity, i.e. the aversive behaviors seen in the tests are reduced by anxiolytic treatment.¹⁶⁶ However, since the aversive behavior also can be augmented with drugs that induces anxiety in humans these tests are also utilized for evaluating anxiety-like behavior in mutant mice.¹⁶¹ Also stress-induced hyperthermia displays predictive validity for anxiolytic drugs, with treatment reducing the increase in body temperature normally observed after exposure to a stressor.¹⁷¹

1.9.3 | CYP2C18/CYP2C19 transgenic mice

Mice transgenic for the whole human *CYP2C18* and *CYP2C19* gene locus were produced at the Astra Zeneca Transgenic Centre in Mölndal, Sweden. The mouse model was created by pronuclear injection in C57Bl/6 eggs with a bacterial artificial chromosome (BAC RP11-466J14), containing the whole human *CYP2C18* and *CYP2C19* gene locus. The 5' end of the inserted gene fragment is located at position -5828 base pairs (bp) from the start codon of the *CYP2C18* gene and the 3' of the insert at +30,869 bp from the end of exon nine of the *CYP2C19* gene, thus also containing potential *cis*-regulatory regions. This created a total gene fragment of 196 kb, incorporated into the mouse genome. The number of copies incorporated was estimated to approximately 12 using real-time polymerase chain reaction (RT-PCR) and human genomic DNA as a reference. The incorporated gene fragments were furthermore analyzed using fluorescent in situ hybridization (FISH) to determine chromosomal location. A single insertion site was found at region C1 on mouse chromosome 2. For further details see Löfgren et al. (2008).¹⁷²

In previous publications CYP2C18/19 transgenic mice are named tg-CYP2C18 & 19. However, in the most recent paper,¹⁷³ included in this thesis, these mice are referred to as CYP2C19Tg mice due to the improbability of any CYP2C18 contribution to the observed phenotype. CYP2C18 displayed high mRNA levels in both male and female transgenic mice (26-31 weeks of age), comparable to CYP2C19 levels, with high expression in liver, kidney and small intestine. However, no CYP2C18 protein was detected in any of the tissues investigated. This confirms previous studies since the human CYP2C18 protein has never been detected in human tissues despite high mRNA levels.^{36,174}

Even though hemizygous CYP2C19Tg (CYP2C19Tg-Hem) mice exhibit around 12 copies of the human genes Western blot (WB) analysis only found an approximately 40 % increase in CYP2C/Cyp2c (human/mouse) protein levels, something that was also confirmed by activity assays in liver microsomes. This is most probably due to background levels from the relatively large mouse Cyp2c family (see 1.9.3). CYP2C19Tg-Hem mice do not display any macroscopic or histologic pathology but do however exhibit some differences in organ weight and clinical pathology with e.g. lower brain weights in both male (4.3 % lower) and female mice (5.9 % lower). The CYP2C19Tg-Hem mice are nevertheless considered viable and healthy.¹⁷⁵

As an attempt to confirm data produced within this thesis two other transgenic founders were also evaluated. The BAC used in the generation of the mice described above was modified by deleting exon 7-9 in the *CYP2C18* gene. This was done in collaboration with Polygene Inc. to further ensure that the phenotype seen was due to CYP2C19 and not CYP2C18 enzymatic activity. The two transgenic founders were produced by Duke University but did unfortunately display low copy number of the genetic insert (3 and 5 copies, respectively) and brain expression could not be detected, indicating that the deleted region could be important for expressional regulation of the *CYP2C19* gene.

1.9.4 | Mouse Cyp2c family

Compared to the human CYP2C-family with only four genes (*CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*) the mouse family has expanded to one of the most complex CYP families with 15 functional genes located on chromosome 19.^{176,177} It is difficult to compare CYP2C genes between humans and mice due to the difference in number and sequence variation. However, the human *CYP2C19* gene is relatively homologous to three genes in the mouse 2c-cluster namely *Cyp2c37*, *Cyp2c50*, and *Cyp2c54* (DNA identity: 77.9-78.5 %).¹⁷⁸ The mouse Cyp2c family is not so well characterized but do however display great variety in tissue distribution and metabolic activity, being mainly involved in endogenous functions like e.g. AA and linoleic acid metabolism.^{179,180}

Recently a mouse Cyp2c knock-out (KO) was created and characterized with 14 out of the 15 Cyp2c genes deleted from the mouse genome. This model can be used as a basis for more humanized mouse models to investigate effects of a single human CYP2C gene insert without the possible background effects of the numerous endogenous

enzymes. The Cyp2c KO has been a basis for a CYP2C9 humanized mouse model intended for investigations of CYP2C9 drug metabolism and drug-drug interactions.¹⁷⁷

2 | Aims

The general aim of this thesis was to study CYP2C19 with regards to a possible endogenous function of this enzyme. The aim was furthermore to investigate the effects of estrogens on CYP2C19 gene regulation and metabolic activity.

The specific aims were:

- I. Develop a cell system stably expressing CYP2C19 that can be used as a screening tool for potential substrates and inhibitors.
- II. Investigate if estradiol and 17 α -ethinylestradiol have any direct effects on CYP2C19 enzyme activity *in vitro*. Furthermore elucidate if and how CYP2C19 gene expression is regulated by these estrogens.
- III. Investigate endogenous functions of CYP2C19 by characterizing a *CYP2C19* transgenic mouse model with regard to brain development and morphology and behavior.

3 | Methodological considerations

The work included in this thesis spans over many aspects of CYP2C19 gene regulation and function, from *in vitro* cell experiments to *in vivo* studies in transgenic mice. In this section methodological considerations are raised for further understanding regarding methods used and validation of animal experiments.

3.1 | Paper I – Regulation of CYP2C19 expression by estrogen receptor α : implications for estrogen-dependent inhibition of drug metabolism

The main aim of stably transfecting HEK293 cells with CYP2C19 cDNA was to develop an *in vitro* system that could be used as a screening tool for potential endogenous CYP2C19 substrates. Several candidates from more high-throughput screenings have been tested in this cell system with results not yet published. In the first paper the HEK293 cell line stably expressing CYP2C19 was produced and used to study the direct effects of 17 α -ethinylestradiol (ETE) and estradiol (EE) on CYP2C19 enzyme activity. Estrogens were also investigated with regards to their effect on CYP2C19 gene regulation, something that is described in further detail in paper I.

3.1.1 | CYP2C19 stable cell line

Establishing a cell line expressing CYP2C19 was achieved by using the Flp-In™ system from Invitrogen and their modified HEK293 cell line, Flp-In™-293, containing a single integrated Flp recombination target (FRT) site ensuring a single integration site of the CYP2C19 gene.

CYP2C19 cDNA was subcloned into the pcDNA5/FRT expression vector and homologous recombination between the FRT sites in the cells and vectors was performed using the Flp recombinase, pOG44. Mock transfected cells were prepared in the same way with the pcDNA5/FRT vector and were used as controls in all experiments. After transfections cells acquired Hygromycin B resistance and resistant clones were sub-cultured and further analyzed. CYP2C19 expression and enzymatic function in the stable cell line was validated by RNA and protein expression by performing real-time polymerase chain reaction (RT-PCR), immunocytochemistry (ICC) and western blotting (WB), and by studying enzyme activity in intact cells.

3.1.2 | Enzyme activity assay

When studying CYP enzyme activity many different systems and assays can be used. The P450 Glo assay from Promega contains the CYP2C19 specific substrate Luciferin-H EGE and was used for measuring the effects of 17 α -ethinylestradiol (ETE) and estradiol (EE) on CYP2C19 enzyme activity. Flp-In™-293/CYP2C19 cells were seeded in 96-well plates and incubated with the CYP2C19 substrate. Estrogens were added 5 minutes prior to substrate incubations and the luminescence produced was proportional to CYP2C19 activity.

3.2 | Paper II - Decreased hippocampal volume and increased anxiety in a transgenic mouse model expressing the human CYP2C19 gene

3.2.1 | Transgenic mice

All mice included in this study were of C57BL/6 background and transgenic for the human *CYP2C18* and *CYP2C19* genes or wildtype (Wt) controls. The mouse model investigated, transgenic for the whole human *CYP2C18* and *CYP2C19* locus were originally developed and produced at Astra Zeneca Transgenic Centre in Mölndal, Sweden. *CYP2C19* transgenic mice hemizygous (CYP2C19Tg-Hem) for the gene insert express approximately 12 copies and have previously been characterized with regards to gene regulation, expression and pathology.^{172,175,181} For a more detailed description see 1.9.3.

CYP2C19 appears to have drastic effects on development at high expression levels since pups homozygous (CYP2C19Tg-Hom) for the insert rarely survive past postnatal day 3 (PND3). CYP2C19Tg-Hom mice were therefore only bred for in the developmental part and for PND0 brain morphology assessments. For all experiments male CYP2C19Tg-Hem mice were investigated and generated by crossing CYP2C19Tg-Hem and wild-type (Wt) mice. For all experiments, except for the tail-suspension test without stress, CYP2C19Tg-Hem females were used to avoid any potential maternal environmental differences between the litters. For the same reason Wt litter mates were always used as controls. Early in the characterization of the CYP2C19Tg-Hem mice a specific motoric phenotype was observed, where one or both hind paws were lifted higher, and sometimes stayed elevated for longer than in Wt mice. This phenotype does not seem to affect the animals overall performance in the behavioral tests as will be described in further details in the results and discussion part. Male mice were used for all experiments apart from the developmental study where genders were unknown. This choice was made to avoid additional variables such as hormonal fluctuations. It has already been shown that *CYP2C19* display a sexually dimorphic expression pattern in the transgenic mice so differences in the phenotype could be expected between genders. All mice were group-housed with a 12h light/dark cycle and with *ad libitum* access to food and water. Every effort was made to minimize animal suffering and number of individuals that had to be sacrificed during the work of this thesis. All animal experiments were approved by the Stockholm Northern Ethics Board of Animal Experimentation.

3.2.2 | Behavioral studies

When starting this thesis work little was known about the phenotype of the CYP2C19Tg-Hem mice in regards of behavior and brain morphology and function. As described above an association has however been discovered between *CYP2C19* genotype, more specifically low enzyme function, and less depressive symptoms.⁹² We therefore decided to start with a behavioral investigation of male CYP2C19Tg-Hem mice at 7 and 15 weeks of age as a first step in elucidating a possible endogenous function for CYP2C19. A battery of behavioral tests was evaluated in the transgenic

mice trying to cover important aspects such as motor function and activity, depressive- and anxiety-like behavior, and stress sensitivity.

To avoid any unnecessary stress for the animals all mice were handled by the experimenter the week before behavioral testing for at least one minute per day for four consecutive days. On test days, all mice were acclimatized to the test room for one hour before proceeding with behavioral tests. All assessments were performed at both ages apart from the Morris water maze (MWM) that was only investigated in 15-week old mice. The tail-suspension test (TST) was performed with and without prior exposure to the MWM on separate groups of mice. All other behavioral tests were performed on the same group of mice at both ages. Mice were left to rest for at least three days between tests that were conducted in the following order: Open-field (OF), light-dark box (LDB), and stress-induced hyperthermia (SIH).

3.2.2.1 | Tail-suspension test (TST)

For investigating and validating antidepressant drugs, the TST is one of the most commonly used tests in drug development today. It is based on the behavioral despair monitored in mice exposed to the short-term stressor of being suspended by the tail above the ground.¹⁶⁰ The forced swim test is also widely used in the same way as the TST but these tests are generally displaying similar outcomes and therefore only the TST was used for the initial screening in this study.¹⁶⁰ The main goal of this study was not to investigate antidepressant drugs but to study any potential phenotype of the CYP2C19Tg-Hem mice when exposed to this test.

All mice were exposed to the TST for 6 minutes and immobility time (>2s), frequency and latency to first immobility were manually calculated for the whole session. The TST was performed on 7-week old and 15-week old male mice (n=6-10/group). Three days after being exposed to the MWM, separate groups of mice were subjected to the TST to investigate if the stress of water maze exposure could potentially change the outcome of this test (Wt: n=10; CYP2C19Tg-Hem: n=9). The test was performed and evaluated in the same way as described above.

3.2.2.2 | Open-field (OF)

The open-field paradigm was used for the evaluation of locomotor activity since the CYP2C19Tg-Hem mice display a walking phenotype, as described in 3.2.1. All mice were placed in the same position in square opaque Plexiglas boxes (50 cm³) without bedding (n=15/group). The OF is furthermore considered an unconditioned conflict test for assessing anxiety-like behavior with the open illuminated arena being potentially threatening for small rodents.^{166,167} To analyze anxiety-like behavior in the OF, the arena was divided into peripheral, intermediary and central regions. Total distance travelled and time spent in each area was calculated using recordings and the behavior analysis software TopScan Lite from Clever Sys Inc.

3.2.2.3 | *Light-dark box (LDB)*

The LDB evaluates mouse aversion to illuminated open areas and the desire of exploring new environments¹⁸² and is one of the most commonly used behavioral tests for assessing anxiety-like behavior in mice.¹⁸³ The light-dark box consisted of two compartments: one dark, closed compartment (25 cm³) and one illuminated, open compartment (25 cm³). All mice were placed in the light compartment facing away from the opening and were allowed to freely explore for 5 minutes (n=15/group). Time spent in and number of transitions between the different compartments was recorded.

3.2.2.3 | *Stress-induced hyperthermia (SIH)*

The SIH, mostly used for its predictive validity by using anxiolytic drugs, was employed to further investigate the stress response in the transgenic mice.¹⁷¹ Rectal temperature was measured twice in each mouse: t=0 (T₁) and t=+10 min (T₂). After the first measurement (T₁), each mouse was placed individually in a novel cage for 10 minutes (n=15/group). The T₁ handling plus the 10-minute exposure to a novel cage was considered stressors, the response to which was analyzed by temperature raise at T₂. The difference in temperature ($\Delta T = T_2 - T_1$) is considered to reflect stress-induced hyperthermia.¹⁷¹ This test was not included in paper II due to space limitations.

3.2.2.4 | *Morris water maze (MWM)*

Spatial navigation learning in the MWM is highly correlated to hippocampal function in mice. This is best described by studying the effects that MWM training has on neurogenesis and survival of GCs in the hippocampus. Several studies have shown that learning in the MWM selectively adds and remove adult-born GCs depending on their maturation stages and functional significance, thus suggesting that these cells and hippocampal plasticity is highly involved in the learning process.^{184,185} Due to the drastic changes in hippocampal size and neuron maturation in the transgenic mice the MWM was employed to evaluate if these changes have any effects on spatial learning, i.e. hippocampal function. This was assessed in 15-week old male mice in a water pool measuring 120 cm in diameter. A transparent platform (10 cm) was placed in the north-west quadrant with four visual cues placed around the pool. For a more detailed description see Figure 9a. All mice were assessed for motivation and swim capacity in a pre-training session, revealing no obvious problems. Three days after the pre-training assessment all mice were trained in the water maze four times a day for five consecutive days. During the training sessions the platform was hidden and all mice were placed in 4 different random positions per day: north, west, south, and east. To be considered a successful response during the training sessions the mice had to stay on the platform for three seconds. During the retention test, 1 day after the last training session, the mice were placed in the south-east corner of the pool and left in the water for 60 seconds. During the retention test the platform was removed and learning and memory evaluation was accomplished by manually calculating time to first platform crossing, number of platform crossings and total time spent in the platform quadrant.

3.2.3 | Acute restraint stress and plasma corticosterone levels

To investigate stress reactivity of the mice, including hippocampal neuronal activation and the function of the hypothalamic-pituitary-adrenal (HPA) axis, both 7- and 15-week-old mice were exposed to acute restraint stress. This was achieved by placing the mice in a 50 ml ventilated Falcon tube for 30 minutes. To collect whole blood mice were either directly decapitated after the restraint stress or placed in their home cage for another 30 minutes before decapitation. As a control, mice were immediately decapitated without stress exposure. All animals in the same age group were decapitated on the same day, between 08:00 and 11:00 a.m. Serum aliquots were analyzed for corticosterone (CORT) content using an enzyme-linked immunosorbent assay specific for mouse/rat corticosterone. To investigate hippocampal activation after stress exposure all hippocampi were dissected and analyzed the expression of the immediate-early gene *c-fos*.

3.2.4 | mRNA expression and RT-PCR

CYP2C19 and *CYP2C18* expression levels were investigated in liver and brain tissue during mouse development, embryonic day 11 (E11), E14, and E18, early post-natal days, post-natal day 0 (PND0), and PND7, and at 7 weeks of age. In four additional E18 transgenic embryos, the hippocampus and cortex were dissected out to investigate specific expression within the different areas. The hippocampi had to be pooled due to their low weight. RT-PCR protocol and primers were obtained from Löfgren et al. (2008).¹⁷²

3.2.4.1 | Human fetal samples

To validate the developmental findings in the *CYP2C19* transgenic mice brain tissue from three human fetuses was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). A total of 8 samples were therefore analyzed for *CYP2C19* mRNA expression. Samples were from 3 different female Caucasian donors; gestational week 19, 24 and 39. Brain samples were from different cortical areas with little or no overlap between donors. All samples were processed and analyzed in the same way as described for human *CYP2C19* expression in the transgenic mice but with the human housekeeping gene: glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.2.5 | Brain morphology studies

3.2.5.1 | Newborn *CYP2C19Tg-Hom* mice

CYP2C19Tg-Hom mice displayed high neonatal lethality and in an attempt to investigate any possible reasons for this extreme phenotype, brain morphology was evaluated at postnatal day 0 (PND0) in *CYP2C19Tg-Hom* (n=4), *CYP2C19Tg-Hem* (n=5) and *Wt* (n=3) pups. To elucidate any differences in brain morphology cresyl violet stained sections were blinded and visually assessed. After this initial assessment sections from three *CYP2C19Tg-Hom* and three *Wt* mice were further analyzed and the

cortex, hippocampus and central regions were manually outlined in all sections to get an estimated over some main morphological findings.

3.2.5.2 | 7- and 15-week old CYP2C19Tg-Hem mice

One of the most severely affected structures in PND0 CYP2C19Tg-Hom mice was the hippocampus and therefore hippocampal size was assessed in 7- and 15-week old CYP2C19Tg-Hem mice and Wt controls. To cover the whole hippocampal formation every 7th coronal section, with a total number of 12 sections starting at Bregma -0.94 mm, was stained with cresyl violet and the hippocampi were manually outlined in all sections, see Figure 3.

3.2.5.3 | Magnetic resonance imaging

In collaboration with Karolinska Experimental research and imaging center (KERIC) a magnetic resonance imaging (MRI) study of 15-week-old CYP2C19Tg-Hem mice brains and Wt controls was performed. This was done to confirm previous size measurements in brain sections and was performed by using a horizontal 9.4 T Varian magnet. The volumetric images were acquired using a 3D Inversion Recovery Fast Spin-Echo Sequence and image analysis was made with the image analysis software ITK-SNAP (www.itksnap.org).¹⁸⁶ Hippocampi and whole brain volumes (including cerebellum) were manually outlined with genotypes blind to the experimenter.

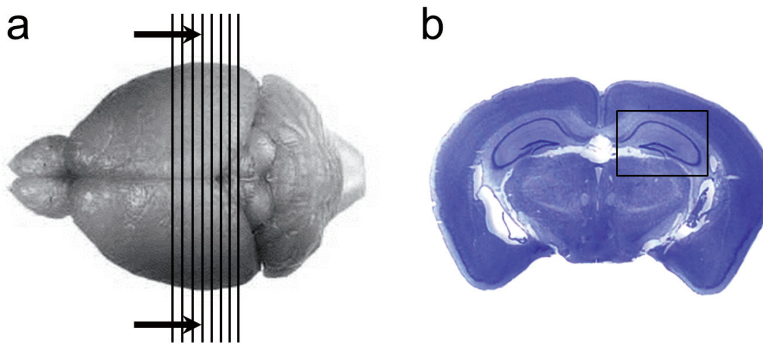


Figure 3 | Image of a whole mouse brain and one coronal section visualizing the hippocampal formation. (a) Mouse brain with approximate lines representing the 40 μ m coronal sections made for immunohistochemical assessments. The most rostral section was located approximately at Bregma -1.46 mm and every 7th section, with a total of 8 sections was processed for different markers. Similar sectioning was done for area measurements of the hippocampal formation but with a total of 12 sections starting at Bregma -0.94 mm. Arrows indicate section seen in (b). (b) Coronal section of mouse brain stained with cresyl violet. As clearly visualized in the section the hippocampal formation is present in both hemispheres. The black box indicates one hippocampus. Brain in (a) adapted from: <http://www.nervenet.org>. Photo: A. Persson.

3.2.6 | Immunohistochemistry

For all brain immunohistochemical (IHC) stainings mice were perfused with 4 % paraformaldehyde to acquire a quick preservation of the tissues and antigens and to avoid any hemoglobin auto-fluorescence. Brains were coronally sectioned into 40 μ m thick sections as seen in Figure 3, to perform all stainings in free-floating. For all IHC markers, total numbers were also corrected for total hippocampal volumes as measured by MRI.

Cell proliferation and maturation of young neurons is important for hippocampal function. It is furthermore suggested that the reduced hippocampal size can be caused by reduced neurogenesis in the DG of the hippocampus.¹⁴² Therefore 7-week-old mice were injected with bromodeoxyuridine (BrdU) to investigate cell proliferation and survival. Ki-67, another proliferation marker was used as a control for 7-week-old mice and for assessing proliferation rates in the DG in adult mice. In 15-week old mice the number of immature, migrating neurons in the DG was assessed by staining for DCX positive cells.

Additionally, number of parvalbumin (PA) positive cells was evaluated in the whole hippocampal formation at 15 weeks of age. Number of cells for all markers was manually assessed in the DG of the hippocampal formation. PA positive cells were also assessed in the CA1+2 and the CA3 regions of the mouse hippocampus.

4 | Results and discussion

In the following section results from the two papers included in this thesis will be discussed. For a more detailed description of the result, including statistics, see respective paper.

4.1 | Paper I – Regulation of *CYP2C19* expression by estrogen receptor α : implications for estrogen-dependent inhibition of drug metabolism

The CYP2C19 enzyme is involved in the metabolism of many drugs on the market and it is therefore important to identify all factors that affect enzyme expression. Polymorphism in the *CYP2C19* gene is well characterized and makes it possible to divide the population into different metabolic phenotypes. However, other factors besides genetic polymorphism also influence CYP activity, including drugs and other substances that are known to either induce or inhibit CYP2C19 gene transcription and enzyme activity. As described in the introduction, estrogens are shown to be metabolized by CYP2C19 and furthermore to inhibit enzyme activity, however by which mechanisms this is achieved and its clinical implications warrants a more in detail investigation. Both transcriptional regulation and direct enzyme inhibition of CYP2C19 could be envisioned and was further investigated in this paper.

4.1.1 | Validation of the CYP2C19 stable cell line

To investigate direct effects of the estrogens ETE and EE on CYP2C19 enzyme activity a HEK293 cell line stably expressing CYP2C19 was established by using the Flp-In™ system (Invitrogen). RT-PCR and WB confirmed CYP2C19 mRNA and protein expression in the HEK293 cells. CYP2C19 protein expression was also confirmed by ICC as visualized in Figure 4. The HEK293 cells originate from transformation of human embryonic kidney (HEK) cells by exposure to sheared fragments of human adenovirus type 5.¹⁸⁷ HEK293 cells are widely used for both transient and stable expression of recombinant proteins due to their efficiency and consistency in transfections and protein expression.^{188,189} This cell line has been further modified by Invitrogen, referred to as Flp-In™-293 cells, to ensure a single integration site of your gene of interest, thus making the Flp-In system ideal for fast, predictable and stable protein expression. I have furthermore used the HEK293 cell line, stably expressing the CYP2C19 enzyme, for inhibitor screening and to investigate possible endogenous substrates for CYP2C19, something that will be addressed in future publications.

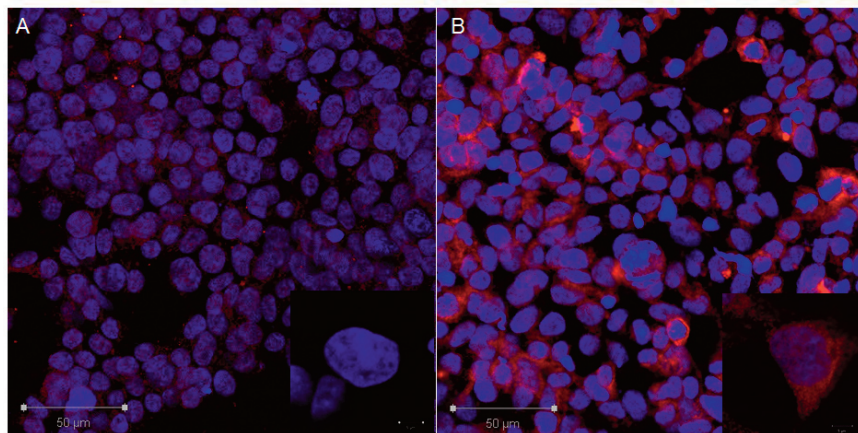


Figure 4 | CYP2C19 protein expression in the modified HEK293 cell line, Flp-In™-293 (Invitrogen). Flp-In™-293 cells were stably transfected with CYP2C19 cDNA and protein expression was confirmed by immunocytochemistry using the anti-CYP2C19 antibody produced in rabbit (Sigma-Aldrich). **(a)** Mock transfected cells were used as controls in all experiments. **(b)** CYP2C19 transfected Flp-In™-293 cells. Scale bar= 50 µm. The inserted images are magnifications (63x) of one representative cell. Scale bar= 5 µm. Photo: A. Persson

4.1.2 | Relatively high estrogen concentrations inhibit CYP2C19 enzyme activity

The estrogens 17 β -estradiol (EE) and 17 α -ethinylestradiol (ETE) are the most commonly used female steroid hormones in hormone replacement therapy and OCs.⁷⁰ Drug interactions caused by these hormones are important to investigate in the development of new drugs since these estrogens are widely prescribed and studies show that female hormones can affect the activity of drug metabolizing enzymes, with some enzymes being induced and others displaying substantial inhibition.^{69,70,72} As described in the Introduction (section 1.3), *in vivo* data shows that the interaction of OCs and CYP2C19 specific substrates seems to be substantial, displaying a marked reduction of CYP2C19 specific metabolites.^{39,50,72-74} In this paper we showed that both EE and ETE have a direct inhibitory effect on CYP2C19 enzyme function, thus confirming previously published data. However, all *in vitro* data published hitherto, including this study, have used rather high ETE concentrations in the micromolar range.⁷⁰ Plasma levels of ETE after using OCs is usually in the pico- to nanomolar range making the published results rather questionable from a clinical perspective.⁷¹ Female sex steroids do however display an extensive enterohepatic circulation which could lead to relatively high hepatic concentrations, despite low plasma levels.⁷⁰ Thus, it is still rather questionable if the high estrogen concentrations needed for significant enzyme inhibition in the CYP2C19 expressing cells can explain or even contribute to the marked effects OCs display on CYP2C19 metabolism in human studies.^{39,50,72-74} It was thus considered important to evaluate effects on the transcriptional level.

4.1.3 | Estrogens affect CYP2C19 enzyme activity through transcriptional regulation

The main focus of this study was therefore to investigate if ETE and EE could affect transcriptional regulation of the *CYP2C19* gene. In this paper, four potential estrogen responsive element (ERE) half-sites were discovered in the promoter region of the *CYP2C19* gene by *in silico* analysis. However, only one site, in the promoter region; position -151/-147, showed binding of estrogen receptor α (ER α) by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis in human hepatoma (Huh7) cells. It was confirmed that ER α is activated by ETE and EE and inhibits *CYP2C19* transcription through the newly identified ERE binding half site (-151/-147) in the proximal part of the *CYP2C19* 5'-flanking region. EE and ETE displayed inhibition of *CYP2C19* gene transcription in a dose-dependent manner with the half-maximal inhibitory effect of 100 and 10 nM, respectively. This was displayed both by luciferase gene reporter assay and by measuring mRNA levels in human hepatocytes. A similar transcriptional regulation has also been suggested for the related *CYP2C9* gene indicating that this regulatory mechanism is rather conserved in the human CYP2C family.¹⁹⁰ However, disrupting the ERE half site in the promoter region only partly restored the *CYP2C19* transcription thus suggesting additional important ERE sites or other regulatory mechanisms by the activated estrogen receptor. ER α is known to affect other transcription factors by stabilizing their DNA binding but also by recruiting other co-activators to the transcription complex.¹⁹¹

It was previously shown by Laine *et al.*, that estradiol did not affect CYP2C19 enzyme activity *in vitro*,⁷⁰ which is not only contradictory to our results but also to the studies suggesting that CYP2C19 metabolizes this specific estrogen.⁷⁵ Even though the effect of EE on CYP2C19 activity needs to be confirmed *in vivo*, our data suggests that also hormone replacement therapy could impact the metabolism of other CYP2C19 substrates. However, the transcriptional inhibition by ETE is observed at much lower concentrations than EE thus suggesting that OCs are more likely than HRT to cause important drug-interactions.

This is the first study showing an effect of estrogens on *CYP2C19* gene regulation and the transcriptional inhibition by ETE and EE was seen at much lower and more clinically relevant concentrations than for direct enzyme inhibition. We can conclude that transcription factor-mediated regulation is probably the major mechanism by which estrogens inhibit CYP2C19 activity.

4.2 | Paper II - Decreased hippocampal volume and increased anxiety in a transgenic mouse model expressing the human *CYP2C19* gene

The human *CYP2C19* gene displays high degree of polymorphism leading to absent, decreased or increased enzyme activity. This polymorphism has a great impact on drugs metabolized by CYP2C19 but has also been associated with depressive symptoms and personality traits.^{85,86,92} So far, no confirmed phenotype has been proposed for humans with regards to CYP2C19 enzyme activity besides the metabolic phenotypes described in the introduction. To elucidate the possible effects of high CYP2C19 expression, a transgenic mouse model expressing the human enzyme was investigated. The study was performed with emphasis on brain development, behavior and characterization of the hippocampal formation.

4.2.1 | CYP2C19 and brain development

4.2.1.1 | Brain morphology at PND0

The hippocampus

Homozygous mice for the human *CYP2C18/CYP2C19* locus displayed high neonatal lethality, thus suggesting that CYP2C19 might affect development. As a part of elucidating possible developmental effects of the genetic insert, brain morphology in CYP2C19Tg-Hom (n=4), CYP2C19Tg-Hem (n=5) and Wt (n=3) mice at PND0 was investigated. Upon visual assessment of brain morphology some rather striking abnormalities could be observed in the CYP2C19Tg-Hom brains. The hippocampal formation displayed an overall smaller appearance (about 60 %, Supplementary Figure 2, paper II) with the different areas and layers appearing less developed as seen in Figure 5b-c (Figure 1, paper II). When comparing with a mouse brain developmental atlas, CYP2C19Tg-Hom hippocampi much resembles developmental stages E17-18.¹⁹² This implicates that the hippocampal development is either terminated at this time-point or, perhaps more likely, that the development of the hippocampal formation is delayed in the transgenic mice. This was however not seen in hemizygous mice thus suggesting a gene-dose effect.

Commissural agenesis

The development of white matter structures are severely affected in CYP2C19Tg-Hom pups. Homozygous pups display total commissural agenesis, as seen in Figure 5a-b (Figure 1, paper II), involving the corpus callosum (CC), hippocampal commissure (HCC) and the anterior commissure (AC). These trajectories are the major connections between the cerebral hemispheres and the defects seen indicate a global defect in the midline crossing of commissural fibers.^{193,194} Such a delayed hippocampal development and total commissural agenesis do however not explain the premature death of these pups. However, induction of callosal agenesis in animal models often triggers agenesis in other major callosal tracts as well and this might also include deficits of the internal capsule that in many cases leads to perinatal death.¹⁹⁵ The internal capsule has not been

investigated in the CYP2C19Tg-Hom mice but malformation of this capsule could however be the explanation for the early death of these mice. Callosal tract development is not the scope of this study since callosal defects are not observed in our visual assessment of the CYP2C19Tg-Hem brains at PND0. We can however not exclude the possibility of a callosal hypogenesis (partial agenesis) in the CYP2C19Tg-Hem mice although preliminary studies do not indicate this to be the case. Investigating commissural fiber thickness and density in adult CYP2C19Tg-Hem mice could be important for further understanding of the displayed adult phenotype. Complete or partial agenesis of the major commissures is one of the most commonly observed congenital malformations of the human brain,¹⁹⁴ with agenesis of the CC occurring in approximately 1:4,000 live births.¹⁹⁶ Commissural agenesis or reduced size and density have been associated to many syndromes and disorders,¹⁹⁴ however mostly studied in autism spectrum disorders (ASD),^{193,196,197} MDD,¹⁹⁸⁻²⁰⁰ and attention-deficit hyperactivity disorder (ADHD).^{193,201}

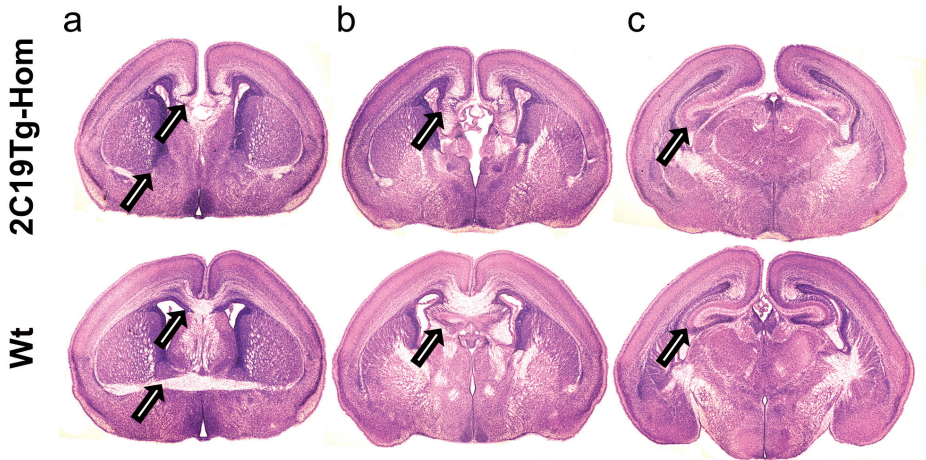


Figure 5 | Brain morphology at PND0 in CYP2C19Tg-Hom pups and Wt controls. Images are cresyl violet stainings of Wt (n=3) and CYP2C19Tg-Hom (n=4) pups at PND0. **(a-c)** The representative images display coronal sections from 3 rostral to caudal positions. **(a-b)** CYP2C19Tg-Hom mice display a distinct morphological phenotype with complete commissural agenesis. As pointed out by arrows on each image, the corpus callosum (top arrow **a**, and **b**), the hippocampal commissure (**b**) and the anterior commissure (bottom arrow **a**), are all essentially lacking axons crossing over the midline of the hemispheres. **(b-c)** The hippocampal formation in CYP2C19Tg-Hom pups much resembles developmental stages E17-18 thus suggesting a stalled or delayed development of this structure. Brain sections from CYP2C19Tg-Hem mice were indistinguishable from those of Wt litter mates. Figure from paper II.

Since CYP2C19Tg-Hom pups only survive for a few days after birth, all adolescent and adult studies were performed on hemizygous mice. None of the above mentioned morphological changes were apparently seen in CYP2C19Tg-Hem pups; however a

more in depth evaluation of hippocampal size and commissural integrity at PND0 has not been performed.

4.2.1.2 | *CYP2C19* is expressed during brain development in *CYP2C19Tg-Hem* mice

As described above, severe morphological changes were found in the brains of PND0 *CYP2C19Tg-Hom* pups. Further investigations on developmental effects were focused on *CYP2C19Tg-Hem* mice and expression of *CYP2C18* and *CYP2C19* was investigated during embryonic development, early postnatal days (gender unknown) and in 7-week-old male mice.

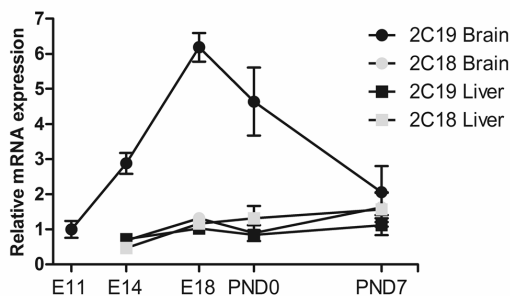


Figure 6 | Expression of *CYP2C19* and *CYP2C18* mRNA in brain and liver tissue during embryonic development and early postnatal days. *CYP2C18* and *CYP2C19* mRNA expression pattern was investigated in liver and brain tissue during embryonic development (embryonic day 11 (E11), E14, and E18) and early postnatal days (postnatal day 0 (PND0) and PND7). The graph displays relative *CYP2C18* and relative *CYP2C19* mRNA expression levels in fetuses and pups of unknown gender. As evident from the graph, there is a brain specific expression of *CYP2C19* mRNA that peaks at E18, with a 6-fold higher brain expression compared to liver. The expression of the *CYP2C18* gene was consistently low in both tissues and no expression of *CYP2C18* or *CYP2C19* mRNA was detected in Wt mice. Four to five mice were analyzed for each time-point and data are presented as mean with S.E.M. Figure from paper II.

Little is known regarding the ontogeny of *CYP2C18* in humans²⁰² and *CYP2C18* protein has furthermore never been detected in either adult (26-31 weeks) *CYP2C18/CYP2C19* transgenic mice¹⁷² or in adult human liver.^{36,203} *CYP2C18* mRNA expression was however investigated during mouse development since the gene might display a specific developmental expression, like e.g. *CYP3A7* in humans.^{204,205} *CYP2C18* mRNA levels were however found consistently low throughout the development in both liver and brain tissue, Figure 1, paper II. Expression analysis of *CYP2C19* however, revealed relatively high mRNA levels in the brain at E14, E18 and PND0, with a peak value of more than 6-fold (E18) of that seen in liver as seen in Figure 6. It is rather difficult to compare mouse and human brain development. However, the E18 expression peak in mouse brain can roughly be translated to gestational week 15 in human brain development.²⁰⁶ Since the hippocampal formation seemed to be one of the most effected structures in the *CYP2C19Tg-Hom* pup, specific hippocampal *CYP2C19* expression was investigated in four E18 *CYP2C19Tg-Hem* embryos. No significant differences were seen in expression between the four pooled

hippocampal samples and the cortex or the rest of the cerebrum indicating that the expression pattern is rather uniform in the developing brain (Supplementary Figure 1, paper II). Hepatic expression of *CYP2C19* was constantly low during the embryonic, fetal and postnatal time-points investigated as clearly visualized in Figure 6. At 7 weeks of age a clear shift in hepatic and brain expression was observed, with silencing of the expression in brain tissue and a clear induction of hepatic expression (Figure 1b, paper II). This is in line with previously published expression data from adult CYP2C19Tg-Hem mice with relatively low brain expression and high hepatic levels of *CYP2C19* mRNA.¹⁷² Mice have a large Cyp2c family of proteins, however no direct homologues to CYP2C18 and CYP2C19,¹⁷⁸ thus making the observed human *CYP2C19* expression specific for the transgenic mice.

The ontogeny of CYP2C19 has been studied in human liver where expression is rather constant, and relatively high, throughout gestation. CYP2C19 expression levels range between 10-20 % of adult liver values.^{205,207} It is hypothesized that CYP2C19, and other CYPs with similar gestation expression patterns, might have important developmental roles that could be different from their functions in adult liver.²⁰⁴ This is interesting in correlation to our data even though the developmental expression pattern in liver is rather different between the species.

4.2.1.2 | Human brain expression of CYP2C19

Expression of CYP2C19 during human brain development has never been investigated. As described above, it is however one of the highest expressed CYPs in the liver during human development thus suggesting that it might have important endogenous functions.^{205,207} In light of the embryonic expression pattern found in the transgenic mice, cerebral cortical brain samples from three different human fetal donors were investigated. *CYP2C19* mRNA quantifications showed that *CYP2C19* can be expressed at a level representing approximately 0.5 % of that found in human adult liver. Two different cerebral cortical samples of the donor from gestational week 24 displayed the highest *CYP2C19* expression, showing approximately 0.3 and 0.6 % of the levels in adult human liver. This was found in two separate regions and is interesting since it corresponds rather well to the expression peak at E18 in the transgenic mouse brain.²⁰⁶ Samples were compared to adult liver expression where CYP2C19 is one of the most abundant CYPs and probably expressed in about 50 % of all hepatocytes. Therefore, CYP2C19 expression in the fetal samples could be rather significant especially since the expression most likely is localized to a specific region and even a specific cell type.

It is of course important to remember that this is a pilot study with an exceptionally limited number of samples. However, studying human fetal samples are difficult since samples are extremely rare and therefore the results presented are important for future investigations of CYP2C19s possible endogenous role in human brain development.

4.2.2 | CYP2C19s effects on behavior in the CYP2C19Tg-Hem mice

4.2.2.1 | Hyperactivity and stress sensitivity

The open-field assessment and the tail-suspension test

One major phenotypic characteristic observed in the CYP2C19Tg-Hem mice is that they display increased handling stress, i.e. they are more difficult to move between cages and do not seem to respond as well to the handling before behavioral testing. As an initial step in the behavioral investigation of the CYP2C19 transgenic mouse model, transgenic mice and Wt controls were exposed to the open-field (OF) test. Many different aspects of behavior can be assessed in the OF and here we studied motor activity and anxiety-related behavior.^{166,167} Mice were exposed to the OF for 30 minutes where CYP2C19Tg-Hem mice displayed an increased locomotor activity, with an overall longer distance travelled during the session, compared to Wt litter mates at both ages investigated as seen in Figure 7a-b (Figure 5a-b, paper II). This suggests that the observed walking phenotype with extended elevation of hind paws, described in section 3.2.1, does not affect their walking abilities. The hyperactive behavior was seen already at 7 weeks of age thus indicating that this phenotype is established early in life.

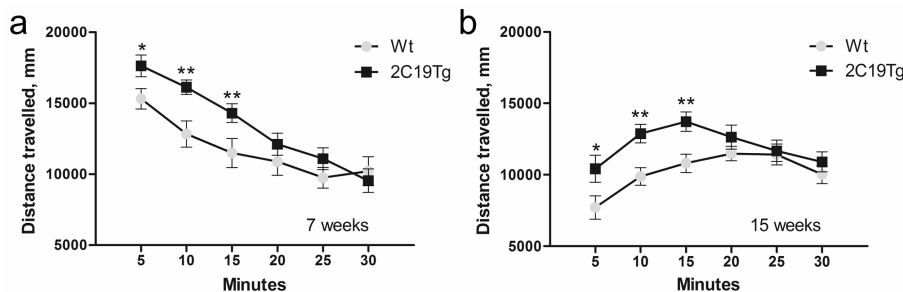


Figure 7 | CYP2C19Tg-Hem mice displayed increased reactivity to the open-field at both 7 and 15 weeks of age. CYP2C19 transgenic mice and Wt controls were assessed in the open-field arena for 30 minutes to investigate motor function. **(a-b)** CYP2C19Tg-Hem mice displayed increased motor activity during the first 15 minutes of the test when investigating distance travelled over time. This was true for both 7- and 15-week-old transgenic mice when compared to Wt controls. Data is presented as distance travelled over time in 5 minute bouts presented with mean \pm S.E.M. The Student's t-test was performed on each bout. * $p < 0.05$ ** $p < 0.01$ Figure adapted from paper II.

For all rodents the open, highly illuminated arena is a potential threat and therefore stressful.^{158,159} Transgenic mice displayed an increased reactivity to the novel environment compared to Wt mice as seen in Figure 7a-b (Figure 5a-b, paper II). However, CYP2C19Tg-Hem mice were not hyperactive for the whole session period. They were habituating to the same activity levels as Wt mice after approximately 20 minutes, which most likely corresponds to when the novelty stress decreases. Similar novelty-induced hyperactivity has been observed in for example bulbectomized rats.²⁰⁸

This hyperactive response differs from animal models of for example ADHD, where the hyperactivity usually starts when the environment gets familiar,²⁰⁹ and normally does not adapt to Wt values.^{210,211} The CYP2C19Tg-Hem mice display a low but significant hyperactive response in the OF and adapt to Wt values after approximately 20 minutes in the open-field suggesting that the hyperactivity is caused by novelty stress rather than by an ADHD phenotype. No difference in anxiety-like behavior was observed at any age when calculating exploration time in different parts of the arena. This aspect is further discussed in section 4.2.2.3.

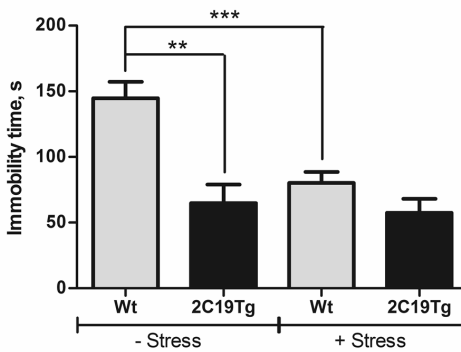


Figure 8 | Immobility time in the tail-suspension test before (-Stress) and after Morris water maze training (+Stress). CYP2C19Tg-Hem mice and Wt controls were subjected to the TST and immobility time was manually calculated during the 6-minute session period. 15-week-old CYP2C19Tg-Hem mice (n=10) displayed 55 % less time immobility time compared to controls (n=6), here referred to as - Stress. A separate group of mice were subjected to the TST after the MWM, here referred to as a stressor (+Stress). Only Wt (n=10) mice were affected by this pre-exposure displaying reduced immobility, comparable with transgenic mice both with (n=8) and without prior stress exposure. ** p<0.01 *** p<0.001 Figure from paper II.

Transgenic and Wt mice were also exposed to the TST to investigate any potential depression-like behavior. The TST is mostly used for its predictive validity of antidepressants and is one of the most commonly used tests in rodents for assessing new antidepressant drugs. Immobility time in the TST is generally considered as depression-like behavior since most antidepressants reverse this phenotype.¹⁶² As seen in Figure 8 (Figure 5c, paper II), 15-week-old CYP2C19Tg-Hem mice exposed to the TST displayed 55 % less immobility than Wt controls, thus not indicating a depressive phenotype. This behavior was not present in younger mice where no differences in immobility time could be observed. Reduced immobility time in the TST is most commonly interpreted as an antidepressant-like behavior, however, when phenotyping transgenic mice it is probably more appropriate to consider this response as increased stress-sensitivity.²¹²⁻²¹⁴ This interpretation is in line with the results from the OF suggesting an increased stress response in the CYP2C19 transgenic mice.

Acute restraint stress and plasma levels of corticosterone

CYP2C19Tg-Hem mice display an increased reactivity to novelty and stressful situations as showed by the OF and TST assessments. This together with the apparent hippocampal phenotype we wanted to further investigate the stress sensitivity of the CYP2C19Tg-Hem mice. Corticosterone (CORT) is the major stress hormone in rodents²¹⁵ and depletion or sustained high levels of CORT is known to affect morphology and survival of hippocampal neurons (reviewed by Hansson and Fuxe, 2008).²¹⁶ Even though the *CYP2C19* transgenic mice displayed a more stress sensitive behavior in these tests including increased handling stress, when exposed to restraint stress, no differences were seen in CORT levels. CORT levels were almost identical in CYP2C19Tg-Hem mice and Wt littermates at both 7 and 15 weeks of age when assessed without stress, after 30 minutes of restraint stress, and after 30 minutes of stress plus a 30 minute recovery period, see Supplementary Figure 7, paper II. It could be hypothesized that these mice have the capacity to sustain a normal hormonal response to stressors when not exposed for a longer period of time, i.e. during the acute restraint stress. In the MWM CYP2C19Tg-Hem mice acquire a behavior similar to the floating/despair-behavior observed in the forced-swim test (FST).^{217,218} In the FST test, increased floating behavior is considered depressive-like, in the same way as immobility in the TST. Therefore it would be interesting to challenge the transgenic mice with a more chronic stress paradigm such as chronic mild stress, including cage tilt, over-night illumination etc. over a period of weeks or months.¹⁶³ It could be hypothesized that *CYP2C19* transgenic mice have difficulties coping with a more chronic stressor thus more easily developing a depressive phenotype. It would also be of value to further evaluate HPA-axis function and adaptation during chronic stress treatment.

The initial objective of this study was to evaluate if the CYP2C19 transgenic mice displayed any depression-like behavior. Due to the early behavioral findings in the mice the project turned on a different track and focused more on trying to evaluate the displayed stress sensitivity and hippocampal phenotype. In retrospect it would have been interesting to evaluate a potential depressive phenotype in more detail. There are many tests that could have been performed and that might add important knowledge and insight into the phenotype observed in these mice. For example the sucrose-preference test investigates any anhedonia related phenotypes correlating to another endophenotypes of human depression.¹⁶²

4.2.2.2 | Spatial learning and despair behavior in the CYP2C19Tg-Hem mice

As an attempt to evaluate spatial learning abilities and hippocampal function adult CYP2C19Tg-Hem mice and Wt litter mates were trained and assessed in the Morris water maze (MWM), see Figure 9. Unexpectedly, CYP2C19Tg-Hem mice developed a rather distinct behavior over the 5 day training week. They displayed a gradually increasing floating or despair behavior that was not seen in the beginning of the training week, something that was furthermore not seen in the Wt controls at any day, see Figure

9b. However, three out of ten transgenic mice did not acquire this despair behavior and displayed similar or perhaps even better spatial memory during the retention test as clearly visualized in Figure 9c-d. However, since the number of individuals in the transgenic group for evaluation of the test got rather small it is difficult to draw any conclusions from the results, but they do however suggest that the CYP2C19Tg-Hem mice have normal learning abilities in the MWM.

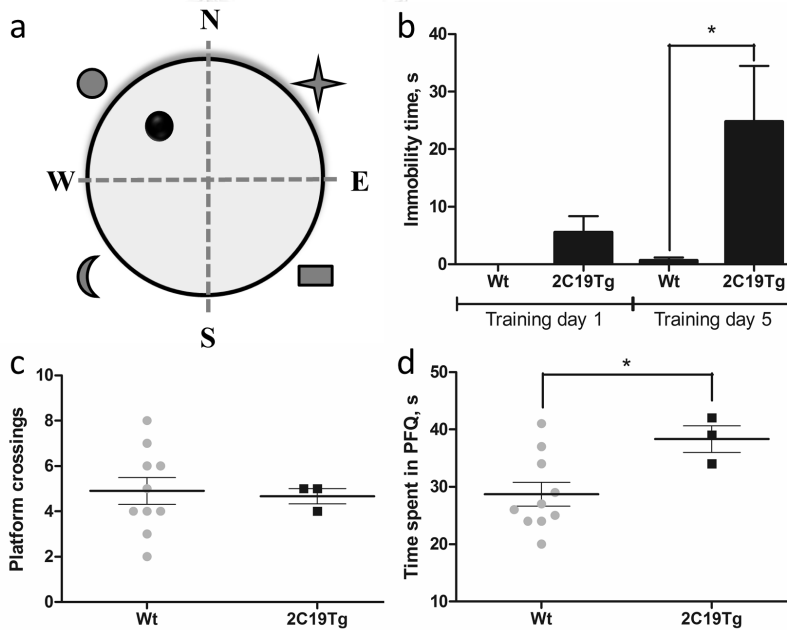


Figure 9 | Spatial learning and hippocampal function was evaluated in the Morris water maze (MWM). (a) Experimental set-up of the MWM. 15-week old male mice were placed in a water pool divided into 4 quadrants named; northwest (NW), northeast (NE), southeast (SE), and southwest (SW). A transparent platform (10 cm) was placed in the NW quadrant with four visual cues placed around the pool, here represented by the star, moon, circle, and rectangle. MWM training sessions were performed with a hidden platform that was removed during the retention test. (b) Over the 5 day training period CYP2C19Tg-Hem mice gradually developed a floating or despair behavior in the water maze. (c-d) During the retention test learning and memory evaluation was performed by manually calculating number of platform crossings and total time spent in the platform quadrant (PFQ), during 60 s. All mice displaying more than 10 s of floating behavior were excluded. CYP2C19Tg-Hem mice do not differ in their ability to learn and navigate in the water maze as displayed by the number of platform crossings and time spent in the PFQ during the retention test. Data are presented as mean with S.E.M. * $p < 0.05$

Spatial navigation learning in the MWM is highly correlated to hippocampal neurogenesis in mice.^{184,185} Even though a drastic reduction of DCX positive cells can be seen in the DG of CYP2C19 transgenic mice (Figure 14) it does not seem to affect the learning abilities in the MWM. However, in correlation to the reduced hippocampal size, these behavioral data support our postulated hypothesis that the CYP2C19Tg-Hem mice display a specific size reduction of the caudal/ventral part of the hippocampus.

This part is thought to be more involved in the regulation of anxiety-like behavior but is not as related to learning abilities and memory function.^{113,115,116}

Rodents react differently to short- and long-term stress and when repeatedly exposed to a stressor the response can either stay the same, habituate (decrease), or increase.²¹⁸⁻²²⁰ The CYP2C19 transgenic mice display hyperactivity in the TST, an intense short term stressor and develop a despair behavior during MWM training, a relatively mild but long-lasting or repeated stressor. The reasons for these different responses remain to be elucidated but it could be hypothesized that the transgenic mice have difficulties in chronic stress coping. Another TST was performed on the MWM mice three days after the retention test to evaluate if this long term stress exposure also would affect their performance in this test. Interestingly, no effect was seen on the CYP2C19Tg-Hem mice that displayed a similar immobility time as seen previously, without the MWM stress; see paper II, Figure 5c. However, acquired despair behavior normally only stays for around 2-3 days, and thus could have vanished by the time they were tested in the TST. Wt mice however, significantly reduced their immobility time down to CYP2C19Tg-Hem levels. My interpretation of these differences in behavior is that the long term mild stressor, i.e. the MWM induced despair behavior in the transgenic mice, which either disappeared over three days resting or is not transferrable between tests. Wt mice on the other hand acquired an increased stress-sensitivity, comparable to transgenic behavior prior stress exposure.

It might be of value to perform a more extensive evaluation of memory and learning ability in the CYP2C19Tg-Hem mice using other hippocampus-related tasks in addition to the MWM. Such a study could for example include Olton's radial arm maze, which is furthermore also considered less stressful than the MWM.²²¹ Although the MWM test did not address memory due to the unexpected behavior of the CYP2C19 transgenic mice, this response instead supports a disturbed affective phenotype.

4.2.2.3 | Anxiety-related behavior

The LDB measures the conflict between rodent curiosity in exploring new environments and their fear of open, highly illuminated places. Spending less time in the light box is considered as increased anxiety-like behavior, mainly since anxiolytic drugs make rodents more prone to investigate the light environment.^{183,222} Fifteen-week-old CYP2C19Tg-Hem mice spent significantly less time in the light compartment thus suggesting a more anxious phenotype. In adult mice this behavior is significant with a reduction of time spent in the light compartment of 42 %, a similar trend was observed in 7-week-old mice as seen in Figure 10 (Figure 5d, paper II).

Interestingly, the CYP2C19Tg-Hem mice do not display any anxiety-related behavior in the OF, where spending less time in the central part of the arena is interpreted as an anxious phenotype.¹⁶⁷ However, results from the OF and LDB do not always overlap even though they are considered similar in their unconditioned avoidance of a potential threat. The differences seen are most probably due to the fact that there seems to be

some construct differences between these tests, i.e. that they are measuring different aspects of anxiety.²²³

Other tests exploring similar aspects of anxiety-like behavior in rodents are available, such as the elevated-plus maze. Many anxiety-related animal models do display anxiety-related behaviors in at least two of these tests. It would therefore be interesting to investigate the CYP2C19Tg-Hem mice in the EPM for a more in depth evaluation of anxiety-related behavior in our model.

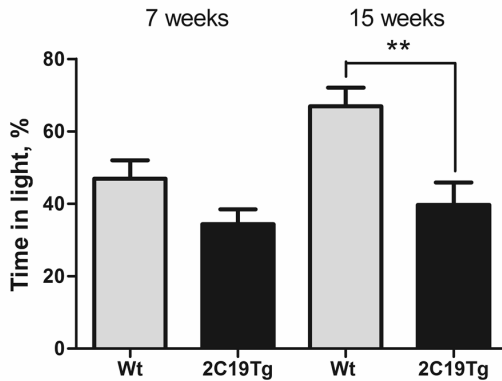


Figure 10 | CYP2C19Tg-Hem displayed anxiety-like behavior in the light-dark box at 15 weeks of age. During 5 minutes CYP2C19Tg-Hem and Wt mice were investigated in the light-dark box test. The time spent in the light and dark compartments was recorded and 15-week-old CYP2C19Tg-Hem mice displayed significantly less time in the light compartment compared to Wt mice (n=15 mice/group), thus indicating an anxiety-like phenotype. At 7 weeks of age a similar trend was observed. ** p<0.01 Figure from paper II.

All mice were also exposed to the SIH, mostly used for evaluating anxiolytic drugs. Anxiolytic drugs reduce the increase in body temperature seen after stress exposure,¹⁷¹ very similar to the way the TST is used for evaluating antidepressant effects of drugs. However, no differences were seen in the increase in body temperature after stress. When subjected to the SIH test, both age groups and both genotypes responded with a similar stress-induced hyperthermia something that was not presented in paper II due to space limitations. As seen in Figure 11, a higher basal body temperature was however observed in CYP2C19Tg-Hem compared to Wt mice at both 7 and 15 weeks of age. This could be caused by different factors, including dysfunctional hypothalamic regulation.²²⁴ Whether the higher body temperature observed in CYP2C19Tg-Hem mice is caused by an increased psychological stress remains to be further investigated but it is an intriguing possibility in combination with the observed behavioral phenotype.

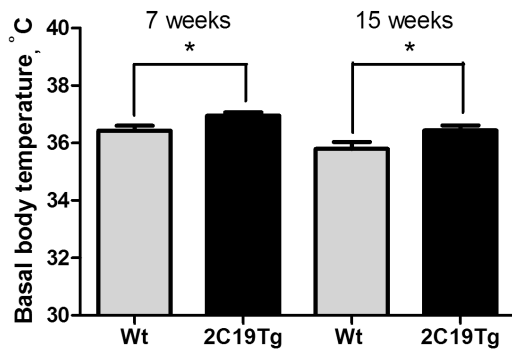


Figure 11 | Basal body temperatures of CYP2C19Tg-Hem mice and Wt controls. Rectal temperatures were measured and a significant increase in basal body temperatures was found between CYP2C19Tg-Hem mice and Wt controls at both 7 and 15 weeks of age. Data are presented as mean with S.E.M. * $p < 0.05$ Figure from Supplementary Information, paper II.

We have not investigated the effects of anxiolytic drugs on the behavior observed in the OF, LDB, and the SIH. However, we are planning to evaluate the anxiety-like behavior seen in the LDB by administrating anxiolytic drugs. This is an important aspect since a positive response of the anxiolytic drugs in the LDB would add predictive validity to our potential anxiety model.¹⁶⁶ Regarding all three behavioral tests, LDB, OF, and TST, we observed that the behavioral phenotype in the CYP2C19Tg-Hem mice was more pronounced in young adult, 15-week old compared to adolescent, 7-week-old mice. So despite the plausible effect of CYP2C19 on brain development, the behavioral effects do not manifest until young adulthood, very much like MDD and other neuropsychiatric disorders in humans where the manifestation normally occurs at this age.^{93,225}

4.2.3 | The hippocampal formation in CYP2C19Tg-Hem mice

Due to the behavioral findings and the drastic developmental effects on hippocampal morphology much focus was spent on this important limbic structure throughout paper II. The hippocampus is mostly known for its important functions in learning and memory formation, stress regulation and emotional processing.^{46, 110,226, 134}

4.2.3.1 | Hippocampal size

To investigate hippocampal volume or size in CYP2C19Tg-Hem mice two methods were used: area measurements of sections and MRI. Sections from both 7- and 15-week old mice were measured using cresyl violet stainings of 12 sections per mouse covering the whole hippocampal formation. A reduction in hippocampal size was seen in CYP2C19Tg-Hem mice at both 7 (12% reduction) and 15 weeks of age (11% reduction). Interestingly, the systematic sectioning revealed that this size reduction was not uniform throughout the structure but rather specific for the caudal-ventral part of the hippocampus as seen in Figure 12c-d (Figure 2b-c, paper II). Fifteen-week-old mice were furthermore also examined using a 9.4 T Varian MRI system to confirm the results from the measured sections. As clearly visualized in Figure 12 a-b these volumetric analyses revealed smaller whole brain (3.1 %) and hippocampal volumes (7.1 %) in CYP2C19Tg-Hem compared to Wt mice. Probably due to the low number of

animals investigated, no correlation was found between whole brain and hippocampal volumes. Hippocampal volumes were therefore not normalized against whole brain volumes. However, it is quite clear that the smaller hippocampal volume is due to a specific reduction of the caudal-ventral part as shown by sectioning analyses and does not affect the whole structure, see Figure 12c-d (Figure 2b-c, paper II). Many studies suggest that the hippocampal formation has distinct functions along its dorso-ventral axis, with the caudal-ventral part of the hippocampus being mostly involved in the regulation of stress response and emotional processing, in particular regulation of anxiety-related behavior in mice.^{116,118} This fits well with the behavioral phenotype observed in the CYP2C19 transgenic mice. That the hippocampus is found to be smaller already in young adolescent mice suggests that this might be a congenital effect, something that is further proven by the drastic changes seen in CYP2C19Tg-Hom mice at PND0.

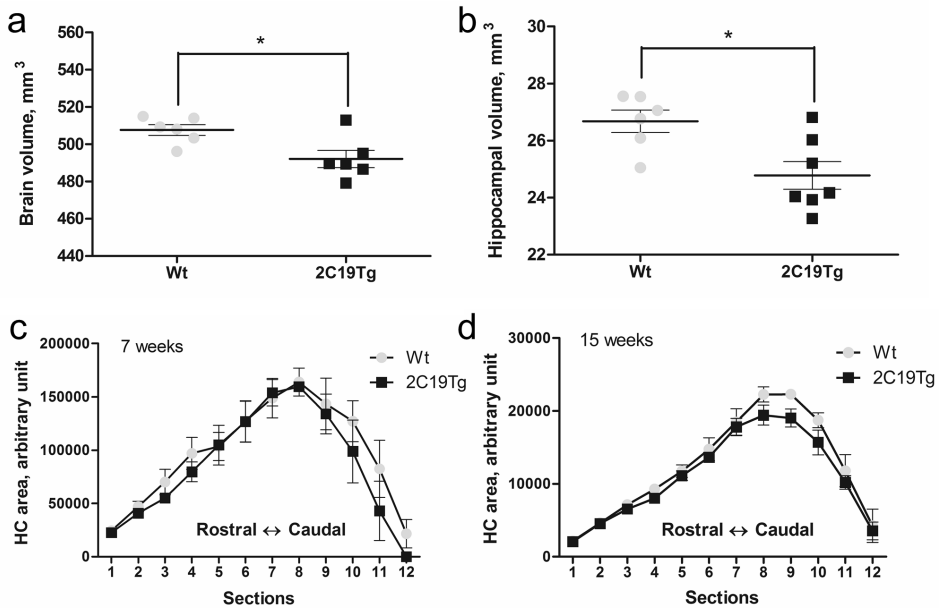


Figure 12 | Smaller brain and hippocampal volumes in CYP2C19Tg-Hem mice. (a-b) Whole brain and hippocampal volumes were investigated in 15-week-old CYP2C19Tg-Hem mice and Wt controls (n=7/group) using a 9.4 T Varian magnetic resonance imaging system. Manual outlining using the software ITK-SNAP revealed smaller brain (3.1 %) and hippocampal volumes (7.1 %) in CYP2C19Tg-Hem mice. (c-d) Hippocampal volumes were also estimated by systematic sectioning measuring the hippocampus in 12 coronal sections, covering most of the hippocampal formation. This clearly visualizes the specific reduction of the caudal-ventral part of the hippocampus in both 7- (c) and 15-week-old mice (d). Data are presented as mean with S.E.M. *p<0.05. HC, hippocampal. Figure from paper II.

4.2.3.1 | The hippocampal formation, neurogenesis and stress coping

Proliferation and DCX positive cells in the dentate gyrus

In the DG of the hippocampal formation in mice, the neurogenesis process has been found to be critical for hippocampal function^{84, 85, 207,208} and reduced neurogenesis has been suggested as a possible explanation for reduced hippocampal volumes in depression and other psychiatric disorders. However, CYP2C19Tg-Hem mice do not display any differences in the classical proliferation markers, BrdU and Ki-67. Thus, no differences in proliferation rates were detected at either 7 or 15 weeks of age in the DG of transgenic mice.^{149,150} Furthermore, no differences in survival of BrdU positive cells in the DG were seen four weeks after BrdU injections in young mice as seen in Figure 13. Total numbers were also corrected for hippocampal size (from MRI study) not changing the results, see Supplementary Figure 5, paper II.

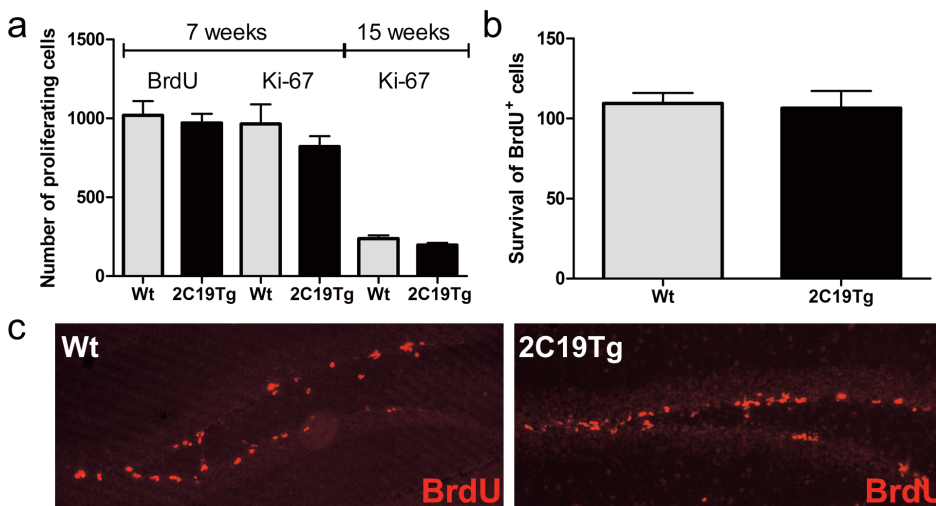


Figure 13 | Investigation of proliferation rates and survival of newborn cells in the dentate gyrus of CYP2C19Tg-hem mice and Wt controls. (a) Number of proliferating cells in the dentate gyrus as measured by BrdU incorporation and Ki-67 expression at 7 weeks of age and Ki-67 at 15 weeks of age. No differences were detected between the genotypes. **(b)** CYP2C19Tg-Hem mice and controls were injected with BrdU and sacrificed 4 weeks later to investigate survival rates of newborn cells. No differences were seen in number of BrdU positive cells at this time point. **(c)** Representative images of proliferating cells in the dentate gyrus of Wt and CYP2C19Tg-Hem mice. Figure adapted from Supplementary Figure 4, paper II. Photo: A. Persson

The neurogenesis process has been intensively studied in mice and displays a complex pattern with many different cell types and specific markers. As a part of the maturation process of new neurons in the DG, cells migrate in to the granule cell layer and are incorporated in the hippocampal circuits. This specific cell type is referred to as migrating immature neurons and can be visualized by the cytoskeleton marker DCX. As

seen in Figure 14 (Figure 4, paper II) adult CYP2C19Tg-Hem mice display a drastic decrease of the DCX expressing cell population in the DG, with a mean reduction of 42 % compared to Wt controls. DCX is sometimes used as a proliferation marker but in the CYP2C19 mice a specific reduction of this marker is seen without any effects on proliferation. Knocked-out neurogenesis, leading to few DCX positive cells in the rodent DG, can lead to impaired negative feed-back on the HPA-axis.¹¹⁰ If this response is dependent on DCX cells is still not known but these cells do have specific properties, displaying increased excitability and are suggested to be important in hippocampal structural plasticity including axonal guidance and dendrite sprouting.¹⁴³⁻¹⁴⁵ As mentioned, we did not see any differences in corticosterone levels when exposing the transgenic mice to acute stress.

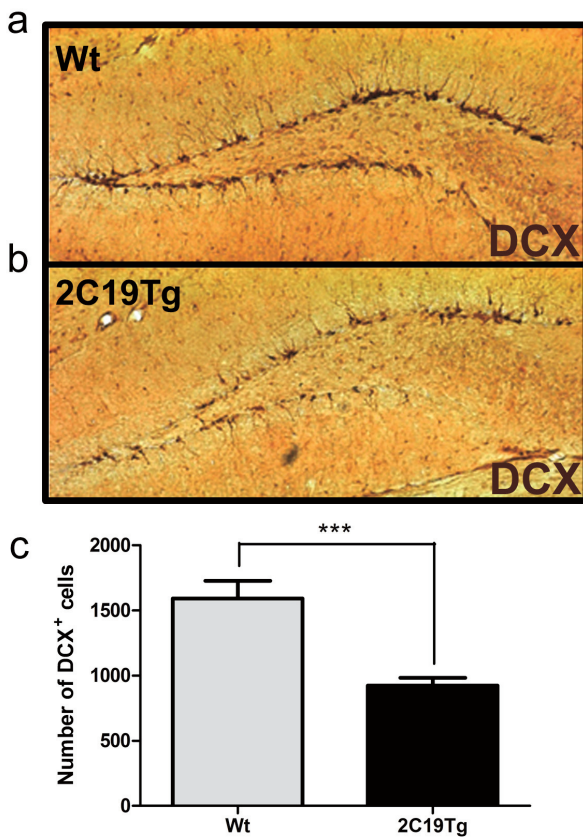


Figure 14 | DCX positive cells are drastically reduced in the dentate gyrus of CYP2C19Tg-Hem mice compared to Wt controls. (a-b) Using immunohistochemistry immature neurons expressing DCX were visualized in the dentate gyrus of Wt and CYP2C19Tg-Hem (2C19Tg) mice. CYP2C19Tg-Hem mice displayed a drastic reduction of immature neurons in the dentate gyrus of the hippocampal formation. **(c)** Number of immature neurons is significantly reduced (42 %) in the hippocampal dentate gyrus of CYP2C19Tg-Hem mice. *** $p < 0.001$ Figure from paper II.

If DCX cells are regulating the release of corticosterone, remaining cells are probably sufficient to respond and regulate the acute stress response. It would however be interesting to investigate how the response might be to a more chronic stressor. DCX positive cells are rather abundant in the DG, as seen in Figure 14 and hippocampal neurogenesis and DCX positive cells are most probably crucial for hippocampal mediated learning.^{114,151} However, despite the drastic reduction of DCX positive cells in the hippocampus of the CYP2C19 transgenic mice, spatial learning does not appear to be deficient in these mice. It could be possible that they have “enough” cells or that the cell population is in fact still inducible in the CYP2C19Tg-Hem mice when exposed to a learning task.

It is difficult to draw any conclusions regarding behavior and the reduction of immature neurons seen in the hippocampi of CYP2C19 transgenic mice. It is however an intriguing discovery and this animal model could be used for further studies and understanding of this specific cell population. Nonetheless, it is rather clear that such a large reduction of this specific cell population would cause changes in hippocampal signal processing and overall function. Since the proliferation rate and the survival of proliferating cells are not affected in the transgenic mice it appears to be a specific reduction of immature neurons or possibly a defect in the neuronal maturation process in the DG.

Inhibitory circuits in the hippocampus

GABAergic interneurons are highly integrated in the hippocampal circuits and are important for overall signal processing in the DG. PA positive cells, one type of GABAergic interneurons, are abundant and critical for inhibitory signaling in the hippocampus.²²⁷ In the DG of 15-week-old CYP2C19Tg-Hem mice, a 22 % reduction was seen of PA positive cells compared to Wt mice as seen in Figure 4, paper II. However, no other difference in this cell population was detected within the CA regions of the hippocampus. The interneurons of the DG are highly integrated in the hippocampal circuits and it is still rather debatable whether all these cells are of developmental origin or if there is a possibility of adult-generated GABAergic interneurons in the hippocampus.²²⁸ This makes it difficult to speculate if the changes we observe in PA cell numbers are developmentally based or modulated in postnatal life. GABAergic interneurons are believed to aid the maturation process of new neurons both during development and in the adult hippocampus so reduced GABAergic input to progenitors within the DG might cause the lower numbers of DCX-cells.²²⁸ The reduced number of PA-positive cells in the DG could also influence overall hippocampal activity due to a reduced inhibition, and hyperactivity of the hippocampus has previously been associated with reduced GABAergic transmission.²²⁹

One issue in counting cells in a structure that is smaller in the transgenic mice is that there might be a bias towards reduced number of cells due to the smaller volume. The differences in cell numbers were however seen in the DG, a part of the hippocampal structure that does not appear smaller. However, as a control for the smaller hippocampal size, corrections for hippocampal volume as measured by MRI was used

with no major changes for any of the IHC markers apart from PA positive cells in the DG that showed border-line significance after size correction (Supplementary Figure 5, paper II).

Stress reactivity in the hippocampal formation of CYP2C19Tg-Hem mice

Another aspect regarding the hippocampus in the CYP2C19 transgenic mice is the hippocampal response to stress. DCX and PA positive cells are involved in the hippocampal stress response in rodents and reduced levels of these cell populations have been implicated in increased stress sensitivity and hyperactivity of this particular brain structure.^{110,229} All mice were exposed to acute restraint stress and even though no differences could be seen in plasma levels of corticosterone between the genotypes, *c-fos* mRNA levels were significantly higher after 60 minutes (30 min restraint stress + 30 min recovery) in CYP2C19Tg-Hem mice. This pattern was seen at both 7 and 15 weeks of age (51 % and 46 %, respectively) as displayed in Figure 5e-f, paper II. There was however no differences in basal *c-fos* levels, but a steeper increase in levels can be seen already after 30 minutes of restraint stress. As mentioned previously, *c-fos* is extensively used as markers for neuronal activity^{230,231} and is induced by glucocorticoids through the glucocorticoid and mineralcorticoid receptors highly expressed in nearly all hippocampal neurons. The expression of *c-fos* is induced by different stressors, leading to increased mRNA and protein levels of c-Fos in the brain. The c-Fos protein dimerizes with members of the Jun family and forms the activator protein-1 (AP-1) complex, affecting the transcription of a wide variety of downstream genes.²¹⁶ Thus, it is impossible to speculate what effects the observed increase in *c-fos* expression in the hippocampus would have on hippocampal function but we can conclude that in response to acute restraint stress CYP2C19Tg-Hem mice display increased neuronal activation in the hippocampus that is not directly correlating to corticosterone levels. Studies in adrenalectomized rats have previously shown that *c-fos* induction is not always dependent on corticosterone but the mechanism behind this response is still not clear.²³² Since our study only measures *c-fos* mRNA levels in the hippocampus at three time points, with the latest measurement 60 minutes after initiation of the stressor, it is difficult to speculate if the increased mRNA expression is persistent for a longer period of time but could be interesting to investigate further.

4.2.4 | CYP2C19s endogenous function

It is well known that CYP2C19 is an important enzyme in drug metabolism and the displayed genetic polymorphism is important to investigate due to its effects on drug plasma levels and treatment outcome. The main hypothesis behind this thesis is that the phenotype observed in the transgenic model is caused by the CYP2C19 dependent metabolism of endogenous substrates during brain development. Before starting this thesis work some studies were suggesting that *CYP2C19* genetic polymorphism affect personality traits and depressive mood in humans.^{85,86,92} The study previously performed in our laboratory showed that loss of CYP2C19 activity was associated with less depressive symptoms.⁹²

CYP2C19 is mainly a hepatic enzyme but here found in the developing fetal brain. Expression of CYP2C19 has to our knowledge never been investigated in human fetal brain and our preliminary human study indicates that this is the case. Thus, a role of CYP2C19 in the metabolism of endogenous substances during brain development seems to be a likely explanation for the phenotypes observed. The nature of the proposed endogenous substrates for CYP2C19 thus constitutes a critical point. Due to the broad substrate specificity of CYP2C19 it is almost impossible to predict substrates from merely studying their structure. Using recombinant CYP2C19 and the HEK293 cell system described in this thesis we are however screening for potential endogenous substrates.

CYP2C19 is suggested to be involved in the metabolism of several different polyunsaturated fatty acids including arachidonic and eicosapentaenoic acid.^{3,81} If developmental brain expression is confirmed in humans it is possible that a local metabolism of these fatty acids could affect brain development and maturation.^{233,234} Most published data suggest that CYP2C19 is involved in the metabolism of steroid hormones including estrogens, progesterone, and testosterone.^{75,76,78} These steroids are known to be neuro-active and important both during brain development but also for postnatal brain maturation and plasticity.²³⁵⁻²³⁷ It is therefore possible that CYP2C19 could be involved in the metabolism of such steroid hormones and that high or low expression of CYP2C19 causes changes in hormone levels that effect brain development and the susceptibility to develop anxiety disorders.

5 | Conclusions

This thesis can be concluded as follows:

- The established HEK293 cell system, stably expressing the CYP2C19 enzyme, can be utilized for the effective and systematic investigation of CYP2C19-specific endogenous substrates and inhibitors
- 17 β -estradiol and 17 α -ethinylestradiol display direct inhibitory effects on CYP2C19 enzymatic activity as shown by the above mentioned *in vitro*-system
- Estrogens cause inhibition of CYP2C19 activity through transcriptional regulation at lower and more clinically relevant concentrations than required for direct enzyme inhibition suggesting transcriptional inhibition as the major mechanism of action of oral contraceptives on CYP2C19.
- A novel estrogen-responsive element was discovered in the CYP2C19 promoter region. ER- α binds to this site and mediates transcriptional inhibition of the CYP2C19 gene.
- CYP2C19 is expressed in the brain during embryonic development in the CYP2C19 transgenic mouse model. Our preliminary data suggests that this is also true for human brain development.
- CYP2C19Tg-Hom mice display high neonatal lethality and severe brain malformations with complete commissural agenesis and delayed hippocampal development at post-natal day 0, further suggesting a developmental role for CYP2C19.
- CYP2C19Tg-Hem mice lack an obvious neonatal phenotype but display increased stress sensitivity and anxiety-like behavior with the behavioral changes being more pronounced in adult compared to adolescent mice.
- CYP2C19Tg-Hem adolescent and adult mice furthermore display a hippocampal phenotype, with reduced size, a drastic reduction of immature neurons, and fewer GABAergic interneurons in the dentate gyrus of the hippocampus. Our data also suggests that the hippocampal formation is more stress-sensitive in CYP2C19Tg-Hem mice with neuronal hyperactivation, as measured by *c-fos* expression, after acute stress.

6 | General summary and future perspectives

Studies regarding CYP2C19 genetic polymorphism and related phenotypes have mostly been done with respect to its important function in drug metabolism. Besides displaying differential enzymatic activity with regards to this polymorphism, also drugs and other substances have shown to affect CYP2C19 activity. It is important to investigate factors that influence CYP2C19 function since this enzyme is involved in the metabolism of around 10 % of the drugs on the market today, and changes in enzymatic activity can lead to both lack of efficacy of drugs but also to serious side effects. Here we present evidence for a new transcription binding site in the *CYP2C19* promoter responsive to estrogen receptor α . Through this new binding site estrogens used in oral contraceptives mostly 17 α -ethinylestradiol, and hormone replacement therapy, mostly estradiol, inhibit transcription of the *CYP2C19* gene thus causing a reduction in enzyme activity. Also a direct effect was seen by these estrogens on enzyme function but the transcriptional regulation was seen at much lower and more clinically relevant levels, thus suggesting that this is the most likely mechanism by which estrogens inhibit CYP2C19-mediated activity. This is important since oral contraceptives are one of the most commonly prescribed drugs for women and several *in vivo* studies have previously shown that 17 α -ethinylestradiol mediates an inhibition of CYP2C19 activity. Less is known regarding the *in vivo* effects of estradiol but our study suggests that it could be important to also investigate the effects of this estrogen on CYP2C19-mediated metabolism.

The major part of this thesis has been dedicated to elucidate a possible CNS related endogenous function of the CYP2C19 enzyme. Human studies have suggested that CYP2C19 genetic polymorphism can predict personality traits and depressive mood. The characterized transgenic mouse model suggests that CYP2C19 might be involved in the metabolism of endogenous substrates involved in important developmental processes that affect brain development, leading to increased stress sensitivity and anxiety-like behavior later in life. It is still too early to suggest a potential substrate but by using recombinant CYP2C19 and the cell system described in this thesis, we are screening for endogenous substrates and are also investigating nirvanol-like structures as specific inhibitors of CYP2C19 activity. These inhibitors will soon be tested *in vivo* to explore if the phenotypic changes observed in the *CYP2C19* transgenic mice can be reversed by these compounds.

CYP2C19Tg-Hem mice display increased handling stress and display increased reactivity when exposed to novelty stress (OF) and a short term stressor (TST). Since the hippocampal formation is highly involved in stress reactivity and negative feed-back on the HPA-axis it is rather intriguing that we find this limbic structure so different in the CYP2C19 transgenic mice. CORT levels during and after acute stress does however implicate that HPA-axis regulation is functioning normally in the transgenic mice. Our hypothesis is that this might not be the case if the mice would be exposed to chronic stress paradigm or perhaps early-life stress, like maternal separation. We are planning to expose CYP2C19Tg-Hem mice to unpredictable chronic mild stress and investigate

HPA-axis reactivity and adaptation but also study the effects this might have on the behavior in e.g. the TST and LDB.

Before studying the *CYP2C19* mouse model any further it would be interesting to investigate *CYP2C19* genetic polymorphism and distribution in different psychiatric cohorts to elucidate and confirm any associations. It would be interesting to look into MDD, anxiety disorders, and possibly also in combination with different stressful life events since increased stress reactivity is one of the major phenotypes seen in the transgenic mice. Mood and anxiety disorders are a major burden to the society today but still the pathophysiology behind these disorders is largely unknown and the pharmacotherapy available today is far from sufficient, with relatively low remission rates. Confirming associations between anxiety and other stress-related disorders and *CYP2C19* genetic polymorphism could lead to increased understanding of the pathogenesis and pathophysiology of these disorders. Use of the transgenic model in drug screening programs could potentially lead to new therapeutic agents.

Animal models in psychiatric disorder have become valuable tools, mostly in the development and evaluation of new pharmacotherapies but also for investigating the pathogenesis and pathophysiology of these disorders. It is important to point out that the data presented in this thesis regarding this transgenic model has only been initially characterized phenotypically and for a stronger validity of this mouse model a link to a human phenotype needs to be confirmed. This would give this model more solid construct validity. Now the construct validity is based on the hypothesis that *CYP2C19* genetic polymorphism is associated to depressive mood and personality traits.

It would be interesting to subject the animals to anxiolytic pharmacotherapy to investigate its construct validity, i.e. if anxiolytic treatment could reverse the anxiety-like phenotype displayed in the light-dark box. However, this mouse model does display some face validity to endophenotypes displayed in both MDD and anxiety disorders. This includes displayed stress sensitivity on a behavioral level and a smaller and dysfunctional hippocampus. If the hippocampus is truly dysfunctional needs to be confirmed but this hypothesis is based on the observed reduction of several cell populations important for the plasticity and stress sensitivity of this limbic structure, and the increased neuronal activation after acute stress. I believe that already now this transgenic model can be used for studying processes affecting hippocampal plasticity and volume, both with regards to possible developmental effects but also pharmacotherapy. However, much more research needs to be done before this model can be considered a true model for anxiety and other stress-related disorders.

7 | Populärvetenskaplig sammanfattning

Båda artiklarna i denna avhandling handlar om CYP2C19. CYP2C19 är ett protein som ingår i en stor familj av proteiner som heter cytokrom P450, så kallade CYP-proteiner. Dessa proteiner, som till största delen finns i levern, har till uppgift att hjälpa kroppen att ta hand om främmande ämnen som vi får i oss via luftvägar och föda. De har t.ex. en väldigt viktig roll i nedbrytning och utsöndring av läkemedel. Vissa CYP-proteiner har även kroppsegna funktioner som till exempel att reglera nivåer av hormoner i kroppen. Ett stort problem, vid t.ex. läkemedelsutveckling, är att nivåerna i kroppen av dessa proteiner skiljer sig väldigt mycket mellan olika människor. Dessa skillnader är till största delen genetiska, d.v.s. de beror på förändringar i våra gener vilket också gör dem ärftliga. För CYP2C19 kan individer ha allt från inget protein till normala nivåer men även extra mycket protein vilket gör att de läkemedel och andra ämnen som bryts ner av CYP2C19 kommer att variera i koncentration i kroppen beroende på hur mycket CYP2C19 man har. För vissa läkemedel kan det därför vara viktigt att först identifiera vilken gen-variant man har av CYP2C19 innan man väljer hur mycket patienten ska få av läkemedlet. För personer med låga nivåer av CYP2C19 kan annars en normal dos ge toxiska biverkningar och en person med höga nivåer kanske inte får någon effekt alls.

CYP2C19 tar hand om mellan 6-10 % av alla läkemedel som finns på marknaden idag och det är därför viktigt att veta vad som påverkar hur mycket protein vi har i kroppen. För att ytterligare komplicera saken kan nivåerna av dessa CYP-proteiner även påverkas av andra ämnen och i synnerhet läkemedel. I artikel I studeras effekten av två olika kvinnliga könshormoner. Dessa hormoner används i stor utsträckning av kvinnor i form av preventivmedel men även i hormonbehandlingar efter klimakteriet. Då dessa behandlingar är så vanliga är det förstås viktigt att veta om de kan påverka nivåerna av andra läkemedel. Tidigare studier har visat att när läkemedel som bryts ner av CYP2C19 tas i kombination med dessa hormoner får vi högre nivåer av läkemedelen än normalt i kroppen. Det är dock fortfarande oklart hur den effekten uppkommer. Vi kan i denna studie visa hur dessa hormoner, via specifika mottagar-proteiner i cellerna direkt kan påverka genernas effektivitet. De hämmar nybildandet av CYP2C19 vilket gör att vi får lägre nivåer än normalt och läkemedlen kan inte brytas ner utan blir kvar i kroppen under en längre tid. Det är fortfarande inte helt klart hur stor effekt detta har men i framtiden kommer vi kanske behöva ta hänsyn till preventivmedel vid dosbestämning av vissa läkemedel.

CYP2C19 bryter bland annat ner ämnen som har effekter på hjärnan som exempelvis antidepressiva läkemedel men även kroppsegna ämnen som hormoner. Dessa hormoner har visat sig ha effekter på hjärnan under fosterutvecklingen men även i vuxen ålder. Nyligen hittade vår forskargrupp att de personer som saknar CYP2C19 har ett mindre deprimerat grundtillstånd, jämfört med personer med normala nivåer. Det verkar alltså som att låga nivåer av proteinet skyddar mot depressiva symptom. Skulle det kunna vara så att höga nivåer ökar risken för depression? Depression och ångest är till stor del ärftligt och forskare har länge letat efter genetiska orsaker som skulle kunna förklara de stora skillnader som finns mellan individer när det gäller känslighet för dessa psykiska

sjukdomar. För att studera detta närmare har vi använt oss av så kallade transgena möss som fått den mänskliga CYP2C19-genen infogad i sin arvs massa. I artikel II har vi studerat dessa möss och hittat att CYP2C19 finns i mössens hjärna under fosterutvecklingen. Detta är besynnerligt då CYP2C19 endast påvisats i lever hos människa. Ingen har dock studerat om detta protein finns i hjärnan under fosterutvecklingen och vi har i denna studie visat att det faktiskt verkar finnas där även hos människa. Vi har dock endast studerat tre olika mänskliga fosterhjärnor men dessa resultat är otroligt intressanta då hjärnan hos de transgena mössen visat sig utvecklas helt annorlunda mot normala möss. Detta tyder på att beroende på hur mycket CYP2C19 en person har kanske hjärnan utvecklas på olika sätt något som skulle kunna påverka hjärnans funktion i vuxen ålder. I vuxna möss har vi studerat beteende för att se om CYP2C19 kan ha en effekt som liknar psykisk sjukdom hos människa. Vi såg att dessa möss uppvisar en ökad stresskänslighet samt ett ångestliknande beteende. Patienter med depression har ofta ångestinslag och ökad stresskänslighet tros vara en av de viktigaste riskfaktorerna för att utveckla depression. Dessa observationer stämmer därför mycket väl överens med de preliminära studierna med minskade depressiva symptom hos individer som saknar CYP2C19. Vi kunde även se att dessa möss hade en mindre och mer stresskänslig hippocampus. Detta område i hjärnan är ofta mindre hos deprimerade patienter och är starkt kopplat till stresskänslighet men också kontroll av känslor.

Vad vi hittat hos dessa möss kan bidra till kunskap om genetiska faktorer för utveckling av ångest och depression och de transgena mössen kan förhoppningsvis användas för att studera vilka mekanismer som kan orsaka uppkomsten av dessa sjukdomar. Mössen skulle även kunna användas för utveckling av nya ångestdämpande läkemedel. I nästa steg kommer vi nu studera CYP2C19 lite närmare under fosterutvecklingen mössen och i människa. Vi vill för försöka ta reda vilket eller vilka ämnen det kan vara som bryts ner av detta protein och som kan forma hjärnan till att bli mer känslig för utveckling av psykisk sjukdom i vuxen ålder. Dessa studier är otroligt viktiga då vi fortfarande vet väldigt lite om orsakerna till psykisk ohälsa hos människa och de läkemedel som finns tillgängliga idag är långt ifrån effektiva för alla som drabbas.

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