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ORIGINAL ARTICLE

Decreased hippocampal volume and increased anxiety in a transgenic mouse model expressing the human *CYP2C19* gene

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Selective serotonin reuptake inhibitors, tricyclic antidepressants, various psychoactive drugs, as well as endogenous steroids and cannabinoid-like compounds are metabolized by the polymorphic cytochrome P450 2C19 (CYP2C19). Absence of this enzyme has been recently shown to associate with lower levels of depressive symptoms in human subjects. To investigate endogenous functions of CYP2C19 and its potential role in brain function, we have used a transgenic mouse model carrying the human *CYP2C19* gene. Here, CYP2C19 was expressed in the developing fetal, but not adult brain and was associated with altered fetal brain morphology, where mice homozygous for the *CYP2C19* transgenic insert had severely underdeveloped hippocampus and complete callosal agenesis and high neonatal lethality. CYP2C19 expression was also found in human fetal brain. In adult hemizygous mice we observed besides decreased hippocampal volume, an altered neuronal composition in the hippocampal dentate gyrus. Reduced hippocampal volumes have been reported in several psychiatric disorders, supporting the relevance of this model. Here we found that adult hemizygous *CYP2C19* transgenic mice demonstrate behavior indicative of increased stress and anxiety based on four different tests. We hypothesize that expression of the CYP2C19 enzyme prenatally may affect brain development by metabolizing endogenous compounds influencing this development. Furthermore, CYP2C19 polymorphism may have a role in interindividual susceptibility for psychiatric disorders.

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INTRODUCTION

Hippocampal dysfunction is believed to have a crucial role in the pathophysiology of both major depressive disorder (MDD) and anxiety-related disorders.[1,2] Reduced hippocampal volumes have been reported in several psychiatric disorders including anxiety-related post-traumatic stress disorder and schizophrenia.[2–4] However, the phenotype has been most extensively studied in MDD patients,[5,6] where hippocampal volume reduction has been shown to correlate with the severeness and duration of the disorder.[7,8]

In a cohort of 1500 European subjects, we recently found using the Center for Epidemiologic Studies Depression Scale a genetic association of individuals lacking cytochrome P450 2C19 (CYP2C19) enzyme function with lower levels of depressive symptoms.[9] CYP2C19 is involved in the metabolism of several psychotropic drugs including the selective serotonin reuptake inhibitors sertraline and citalopram, the tricyclic antidepressants amitriptyline and clomipramine,[10] as well as benzodiazepines.[11] This indicates that CYP2C19 is able to metabolize substrates with a CNS drug-effect profile. Endogenous compounds suggested as substrates for CYP2C19 based on *in vitro* studies include psychoactive steroid hormones such as estradiol,[12] estrone,[13] progesterone and testosterone.[14]

The *CYP2C19* gene is polymorphic and the *CYP2C19*17* allele, present at 18–27% in different European populations,[15–17] causes higher gene expression and more rapid drug metabolism,[15] whereas *CYP2C19*2* with an allele frequency of around 15% in Caucasians[18] is the most common null allele.[10] In Asian populations, the allele frequency of the *CYP2C19*17* allele is approximately 6% and the *CYP2C19*2* allelic variant has a frequency of about 30%.[18] *CYP2C19* polymorphism impacts not only drug plasma levels,[19–21] but also the therapeutic outcome of psychotropic drugs.[22]

In light of the association of *CYP2C19* polymorphism with depressive symptoms,[9] we investigated brain-related effects of transgenic expression of the human *CYP2C19* gene in mice. We found that the *CYP2C19* gene is expressed in mouse and human fetal brain. The transgenic animals exhibited altered hippocampal morphology both at young and adult age, as well as changes in hippocampal neuronal composition in adult life. In line with these findings, we observed behavioral changes related to stress and anxiety in adult mice. Our data suggest a role for CYP2C19 in brain development, and the human CYP2C19 transgenic mouse model might provide a useful tool for studies of hippocampal function and stress and anxiety-related phenotypes.

MATERIALS AND METHODS

For detailed methodological descriptions, see Supplementary Information 1.

Transgenic mice

A C57BL/6 mouse line transgenic for a BAC insert containing the human *CYP2C18* and *CYP2C19* genes has previously been produced and estimated to hemizygously (CYP2C19Tg-Hem) carry 12 copies of the insert at region C1 of mouse chromosome 2.[23,24] All experiments were carried out on male mice, with the exception of unknown sex status of mice during fetal

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development. All experiments were approved by the local ethical committee; Stockholm Northern Ethics Board of Animal Experimentation.

CYP2C18 and *CYP2C19* expression in transgenic mice and human fetal brain

In CYP2C19 transgenic mice, *CYP2C18* and *CYP2C19* mRNA expression was investigated in brain and liver tissue during embryonic development (embryonic day 14 (E14) and E18), early postnatal days (PND0 and PND7) and at 7 weeks of age, using real-time PCR protocols obtained from Löfgren *et al.*[24] Four to five mice were analyzed for each time point. Human brain tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, USA and were from three different female Caucasian donors; gestational week 19, 24 and 39.

Brain morphology

Mice (PND0, 7 and 15 weeks) were anesthetized and perfused transcardially with 4% paraformaldehyde solution, after which the brains were post-fixed, cryoprotected and snap-frozen. Coronal sections (40 μm) were stained with cresyl violet for morphological assessment. For PND0 pups, three wild-type (Wt) brains were compared with four CYP2C19Tg-Hom brains. In 7- and 15-week old mice, 5–7 brains were analyzed per group.

Magnetic resonance imaging

In vivo magnetic resonance imaging (MRI) was conducted in anesthetized 15-week-old mice using a horizontal 9.4T Varian magnet ($n = 7$ per group). Hippocampal and whole-brain volumes (including cerebellum) were manually outlined using ITK-SNAP (www.itksnap.org).[25]

BrdU injections and immunohistochemistry analyses of the hippocampus

Bromodeoxyuridine (BrdU) is widely used to evaluate hippocampal proliferation rates.[26] Seven-week-old mice were intraperitoneally injected with 75 mg kg^{-1} BrdU; either twice, with a 16-h gap, and killed 2 h after the last injection, or injected once a day for 3 consecutive days and killed 4 weeks after the first injection. Brain sections were stained by immunohistochemistry (IHC) using Ki-67 or BrdU antibodies. Antibodies for parvalbumin and double-cortin (DCX) were used for IHC analyses of brain sections of 15-week-old mice not subjected to BrdU injections. Five to eight mice were analyzed per group. For all IHC markers, total numbers were also corrected for total hippocampal volumes as measured by MRI.

Behavioral tests

Behavioral tests were conducted in adolescent (7-week-old) and adult (15-week-old) mice. Depression-like behavior was evaluated in the tail-suspension test (TST; $n = 6$–10 per group) and spatial learning was evaluated in 15-week-old mice using the Morris water maze (MWM) with a subsequent TST (Wt: $n = 10$; CYP2C19Tg-Hem: $n = 9$). Motor activity and anxiety levels were studied using the open-field and the light–dark box test ($n = 15$ per group). Stress response was studied by subjecting mice to 30 min of acute restraint, followed by direct decapitation or a 30-min recovery period before decapitation. Mice not subjected to stress were decapitated directly and served as controls. Serum corticosterone (CORT) was quantified and hippocampal *c-fos* mRNA expression was analyzed.

Statistical analysis

Statistical analyses were carried out using unpaired, two-tailed Student's *t*-test in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± s.e.m. For correlation studies, Pearson's correlation test was performed. Statistical significance was defined as $P < 0.05$.

RESULTS

CYP2C19 expression in brain and liver tissue of transgenic mice and in human fetal brain

Human CYP2C18 and CYP2C19 mRNA and protein expression has previously been examined in adult transgenic (previously named CYP2C19Tg) mice (26–31 weeks). The highest mRNA expression was found in liver for both genes, whereas the protein could only be detected for CYP2C19 and not for CYP2C18,[24] similar to the relative protein expression of human liver.[27,28] Examination of *CYP2C19* mRNA expression during development revealed relatively high levels in the brain at E14, E18 and PND0, with no significant hepatic expression at the same time points (Figure 1a). The mRNA expression in hippocampus, cortex and cerebrum were similar at day 18 (Supplementary Figure 1). At 7 weeks of age, the tissue-specific *CYP2C19* mRNA expression was silenced in brain and induced in liver (Figure 1b). Expression of the *CYP2C18* gene was found consistently low in the brain irrespective of the age (Figures 1a and b). Mice do not have CYP2C19 or CYP2C18 homologs, and thus, the observed human *CYP2C19* expression is specific for the transgenic mice. Quantification of *CYP2C19* mRNA in human fetal brain regions from three donors showed that *CYP2C19* can be expressed at a level representing slightly $> 1/200$ of human adult liver. Three fetal human brain donors, gestational weeks 19, 24 and 39, with a total of seven small cerebral brain pieces were analyzed for mRNA expression. The donor with the highest *CYP2C19* expression was in gestational week 24 and showed approximately 0.3% and 0.6% of the levels in adult human liver in two separate regions, respectively.

Altered brain morphology in CYP2C19 transgenic mice

Homozygous mice (CYP2C19Tg-Hom) did not survive past PND3, and therefore brain morphology was investigated at PND0 in Wt, CYP2C19Tg-Hem and CYP2C19Tg-Hom pups. CYP2C19Tg-Hom pups were found to display distinct morphological abnormalities. The hippocampus in CYP2C19Tg-Hom pups was severely malformed (Figures 1e and f). Furthermore, connections between the hemispheres were severely underdeveloped in the CYP2C19Tg-Hom pups, with complete callosal agenesis (Figures 1c–e). The morphology of CYP2C19Tg-Hem brains were not different from Wt counterparts (data not shown).

Based on the abnormal brain morphology of newborn CYP2C19Tg-Hom mice, with a severely reduced hippocampal area (about 60%, Supplementary Figure 2), we performed volume measurements in adolescent and adult CYP2C19Tg-Hem male mice using both histology and MRI. In brain sections, a reduced hippocampal area was observed in CYP2C19Tg-Hem compared with Wt mice, both at 7 weeks (12% reduction; $P = 0.0079$) and 15 weeks of age (11% reduction; $P = 0.0082$; Figure 2a). Systematic sectioning revealed a prominent reduction in the caudal-ventral part of the hippocampus (Figures 2b and c). At both 7 and 15 weeks of age, a respective 5.4% ($P = 0.007$) and 5.6% ($P = 0.0004$) reduction of total brain weight was recorded in CYP2C19Tg-Hem compared with Wt mice (Supplementary Figure 3).

Fifteen-week-old CYP2C19Tg-Hem and Wt male mice were also examined using a 9.4 T Varian MRI system. Volumetric analyses showed decreased whole-brain (3.1%; $P = 0.018$) and hippocampal volumes (7.1%; $P = 0.013$) in CYP2C19Tg-Hem compared with Wt mice (Figures 3c and d). We found no correlation between whole-brain and hippocampal volumes ($r^2 = 0.276$; $P = 0.526$, Pearson's correlation test). Hippocampal volumes were therefore not normalized against whole-brain volumes.

Impact of human CYP2C19 expression on mouse hippocampal cell populations

To investigate hippocampal plasticity in transgenic mice, IHC analyses assessing immature neurons were performed by detecting DCX-positive cells in 15-week-old male mice. We observed a marked decrease in this cell population, with a mean reduction as high as 42% ($P = 0.0002$) in CYP2C19Tg-Hem compared with Wt mice (Figures 4a, b and e). No difference in cell proliferation, as measured by BrdU and Ki-67-positive cells in the dentate gyrus (DG) of the hippocampus was observed in 7- or 15-week-old mice (Supplementary Figure 4).

Furthermore, we observed a reduction in the parvalbumin-positive γ-aminobutyric acid (GABA) interneuron population in the DG of 15-week-old CYP2C19Tg-Hem mice compared with Wt mice (22%, $P = 0.017$; Figures 4c, d and f) but no difference within the CA regions of the hippocampus (data not shown). Correction for mean hippocampal volume as measured by MRI (Supplementary Figure 5) revealed retained significance differences for all IHC markers with the only exception of parvalbumin-positive cells in the DG that showed border-line significance after size correction ($P = 0.071$; Supplementary Figure 5).

Behavioral phenotypes observed in transgenic mice

Using the TST, a classical test for assessing antidepressant drugs,[29] we found that 15-week-old CYP2C19Tg-Hem mice had a 55% lower immobility time compared with Wt controls ($P = 0.0017$; Figure 5c), whereas no differences were observed between groups of 7-week-old mice.

In the MWM, 15-week-old CYP2C19Tg-Hem mice acquired a despair-related behavior during the training week as evident by increased floating time compared with Wt mice (Supplementary Figure 6). Owing to this behavior, conclusive results on spatial

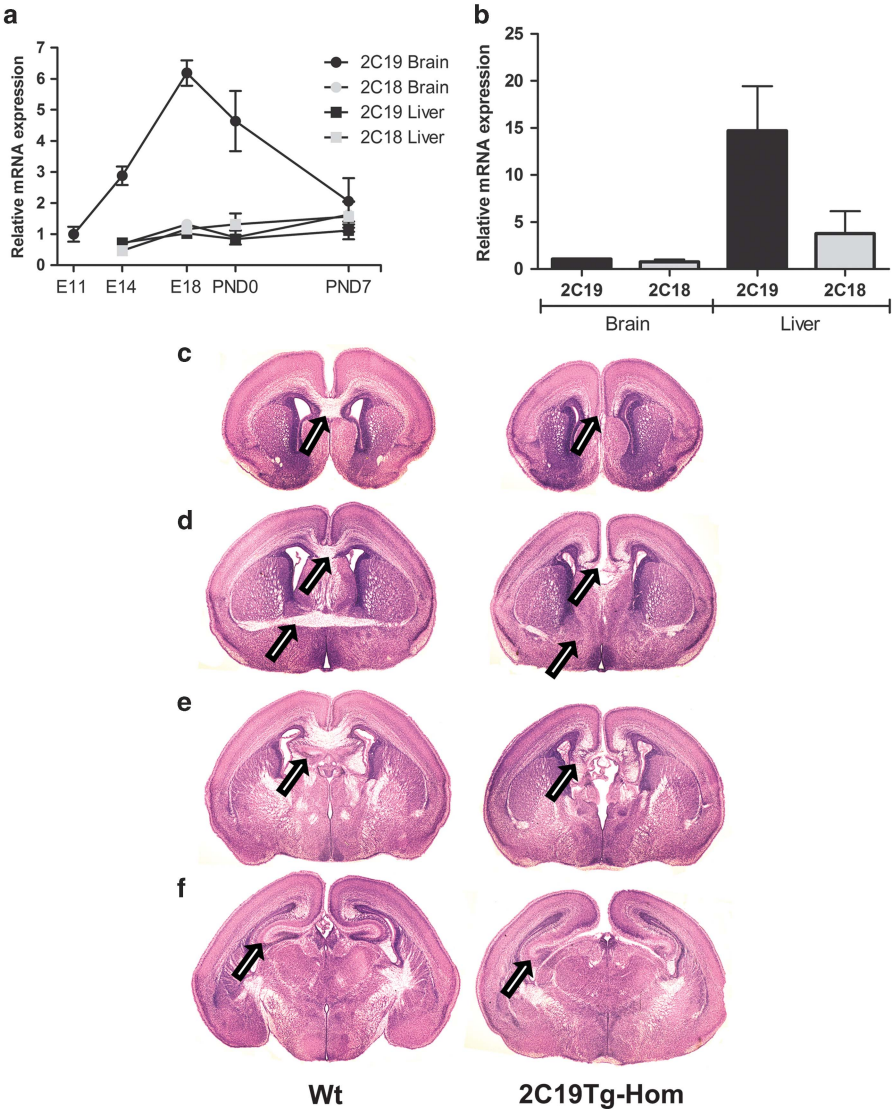

Figure 1. Human *CYP2C19* mRNA expression in developing *CYP2C19* transgenic (CYP2C19Tg) brain and the morphological brain phenotype at postnatal day 0 (PND0). (**a**) *CYP2C18* and *CYP2C19* mRNA expression pattern was investigated in brain and liver of hemizygous transgenic (CYP2C19Tg-Hem) mice during embryonic development (embryonic day 11 (E11), E14 and E18), early postnatal days (PND0 and PND7). The graph displays relative *CYP2C18* and relative *CYP2C19* mRNA expression levels in fetuses and pups of unknown gender. Brain *CYP2C19* mRNA expression peaks at E18, with a sixfold higher expression in brain compared with liver. The expression of the *CYP2C18* gene was consistently low in both tissues. (**b**) Relative *CYP2C18* and relative *CYP2C19* mRNA expression levels in 7 weeks old male mice. As evident from the graph, there is a brain-specific expression of CYP2C19 mRNA in fetal life, which at the age of 7 weeks is silenced and hepatic expression induced. No expression of *CYP2C18* or *CYP2C19* mRNA was detected in wild-type (Wt) mice. Four to five mice were analyzed for each time point. Data are presented as mean with s.e.m. (**c**–**f**) Images are representative cresyl violet stainings of Wt ($n = 3$) and CYP2C19Tg-Hom ($n = 4$) pups brain sections at PDN0. Brain sections from CYP2C19Tg-Hem mice were indistinguishable from those of Wt animals. The representative images display coronal sections from four rostral to caudal positions (**c**–**f**) per genotype. CYP2C19Tg-Hom mice display an extensive morphological phenotype with complete callosal agenesis (**c**–**e**). As pointed out by arrows on each image, the corpus callosum (**c** and top arrow **d**), the hippocampal commissure (**d, e**) and the anterior commissure (bottom arrow **d**), are all essentially lacking axons crossing over the midline of the hemispheres. Furthermore, the development of the hippocampal formation appears stalled or delayed in CYP2C19Tg-Hom mice (**e, f**), with the different layers of the hippocampus being less distinct, in addition to an overall smaller appearance.

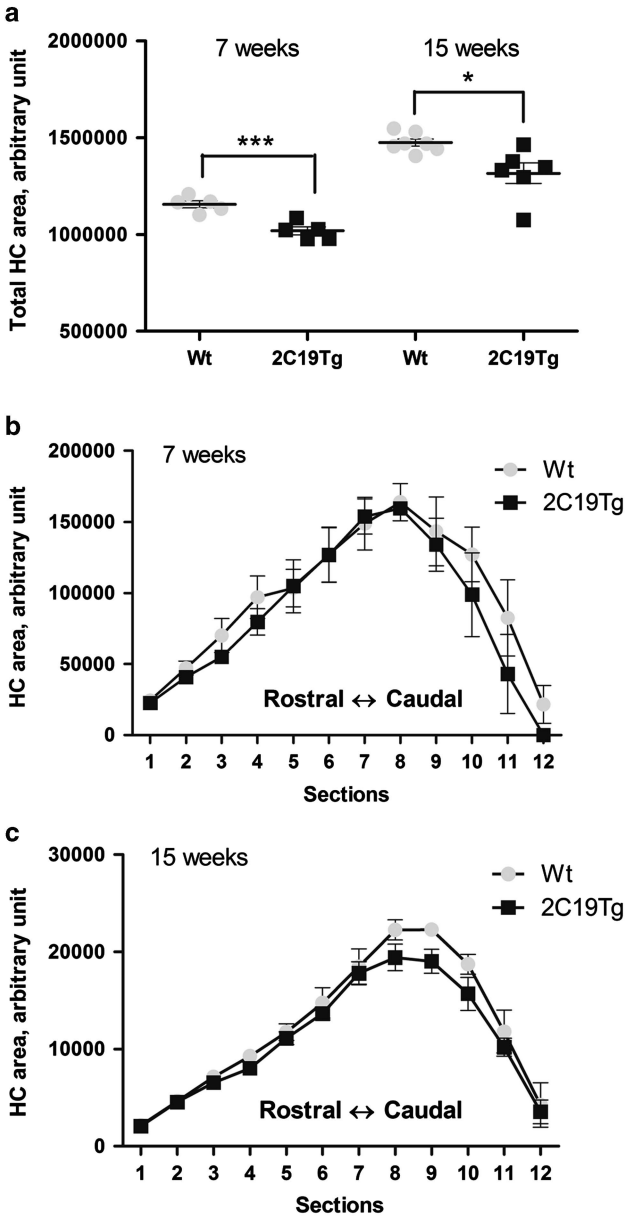

Figure 2. Decreased hippocampal area in adolescent and adult CYP2C19Tg-Hem compared with wild-type (Wt) mice. To investigate hippocampal morphology in young (7 weeks) and adult mice (15 weeks), 12 coronal brain sections from every mouse were stained with cresyl violet and the hippocampal area outlined and measured for each section. (**a**) A reduced total hippocampal area was observed in CYP2C19Tg-Hem compared with Wt male mice, both at 7 weeks (11.9% reduction; $P = 0.008$) and at 15 weeks of age (10.7% reduction; $P = 0.008$). (**b, c**) In the same mice as above (**a**) the hippocampal area is visualized per section in a rostral to caudal manner in 7- and 15-week-old mice, which highlights the specific reduction in size in the caudal part of the hippocampus at both ages. Data are presented as mean with s.e.m. $^*P < 0.05$; $^{***}P < 0.001$. HC, hippocampal.

learning could not be obtained from the MWM test. However, when excluding transgenic mice with >10 s of floating behavior in the retention test of the MWM no differences in spatial learning could be seen between Wt ($n = 10$) and CYP2C19Tg-Hem ($n = 3$) mice. Spatial learning was evaluated by measuring time to first platform crossing, number of total platform crossings and total time spent in the platform quadrant.

Furthermore, when exposing the same set of mice to the TST 3 days after the last session (retention test) of the MWM, the difference previously seen between the groups (no stress, Figure 5c) was attenuated, with a significantly reduced immobility time for the Wt compared with the CYP2C19Tg-Hem mice ($P = 0.0005$; MWM considered as stressor, Figure 5c). Notably, CYP2C19Tg-Hem mice displayed very similar immobility time in TST, independent on the exposure to prior MWM stress.

In the open-field test, evaluation of the total distance travelled over 30 min in the novel environment showed significantly increased locomotor activity in 15-week-old CYP2C19Tg-Hem mice compared with Wt mice ($P = 0.027$), which was already trending toward significance at 7 weeks of age ($P = 0.056$; Figures 5a and b). Moreover, at both ages, an increased response to the novel environment was found in CYP2C19Tg-Hem compared with Wt mice, as evident from increased distance travelled measured over the first 15 min (7 weeks: $P = 0.0048$; 15 weeks: $P = 0.0032$) with no difference in habituation between the genotypic groups (Figures 5a and b).

Adult CYP2C19Tg-Hem male mice subjected to the light–dark box test spent significantly less time in the exposed light compartment compared with Wt controls (41%; $P = 0.0024$; Figure 5d), thus suggesting an anxious phenotype. A similar trend was observed in 7-week-old mice ($P = 0.065$; Figure 5d).

Effect of acute restraint stress on serum CORT levels and expression of *c-fos* in the hippocampus

An independent set of mice that had not been exposed to behavioral testing was subjected to 30 min of acute restraint stress to assess any differences in stress response between genotype groups. CORT is the major stress hormone in rodents,[30] but no difference in serum CORT levels could be detected between CYP2C19Tg-Hem mice and Wt controls at either 7 or 15 weeks of age (Supplementary Figure 7). Although there was no difference in basal neuronal activation as measured by *c-fos* expression, at either age, *c-fos* mRNA levels were significantly higher after 60 min (30-min restraint stress + 30-min recovery) in CYP2C19Tg-Hem compared with Wt mice at both 7 and 15 weeks of age (51%, $P = 0.0030$ and 46%, $P = 0.026$, respectively; Figures 5e and f).

DISCUSSION

Previous studies have indicated that *CYP2C19* polymorphism affects personality traits[31,32] as well as depressive symptoms in humans,[9] thus suggesting that high or low CYP2C19 enzyme activity might affect brain functions and influence the risk of developing psychiatric disorders. Thus, the primary aim of this study was to investigate if expression of the human *CYP2C19* gene in mice could influence brain function or animal behavior.

By analyzing mRNA levels in transgenic mouse brains, we found high *CYP2C19* expression in the developing brain, whereas the brain expression in 7-week-old mice was low, as has also been previously shown in the adult transgenic brain.[24] The developmental expression of *CYP2C19* in brain is particularly interesting since CYP2C19 is one of only a few human CYP enzymes expressed in the human liver at relatively high levels during the prenatal period, thus suggesting an important developmental function.[33] Analysis of expression of *CYP2C19* in human fetal brain regions from three different donors showed that *CYP2C19* can be expressed at a level representing slightly >1/200 of human adult liver, which is high taking into account that the P450 expression in liver is very high and present in 70% of all cells.

We observed extensive alterations in brain morphology of newborn CYP2C19Tg-Hom mice, including severely reduced hippocampal size and complete callosal agenesis. As a result of the lethality of the homozygous genotype, we carried out further investigations in CYP2C19Tg-Hem mice.

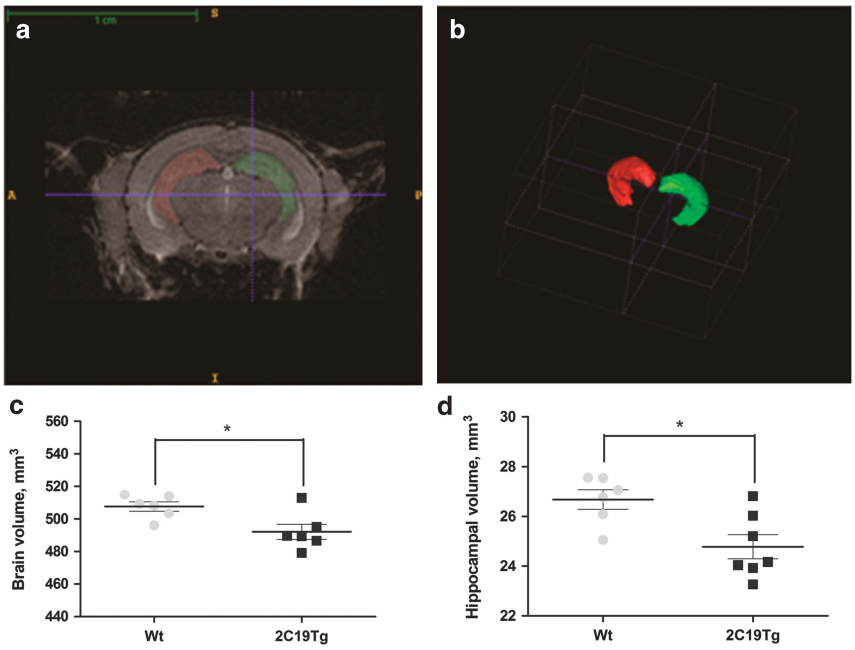

Figure 3. Brain and hippocampal volumes are decreased in adult CYP2C19Tg-Hem compared with wild-type (Wt) mice as determined by magnetic resonance imaging (MRI). Adult (15 weeks) CYP2C19Tg-Hem ($n=7$) and Wt ($n=7$) mice were examined using a 9.4 T Varian MRI system. Whole-brain and hippocampal volumes were manually outlined using the software ITK-SNAP. (**a, b**) Manual outlining of a representative Wt left (green) and right (red) hippocampus, the right image displays a three-dimensional illustration of the whole hippocampus after outlining. (**c**) A mean 3.1% ($P=0.018$) lower total whole-brain volume was observed in CYP2C19Tg-Hem compared with Wt mice. (**d**) Hippocampal volumes were more profoundly reduced than total brain volume with a mean reduction of 7.1% ($P=0.013$) in CYP2C19Tg-Hem compared with Wt mice. Measurements from two mice had to be excluded because of poor positioning and thus incomplete field of view during the imaging. Data are presented as mean with s.e.m. *$P<0.05$.

The hippocampal formation is one of the most studied limbic structures with a critical role in learning and memory,[34] stress adaptation and sensitivity,[35] and emotional processing.[1] Furthermore, hippocampal dysfunction is believed to have a major role in the pathophysiology of both MDD and anxiety-related disorders.[1,2] We investigated hippocampal integrity in adult CYP2C19Tg-Hem mice (15-week-old) using area measurements of brain sections, and MRI on anesthetized animals. Both methods showed that the hippocampal formation was 7–11% smaller in CYP2C19Tg-Hem mice compared with Wt controls, whereas a 3.1% size reduction was seen in whole-brain volumes. As the hippocampus and whole-brain volumes did not correlate as measured by MRI, we did not normalize hippocampal volumes with whole-brain volumes. Corpus callosum integrity was not further investigated in adult mice. However, the callosal agenesis in CYP2C19Tg-Hom new-born pups further suggests that important, MDD-related brain functions, such as white matter integrity are affected in the transgenic mice during brain development.[36–38]

We found that the hippocampal volume reduction was specific for the caudal-ventral part, and did not affect the whole hippocampal formation. Furthermore, the specific and severe malformation and 60% shrinkage of the hippocampus in CYP2C19Tg-Hom mice at PND0 also suggests a specific effect of CYP2C19 on this brain structure. As reviewed by Fanselow and Dong,[39] the caudal-ventral part of the hippocampus is highly implicated in the regulation of emotions and stress responses supporting this morphology finding in correlation to our behavioral data.[39,40] The hippocampal area was also measured in sections from 7-week-old mice with a similar result, thus suggesting that the smaller hippocampal volume is established early in life, most probably during fetal life as based on the drastic changes in CYP2C19Tg-Hom mice at PND0. As mentioned, reduced hippocampal volumes have been reported in several psychiatric disorders in humans. Hippocampal volume measurements have been most extensively studied in MDD patients with reductions ranging between 5 and 10%[5,6,41] resembling effects observed in the adult CYP2C19Tg-Hem mice (7–11%).

The DG of the hippocampus is a neurogenic niche and adult neurogenesis is critical for hippocampal function.[42,43] When investigating the number of immature DCX-positive neurons in adult (15-week-old) mice, we found a 42% reduction of this specific cell population in CYP2C19Tg-Hem mice. Besides being a step in the maturation process of new neurons, DCX-positive cells have unique electrophysiological properties, important for hippocampal signal processing. Immature neurons are highly excitable, display greater synaptic plasticity than mature neurons and long-term potentiation is more easily induced in this young neuron population.[44,45] The large pool of DCX-positive cells in the DG is thought to be available for encoding new experiences[46,47] and are furthermore suggested to be of importance in regulating stress reactivity both by negative feed-back on the hypothalamic–pituitary-adrenal–axis and in stress and depression-related behavioral tasks.[35] Since the proliferation rate in the DG, as measured by BrdU and Ki-67, was not affected at 7 or 15 weeks of age, the reduction of immature neurons appears to be specific, and could reflect a defect in the neuronal maturation process. GABAergic interneurons support the maturation of young neurons during development and in the adult hippocampus, thus suggesting that reduced GABAergic input to immature neurons in the DG could cause the reduction of DCX-positive cells.[48,49] The reduced number of GABAergic interneurons in the DG could also influence overall hippocampal activity because of a reduced inhibition of incoming signals, consequently leading to a hyperactivated hippocampus.[50] As the hippocampus is highly involved in the response to stress,[35,39] the reduction in hippocampal volume, as well as in DCX-positive cells in the DG of CYP2C19Tg-Hem mice, could implicate an altered stress response in these mice. In fact,

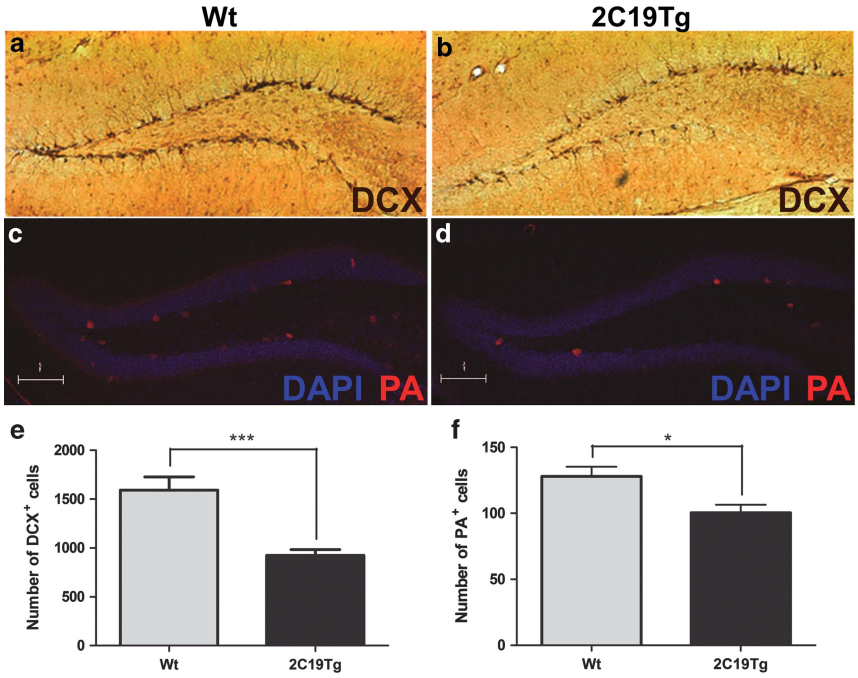

Figure 4. Reduced numbers of immature (double-cortin (DCX)-positive) neurons and GABAergic (parvalbumin (PA)-positive) interneurons in the hippocampal dentate gyrus (DG) of adult CYP2C19Tg-Hem compared with wild-type (Wt) mice. Distribution of immature DCX-positive neurons and PA-positive GABAergic interneurons in the DG of the hippocampus was addressed by immunohistochemistry in 15-week-old CYP2C19Tg-Hem and Wt mice. A total of eight coronal brain sections were analyzed from each mouse for an estimation of the total distribution of specific cells throughout the DG. (**a, b**) Representative images of the hippocampal DG with DCX-positive cells in brown. Whereas DCX neurons were smoothly lining the sub-granular zone of Wt mice, DCX neurons of CYP2C19Tg-Hem mice were frequently found integrated in the granular cell layer. (**c, d**) The hippocampal DG with representative images from PA stainings of 15-week-old CYP2C19Tg-Hem and Wt mice. 4,6-Diamidino-2-phenylindole (DAPI) is used as nuclear counter stain. (**e**) We observed a marked reduction in the total number of DCX-positive neurons in the DG of CYP2C19Tg-Hem ($n=9$) compared with Wt ($n=6$) mice that amounted to a 42% difference between genotypes ($P=0.0002$). **(f)** A 22% reduction ($P=0.017$) of the total number of PA-positive cells in the DG of CYP2C19Tg-Hem ($n=8$) compared with Wt ($n=5$) mice was found. No difference in the number of PA-positive cells in other hippocampal areas was found (data not shown). Data are presented as mean with s.e.m. $^{*}P<0.05$; $^{***}P<0.001$.

although CORT levels did not differ between genotypes, we observed an increased neuronal activation, as measured by *c-fos* expression,[51,52] in the hippocampus of CYP2C19Tg-Hem compared with WT mice 30 min after acute restraint stress. Hence, the response and adaptation to stress in CYP2C19Tg mice hippocampi appear to be impaired.

Besides displaying an impaired stress response, *CYP2C19* transgenic mice also exhibited a behavioral phenotype when exposed to a novel environment (open-field) or a short-term stressor (TST), both representing stressful stimuli. Indeed, CYP2C19Tg-Hem mice (both 7- and 15-week-old) displayed an increased reactivity to the open-field during the first 15 min of the test when the novelty stress is the highest. Furthermore, 15-week-old CYP2C19Tg-Hem mice showed a significantly shorter immobility time compared with Wt mice in the TST, which could be interpreted as increased stress sensitivity. As a matter of fact, exposure of the mice to a TST after the MWM revealed that Wt mice decreased their immobility time by 45% approaching the stable level of the transgenic mice. These data indicate that CYP2C19Tg-Hem mice are more stress sensitive, which furthermore also could be an explanation for the acquired despair (floating) behavior in response to 5 days of swim maze training. This despair phenotype resembles depressive-like behavior in the forced-swim test.[53,54] In combination with increased stress sensitivity, *CYP2C19* transgenic mice also displayed increased anxiety behavior in the light–dark box test. The comorbidity of generalized anxiety disorder and MDD is high, with environmental stressors and increased stress sensitivity being major risk factors for these disorders.[55,56] The CYP2C19 transgenic mice display a behavioral phenotype relevant with regards to CYP2C19s recent association with depressive symptoms in human subjects.[9]

An important question concerns by which mechanism the increased levels of CYP2C19 might cause the mouse phenotype and an obvious point is by metabolism of endogenous compounds. CYP2C19 has been shown to metabolize many different compounds including polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid[57,58] and several psychoactive sex hormones such as estradiol,[12] estrone,[13] progesterone and testosterone.[14] The effects of polyunsaturated fatty acids and steroid hormones on brain function have been extensively studied, and shown to affect not only embryonic development but also adolescent brain maturation.[59–65] CYP2C19 has furthermore been shown to metabolize the exogenous cannabinoid cannabidiol,[66] as well as having a high affinity for cannabinoid-related compounds.[67] These findings support the possibility of an endogenous role for CYP2C19 in the metabolism of compounds influencing brain development, although the identity of such compounds requires further research.

Besides CYP2C19, also the polymorphic enzyme CYP2D6, metabolizes antidepressants and psychoactive compounds.[68] Recently, higher frequencies of subjects carrying multiple active *CYP2D6* genes (ultrarapid metabolizers) have been found in suicide cases[69] and subjects with increased suicidal risk or suicidal behavior.[70,71] Thus, classical drug-metabolizing enzymes do have the potential to be important modulators of brain function.

In conclusion, our data indicate that expression of the human *CYP2C19* gene in transgenic mice causes developmental

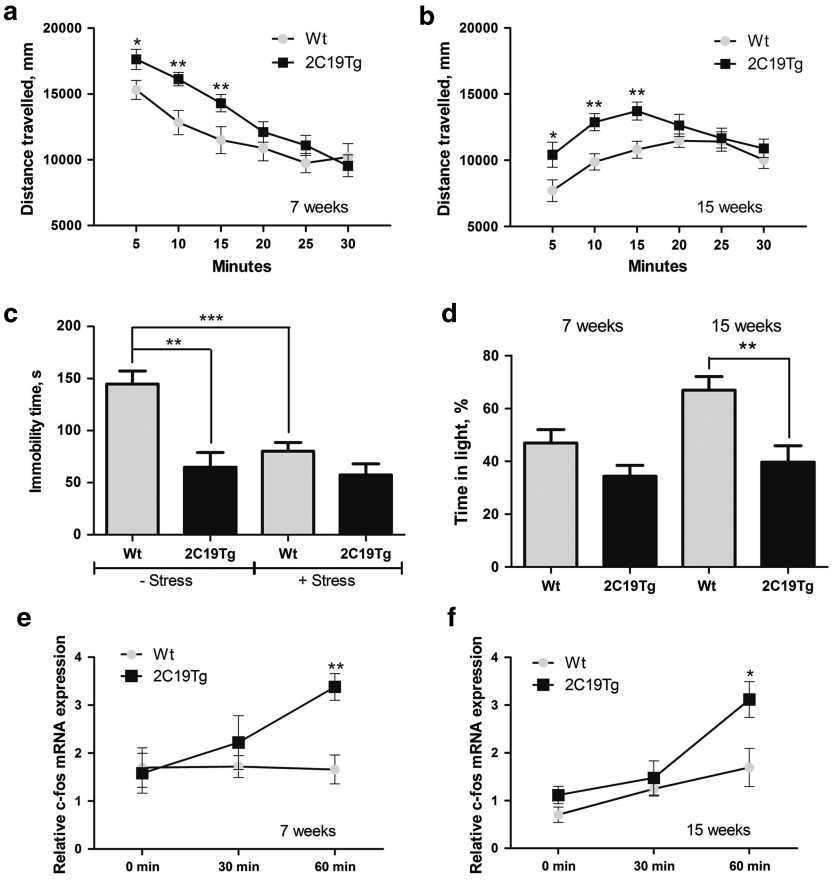

Figure 5. The behavioral phenotype of CYP2C19Tg-Hem mice is indicative of increased stress-sensitivity and anxiogenic-like behavior. (**a, b**) Mice were subjected to the open-field test for 30 min at both 7 and 15 weeks of age to investigate motor activity and reaction to a novel environment. Fifteen-week-old CYP2C19Tg-Hem mice displayed an increased total distance travelled compared with wild-type (Wt) controls ($P = 0.027$) with a similar trend at 7 weeks ($P = 0.056$; $n = 15$ mice per group at both ages; data not shown). Data are presented as distance travelled over time in 5-min bouts and the Student's *t*-test was performed on each bout. (**a**) Distance travelled over time in 7-week-old mice. CYP2C19Tg-Hem mice displayed increased motor activity compared with Wt controls during the first 15 min of the test ($P = 0.0048$). (**b**) At 15 weeks of age, CYP2C19Tg-Hem mice displayed the same increased reactivity to the novel environment as adolescent animals; CYP2C19Tg-Hem mice showed an increased total distance travelled compared with Wt mice during the first 15 min ($P = 0.0032$). The Student's *t*-test was performed on the total time travelled during the first three time points. (**c**) Mice were subjected to the tail-suspension test (TST) and immobility time was recorded during a 6-min session. There was no difference in immobility time between CYP2C19Tg-Hem and Wt mice at 7 weeks of age but a significant reduction in immobility time was recorded in 15-week-old CYP2C19Tg-Hem mice ($n = 10$) compared with controls ($n = 6$; 55%; $P = 0.0011$) here referred to as—stress. A separate group of mice were subjected to the TST after the Morris water maze (MWM), here regarded as a stressor (+ stress). Exposure of the mice to a TST after the MWM revealed that Wt mice ($n = 10$) decreased their immobility time by 45% down to the level of CYP2C19Tg-Hem mice ($n = 8$), whereas CYP2C19Tg-Hem mice displayed the same low immobility time with or without prior MWM stress. (**d**) CYP2C19Tg-Hem and Wt mice were subjected to the light–dark box test at 7 and 15 weeks of age. The time spent in the light and dark compartments was recorded during a test session of 5 min. At 15 weeks of age, CYP2C19Tg-Hem mice spent significantly less time in the light compartment than Wt mice (41% less; $P = 0.0024$, 15 mice per group), thus indicating an anxiogenic-like phenotype. At 7 weeks of age CYP2C19Tg-Hem mice displayed a trend toward spending less time in the light compartment ($P = 0.065$). (**e, f**) CYP2C19Tg-Hem and Wt mice at both 7 and 15 weeks of age were subjected to 30 min of acute restraint stress followed by immediate decapitation (30 min) or a 30-min recovery period in home cages before decapitation (60 min). Mice for baseline measurements were directly decapitated (0 min). Hippocampi were isolated for analysis of *c-fos* mRNA expression ($n = 5$–6 mice per group). CYP2C19Tg-Hem mice showed significantly higher *c-fos* mRNA levels than Wt mice at the 60-min time point at both ages, indicating increased neuronal activation in the hippocampus in response to acute stress. Data are presented as mean with s.e.m. $^*P < 0.05$; $^{**}P < 0.01$.

alterations in the brain. This might be explained by CYP2C19-dependent metabolism of endogenous compounds leading to increased stress-sensitivity at behavioral, morphological and molecular levels, many of which are related to the altered hippocampal formation. The mechanistic basis for the alterations observed in the transgenic mouse model requires further studies that could lead to increased understanding of mechanisms and endogenous factors of importance for the development of hippocampus-related morphological and behavioral phenotypes. It can also be suggested that genetic *CYP2C19* polymorphism, which determines the presence or absence of the enzyme, is an important factor in determining the individual susceptibility to stress- and anxiety-related disorders because of its influence on brain development. Such knowledge could be of importance for future drug-based interventions and the mouse model here presented used as a tool in drug discovery and development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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