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Null mutation in *P4h-tm* leads to decreased fear and anxiety and increased social behavior in mice

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ABSTRACT

36
37 HIF prolyl 4-hydroxylases (HIF-P4Hs, also known as PHDs and EGLNs) are crucial
38 enzymes that modulate the hypoxia inducible factor (HIF) response and help to maintain
39 cellular oxygen homeostasis. This function is especially well-known for cytoplasmic or
40 nuclear enzymes HIF-P4H-1-3 (PHDs 1-3, EglNs 2, 1 and 3, respectively), but the
41 physiological role is still obscure for a fourth suggested HIF-P4H, P4H-TM that is a
42 transmembrane protein and resides in the endoplasmic reticulum. Recently however,
43 both experimental and clinical evidence of the P4H-TM involvement in CNS physiology
44 has emerged. In this study, we first investigated the expression pattern of P4H-TM in
45 the mouse brain and found a remarkably selective abundance in brains areas that are
46 involved in social behaviors and anxiety including amygdala, lateral septum and bed
47 nucleus of stria terminalis. Next, we performed behavioral assays in *P4h-tm*^{-/-} mice to
48 investigate a possible phenotype associated to these brain areas. In locomotor activity
49 tests, we found that *P4h-tm*^{-/-} mice were significantly more active than their wild-type
50 (WT) littermate mice, and habituation to test environment did not abolish this effect.
51 Instead, spatial learning and memory seemed normal in *P4h-tm*^{-/-} mice as assessed by
52 Morris swim task. In several tests assessing anxiety and fear responses, *P4h-tm*^{-/-} mice
53 showed distinct courageousness, and they presented increased interaction towards
54 fellow mice in social behavior tests. Most strikingly, *P4h-tm*^{-/-} mice practically lacked
55 behavioral despair response, a surrogate marker of depression, in forced swim and tail
56 suspension tests. Instead, mutant mice of all other *Hif-p4h* isoforms lacked such a
57 behavioral phenotype. In summary, this study presents a remarkable anatomy-
58 physiology association between the brain expression of P4H-TM and the behavioral

phenotype in *P4h-tm*^{-/-} mice. Future studies will reveal whether P4H-TM may serve as a novel target for anti-depressant and anti-anxiety pharmacotherapy.

Keywords: anxiety, behavioral despair, depression, hyperactivity, hypoxia-inducible factor, prolyl-4-hydroxylases, psychopharmacology

1. Introduction

Prolyl-hydroxylases are enzymes that modulate collagen synthesis and oxygen balance (Myllyharju and Koivunen 2013). According to these roles, they are classified into two main categories: collagen prolyl 4-hydroxylases (collagen-P4Hs) and hypoxia-inducible factor (HIF) prolyl 4-hydroxylases (HIF-P4Hs). The common denominator for these two subtypes is that they both share structural similarity and utilize iron, ascorbate and 2-oxoglutarate as cofactors. Collagen-P4Hs reside inside the endoplasmic reticulum (ER) and catalyze the formation of hydroxyproline in collagen. HIF-P4Hs play a vital role in our bodies modulating the HIF response in an oxygen-dependent manner. In normoxia, HIF α is continuously and quickly degraded by HIF-P4Hs, but in hypoxia the catalytic activity of HIF-P4Hs is decreased leading to its stabilization and formation of a functional HIF $\alpha\beta$ dimer. Once HIF is stabilized, it induces a robust modulation in a large number of genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes and glucose transporters (GLUTs), leading to increased oxygen transport and decreased oxygen demand by cells. Four HIF-P4Hs have been found so far: HIF-P4H-1, HIF-P4H-2, HIF-P4H-3 and a transmembrane form known as P4H-TM [enzyme-family reviewed in (Myllyharju and Koivunen 2013)]. P4H-TM is an oddball enzyme in the classification for several reasons. (1) HIF-P4Hs 1-3 are localized in the cytoplasm or nucleus, whereas P4H-TM resides in the ER and its catalytic side faces the lumen. (2) The amino acid sequence of P4H-TM resembles more that of collagen-P4Hs than HIF-P4Hs, but P4H-TM is unable to hydroxylate procollagen-polypeptides. (3) While P4H-TM can hydroxylate the prolines in HIF α targeted by HIF-P4Hs *in vitro*, it also hydroxylated to a small extent HIF α in which these prolines were

mutated to alanines suggesting it may have additional substrates to HIF α (Koivunen et al. 2007). Finally, the physiological role of collagen-P4Hs and HIF-P4Hs 1-3 is established (Myllyharju and Koivunen 2013), but the role and function of P4H-TM remain still largely unknown (Leinonen et al. 2016), although its inhibition associates with renal EPO production and induction of erythropoiesis similarly to inhibition of HIF-P4Hs 1-3 (Laitala et al. 2012).

Genetic studies on *P4h-tm* gene homolog in morpholino knockdown zebrafish provided the first evidence of the significance of P4H-TM in living vertebrates (Hyvarinen et al. 2010), later followed by mouse knockout studies (Leinonen et al. 2016). *P4H-tm* gene is not vital since *P4h-tm*^{-/-} mice are fertile and have a normal lifespan (Leinonen et al. 2016). However, both the morpholino knockdown zebrafish and *P4h-tm*^{-/-} mice display abnormalities that coexist between these species: structural alterations of the kidneys and proteinuria, and abnormalities in the eye structure and function (Hyvarinen et al. 2010; Leinonen et al. 2016). In both species, the expression level of P4H-TM mRNA is high in the eyes and brain, and moderate in kidneys. Specifically, the expression of P4H-TM is highest in the retinal pigment epithelium, and amygdala, hypothalamus and CA3 area of hippocampus in the mouse brain (Leinonen et al. 2016). Lack of *P4H-tm* stabilizes HIF-1 α in mouse cortical neurons and leads to induction of HIF target genes in several tissues. In the mouse retina, it leads to an unusual phenotype where cone-mediated visual function shows an age-independent impairment that is associated with decelerated signal transmission in the cone-pathway with no clear-cut cone photoreceptor degeneration. Therefore, the previous study

proposed that a universal defect might exist in neurotransmission and CNS physiology in *P4h-tm*^{-/-} mice.

In the only published clinical report on *P4H-TM* mutations, six patients presenting with a novel type of intellectual disability syndrome were clinically and genetically assessed (Kaasinen et al. 2014). In addition to the intellectual disability, the clinical features of these patients included hypotonia, strabismus, difficulty for visual focusing, planovalgus (flat feet), mild contractures in elbow joints, interphalangeal joint hypermobility and coarse facial features that developed during childhood. Therefore, the syndrome was coined HIDEA (Hypotonia-Intellectual Disability-Eye Abnormalities). Genetic mapping of HIDEA patients identified a novel ID predisposition locus with three candidate genes including *P4H-TM*. More recently, a whole exome sequence analysis found five HIDEA patients with gene variants only in the *P4H-TM* gene demonstrating that it indeed is the causative gene for HIDEA (Koivunen P, personal communication).

We have earlier shown that *P4h-tm*^{-/-} mice recapitulate some aspects of the visual disturbances found in HIDEA patients (Leinonen et al. 2016). In the same study, we found a high expression of P4H-TM in limbic areas of the brain: amygdala, hypothalamus and CA3 area of hippocampus suggesting its direct involvement in fundamental behaviors such as fear, anxiety, motivation, learning and memory. In this study, we repeated the expression analysis of P4H-TM in the brain and performed a comprehensive behavioral characterization of *P4H-tm*^{-/-} mice. Our data suggests that P4H-TM is a potential novel target for anti-depressant and anti-anxiety pharmacotherapy.

2. Material & Methods

2.1 Animals

We used *P4h-tm*^{-/-} mice generated by inserting a lacZneo cassette into exon 3 of the *P4h-tm* gene via homologous recombination leading to a truncated transcript (Leinonen et al. 2016). The mice had been back-crossed for 10 generations into C57BL/6J OlaHsd background. Most experiments were performed in 5-month-old male mice (cohort 1: WT, n=10; -/-, n=10 and cohort 2: WT, n=6; -/-, n=12). A few supplementary experiments (noted in figure legends and Table 1) were performed in other cohorts of 2.5- and 14-month-old male *P4h-tm*^{-/-} (n=11 and n=6, respectively) and WT (n=10 and n=4, respectively) mice. Social behavior tests were performed in a cohort consisting both male (WT, n=4; -/-, n=5) and female (WT, n=4; -/-, n=4) mice. Additionally, some key experiments were performed also in male *Hif-p4h-1*^{-/-} (WT, n=10; -/-, n=10), *Hif-p4h-2*^{gt/gt} (WT, n=13; -/-, n=12), and *Hif-p4h-3*^{-/-} mice (WT, n=12; -/-, n=12). Generation of *Hif-p4h-2*^{gt/gt} (a hypomorphic line), *Hif-p4h-1*^{-/-} and *Hif-p4h-3*^{-/-} mice has been previously described (Laitala et al. 2012; Hyvarinen et al. 2010). Table 1 summarizes the mouse cohorts (age, sex and number of replicates) used in each experiment. As controls in all experiments, we used WT mice derived from heterozygous breeding. The experiments were performed with the experimenter blinded to the mouse genotype. Female mice were group caged most of the time but placed

temporarily in individual cages for certain tests such as novelty suppressed feeding. Males were separated into individual cages 3-5 days before the testing period and kept isolated throughout the test period to avoid fighting. The mice were fed *ad libitum* and maintained at 22 °C, 30-55 % room humidity and 12h/12h light-dark cycle (lights on 7am-7pm) unless otherwise noted. All animal experiments were conducted according to the Council of Europe guidelines (Directive 86/609) and approved by the Animal Experiment Board in Finland.

2.2 Histology

Mice were deeply anesthetized with pentobarbital-chloralhydrate mixture and transcardially perfused with 0.9% saline and 4% paraformaldehyde. The brains were removed and post-fixed with 4% paraformaldehyde for 4 h, then immersed overnight in 30% sucrose and stored in antifreeze at -20°C until cut into 35 µm coronal sections. β -galactosidase activity was detected by 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) staining.

2.3 Spontaneous activity tests

Three different testing arenas were used in recordings of spontaneous activity. To avoid odor traces the test arena was cleaned with 70% ethanol before each mouse. Cohort 1 was tested in a square plastic box (dimensions 53 cm x 31 cm) that was air-tightly sealed from the ceiling with a large glass pane. The field was evenly illuminated by a ceiling light and an additional spot light that were directed to the room walls. The trial started with the mouse placed in one of the field corners. The mouse was allowed to explore the field for 5 min. Cohort 2 was tested twice in an observation cage (26 x 26

× 39 cm) with white opaque walls using infrared photobeam detection method coupled with an automated activity monitor (TruScan®, Coulbourn Instruments, CO, USA). The system was designed to enable separate monitoring of horizontal (XY-move time) and vertical activity (rearing). The test session took 10 min and was replicated after 48 h to assess the extent of habituation to the test cage. Cohort 2 was additionally tested once in a large round open-field (empty wading pool of white plastic, diameter 120 cm, wall height 15 cm) in a 5-min trial. Another cohort of 14-month-old mice was also tested once in the large open field to study aging-effect on spontaneous activity. When no infrared detection was used, mouse locomotion was recorded with a HD camera (LifeCam Cinema, Microsoft) inserted over the arena. Locomotor activity was analyzed by EthoVision XT 7 software (Noldus, Wageningen, Netherlands).

2.4 Elevated plus-maze test

Elevated plus-maze apparatus comprised of four arms (length 30 cm and width 5 cm), of which two opposing arms were sheltered with 15-cm high walls and the two others were left open. The maze was painted black and covered with light brown paper to allow contrast between the black mice and the background. The maze was elevated 50 cm from room floor and illuminated steadily at ~250 lux from the room ceiling. The mouse was placed in the maze center, the snout facing a sheltered arm, and allowed to freely explore the maze for 5 min. Mice that fell off the arena were removed from analysis. The session was video-recorded with an overhead HD camera, and the videos were analyzed by EthoVision software (Noldus, Wageningen, Netherlands). Only mice that made five or more arm entries during 5 min were included in the analysis. We used the relative time spent in the open arms as an indicator of anxiety.

2.5 *Panic test – Freezing behavior in 10 % CO₂ environment*

The test was conducted in a rectangular plastic arena (dimensions 53 cm x 53 cm x 31 cm) that was air-tightly sealed from the top with a large glass pane. The arena was illuminated by a ceiling light and additional spot lights directed toward room walls. The mouse behavior was recorded with an overhead HD camera (LifeCam Cinema, Microsoft). Freezing (mouse completely immobile) was timed manually from the videos by an experimenter blinded to the mouse phenotype. The mouse was allowed to explore the field for 5 min. During the first test session, the sealed box was ventilated with normal room air. Ten days later, the mice were tested again with the sealed box ventilated with a gas mixture of 10 % CO₂ and 90 % O₂ (Oy AGA Ab, Finland). The CO₂ content in the box was measured at 8-10 % (Capnomac Ultima, Datex-Engstrom, Finland). (The CO₂ concentration slightly dropped when the glass pane was elevated for the mouse to enter but soon reached the external source concentration).

2.6 *Novelty suppressed feeding (neophobia) test*

The novelty suppressed feeding (NSF) test was started after fasting the mice for 20 h. During fasting, the mice were maintained in their home cages with *ad libitum* access to drinking water. The test arena was a transparent Plexiglas rodent cage (dimensions 31 cm x 21 cm x 15 cm), with the floor covered with 1 cm layer of bedding. A pre-weighted rodent food pellet (Teklad 2016S) was placed on a piece of round white filter paper (diameter ~10 cm) in the cage center. The mouse was placed in one of the

corners of the cage and allowed to roam freely. First contact time (including brief touch) and first feeding time (actual bite) were recorded by an experimenter. When the mouse started feeding, it was immediately transferred back to its home cage with the food pellet available for additional 5 min. After this time, the pellet was re-weighted to control for the appetite.

2.7 Marble burying test

In marble burying test, nine glass marbles (diameter ~1 cm) were laid into a 3 x 3 array into a standard mouse cage with 2 liters of clean bedding. The mouse was left in the cage with the marbles overnight. Visible marbles were counted in the morning. A high number of covered marbles were considered a sign of high anxiety.

2.8 Novel object recognition test

A pair of identical objects (Duplo^R blocks, half of mouse size) was placed in the mouse home cage overnight. The objects were removed next morning, and the actual test ensued after 4 h. In the test, two objects were placed on the front end of the cage. One object was an identical copy of the familiar one, the other was a novel object. The side of presentation of the novel object was random. The mouse was observed for 5 min and the approaches toward the objects were counted. The test was repeated with another pair of objects the next day. The new sample objects were again placed in the home cage overnight and removed the next morning. The second test followed 4 h later in the afternoon.

2.9 Social approach test

In the social approach test, we used 4-month-old female mice as stranger mice for the experimental mice (both male and female). The stranger mice were habituated to stay calm inside a cylinder made of metal grid (grid size 1 cm) one day prior to experiments. Each stranger mouse was used for 2-3 times in the actual testing. Test was done in the mouse's home cage (40 cm x 24 cm x 15 cm) with bedding and two cylindrical cages (height 9 cm, diameter 8 cm, half-filled 60 ml glass beaker on the top to add weight) at the opposite ends of the cage. The cages were separated by an opaque plastic wall with a mouse-size opening. After a 10-min habituation period, a stranger mouse was placed in one of two cages. The actual test took 10 min and was video-recorded. The number of nose contacts with the cages and the time sniffing were calculated from the recording.

2.10 Social interaction test

The test was conducted in an open white plastic rectangular arena (79 cm x 57 cm) with 15 cm walls. The test mouse was placed in this arena with a younger stranger mouse (wild-type) of the same sex for 10 min. The stranger mouse was marked with white TiO₂ on the back. An experimenter blinded to the test mouse genotype counted the number of contacts and the time in contact (distance < 2 cm between the nose of the test mouse and the body of the stranger mouse).

2.11 Odor recognition test

In the beginning of the odor recognition test, the experimental mice had been individually housed and without cage changes for 5 days leaving their own odor on the cage bedding. Similarly, the odor donor mice of same sex, but younger, had resided in

their home cage without cage changes. All experimental mice were given a wooden ball (diameter ~2 cm) into their cage overnight while the donor mice were given several balls. Next morning, the balls were carefully collected individually into small plastic bags (Minigrip®) avoiding contamination and evaporation of odors. Before starting the test, cage enrichment (nesting material and a plastic igloo) was removed from each cage, which generally make mice temporarily active. A mouse cage was placed under a video-camera and the two odor balls (one having the mouse's own odor and the other one odor of a stranger) were placed at one end of the cage ~15 cm from each other. The mouse behavior was tracked for 2 min. Cumulative sniffing time at both odor balls were recorded by an experimenter blinded as to the phenotype. A couple of days later the mice were presented a choice between a wooden ball odorized with another unknown donor mice and a ball odorized with banana extract. Otherwise the test setup was identical to the first one.

2.12 Forced swim and tail suspension tests

To test behavioral despair, we used the well-established forced swim and tail suspension tests. The forced swim test was run in a white round bucket (diameter 19.5 cm, depth 21 cm) filled with 22 °C water. The mouse was neither able to escape nor reach the floor. The test time was 6 min. The mouse behavior was recorded with an overhead HD camera and cumulative mobility/immobility time was analyzed using EthoVision software.

In the tail suspension test (TST), a mouse was suspended from its tail without possibility to escape. The distal third of the tail was attached onto a flexible 50 cm flat rubber band hanging from a tableside. We observed that a flexible band, as compared

to a rigid string, prevented the known ability of C57B6 mice to turn around and grasp the string. The 6-min test session was video-recorded with a HD camera from the side. Cumulative mobility/immobility time was subjectively analyzed by an experimenter blinded to the phenotype.

2.13 Morris swim task

Morris swim task ('watermaze') was performed in a plastic white circular pool (diameter 120 cm, wall height 15 cm) with a transparent 14 cm x 14 cm platform submerged 1 cm below the surface in a fixed location. The platform served as the only escape route from the pool. Water temperature was kept at the room temperature (20 ± 0.5 °C). The mouse was placed into the pool alternatingly between four starting position along the pool edge facing the wall, using the same start position for all mice in a given trial. The maximum swim time was set at 60 s, and if a mouse failed to find the platform within this time, it was placed on the platform for 10 s by the experimenter. Mice that found the platform within the 60 s trial-time were also allowed to stay on the platform for 10 s. The acquisition phase consisted of five daily trials with 10-min intervals between trials for the first 4 days, and three training trials on the 5th day. The first and the last trial of the 5th day were performed without the platform to test for a potential search bias as an index of spatial memory. Between the trials, the mouse was placed in a clean cage under a heat lamp to prevent hypothermia. The mouse was video-recorded with a ceiling camera and tracked with the EthoVision program. We computed swimming speed, path length, time spent in the wall zone (10 cm from the wall), and time spent near the platform zone (diameter 30 cm).

2.14 Statistical analysis

Single parameters for which the two groups were compared were analyzed using Student's t-test (equal variances between groups assumed) or Mann-Whitney U-test (unequal variances). Two-way analysis of variance (2-way ANOVA) was used when dataset had two main factors: sex and genotype. ANOVA for repeated measures (ANOVA-RM) was used when the same experimental parameter was tested on several days on the same subjects. Genotype was used as the between-subjects factor and experimental day as the within-subjects factor. ANOVAs were followed by Bonferroni post-hoc tests. The analyses were performed with SPSS 25 and GraphPad Prism 8 software, and graphs drawn with GraphPad Prism 8. All data are presented as mean $\pm$ SEM. Statistical significance was set at $P<0.05$.

3. Results

3.1 High expression of P4H-TM is observed in brain areas involved in anxiety, social behavior and exploration

The use of LacZ motive for β -galactosidase in the inactivating gene construct for *P4h-tm* allowed us to observe the P4H-TM protein expression pattern using XGal staining. Figure 1 summarizes the brain regions with the observed highest expression of P4H-TM. Notably, most nuclei of amygdala (Fig. 1A,E), lateral septum (Fig. 1A,D), and bed nucleus of stria terminalis (Fig. 1D) and cingulate cortex (Fig. 1 B,D), which are all linked to anxiety and social behavior (Lebow and Chen 2016), showed a high expression. Similarly, the entire hypothalamus (Fig. 1 A,B) and insular cortex (Fig. 1A), which have been associated with monitoring of body homeostasis and elementary

behaviors (Sternson 2013; Gogolla 2017), were positive. Further, high expression was observed in hippocampus (area CA3, Fig. 1 B,C), and laterodorsal and mediodorsal thalamic nuclei (Fig. 1B), which are involved in spatial processing and memory (Gilbert and Brushfield 2009; van Groen, Kadish, and Wyss 2002; Mitchell and Chakraborty 2013). Interestingly, the neocortical expression was widespread but limited to pyramidal layers (L2/3 and 5; Fig. 1 C). In addition, there was a moderate expression in deep cerebellar nuclei and vestibular nuclei, which are involved in motor coordination and balance (Morton and Bastian 2004).

3.2 *P4h-tm^{-/-}* mice are hyperactive

We assessed spontaneous activity of *P4h-tm^{-/-}* and WT mice using three different cohorts and three different test environments (cohorts 1-2 consisted of 5-month-old males, and cohort 3 was tested at 14-month of age). Cohort 1 was tested in a novel 53 cm x 31 cm box sealed with a glass pane. In this environment, the *P4h-tm^{-/-}* mice were clearly more active than WT mice ($t_{17}=4.97$, $P<0.0001$) and traveled ~30% longer distance in a 5-min trial (Fig. 2A). Cohort 2 was tested twice in a smaller 26 cm x 26 cm open-top box with infrared detectors (Fig. 2B). Here as well, the *P4h-tm^{-/-}* mice were significantly more active than WT mice both during their first encounter with the test environment (session 1, $t_{32}=3.21$, $P=0.006$) and a second test session conducted two days later (session 2, $t_{32}=4.61$, $P<0.0001$). Both genotypes showed habituation to the test environment by decreasing their exploratory activity ($F_{1,16}=76.2$, $P=0.0006$), so that there was no test day x genotype interaction ($F_{1,16}=3.00$, $P=0.10$). In additional trials conducted in a large round open-field, 5- and 14-month-old mice displayed practically the same genotype difference in their activity (Fig. S1), implying that the age is not a

determinant for this behavior. These results indicate that *P4H-tm^{-/-}* are in general hyperactive, not only in novel situations.

3.3 *P4H-tm^{-/-}* mice display suppressed fear and anxiety

Next, we performed a classical anxiety paradigm, elevated plus-maze test (EPM), which is widely used in assessing anxiolytic drugs (Dawson and Tricklebank 1995). We used several cohorts of *P4h-tm^{-/-}* and WT mice at 2.5, 5 and 14 months of age. In the EPM test, the mouse is placed into an elevated platform consisting of two closed and two open arms. The mice naturally avoid the open arms while exploring the closed arms that provide a shelter. The main output of the paradigm is the time spent in the open arms, a sign of decreased anxiety. We found that *P4H-tm^{-/-}* mice spent consistently more time in the open arms than WT mice (Fig. 3, genotype: $F_{1,42}=15.9$, $P=0.0003$; interaction: $F_{2,42}=1.0$, $P=0.36$). Other testing paradigms for panic reaction, fear and anxiety led to the similar conclusions. Panic in prey animals causes them to become immobile, or to “freeze”, and elevated CO₂ triggers panic reaction in all animals. The freezing behavior of WT and *P4h-tm^{-/-}* mice was recorded in an open arena first in room air, and then ten days later in an elevated CO₂ environment. We found that WT mice display significantly more robust panic reaction towards 10 % CO₂ compared to *P4h-tm^{-/-}* mice (genotype – test day interaction: $F_{1,17}=68.1$, $P<0.0001$). In the novelty suppressed feeding test, where an animal is balancing between the feeling of hunger and anxiety towards novelty (Ramaker and Dulawa 2017), *P4h-tm^{-/-}* mice approached and bit a food pellet in a novel environment significantly faster than WT mice after overnight fasting (Fig. 4B, $t_{17}=2.44$, $P=0.03$). A difference in appetite unlikely explains this result since the WT mice ate more when given free access to food for 5 min in their

home cage immediately after the NSF test (Fig. 4B insert, $t_{17}=2.83$, $P=0.01$). In an overnight marble burying test, where novel objects were introduced into mouse home cage, $P4h-tm^{-/-}$ mice covered fewer marbles with bedding than WT mice (Fig. 4C, $t_{16}=7.08$, $P<0.0001$), which is another sign of decreased anxiety towards novelty (Nicolas, Kolb, and Prinssen 2006). Furthermore, in the novel object recognition (NOR) task, which is generally considered a recognition memory task (Antunes and Biala 2012), 3-month-old male $P4h-tm^{-/-}$ mice did more exploratory visits toward the novel object on test day 1, but after familiarization to the objects on day 2 the exploratory visits declined to the same level in both genotypes (Fig. 4E; genotype – test day interaction: $F_{1,19}=4.94$, $P<0.05$). To control for hyperactivity, we analyzed exploratory visits towards a familiar object as well. $P4h-tm^{-/-}$ mice were not any more interested in exploring the familiar object than WT mice were (Fig. 4D; genotype main effect: $F_{1,19}=0.13$, $P=0.72$; genotype – test day interaction: $F_{1,19}=0.11$, $P=0.74$). Collectively, $P4h-tm^{-/-}$ appeared less fearful and anxious in all behavioral paradigms tested, although hyperactivity and increased curiosity towards novelty on the other hand may complicate conclusions especially with regard to EPM and NSF tests.

3.4 $P4h-tm^{-/-}$ mice show increased social behavior

Since $P4h-tm^{-/-}$ mice showed increased exploratory activity and decreased anxiety in several testing environments, we were interested to see if they would also show less fear and/or more interest toward fellow mice. In the social approach test, a standard mouse cage was divided into two compartments, one housing an empty cylindrical cage and the other a cage with a stranger mouse inside. A cohort consisting of 5-month-old female and male $P4h-tm^{-/-}$ and WT mice was tested. $P4h-tm^{-/-}$ mice spent

> 30% more time exploring the caged mouse than WT mice (Fig. 5A, $F_{1,13}=13.10$, $P=0.003$). There was no sex main effect ($F_{1,13}=1.16$, $P=0.30$) nor sex by genotype interaction ($F_{1,13}=0.71$, $P=0.41$). In the social interaction test where two mice were freely moving in a large, neutral cage, $P4h-tm^{-/-}$ mice displayed > 30% more close interactions, such as sniffing, with the stranger mouse, although the genotype effect only approached statistical significance (Fig. 5B, $F_{1,13}=4.51$, $P=0.05$). In another test for social approach (odor recognition), $P4h-tm^{-/-}$ mice sniffed objects paired with stranger mouse odors significantly more than WT mice did on test day 2 (Fig. 5F, $F_{1,13}=27.8$, $P=0.0002$). There was also a sex effect, such that male mice had a higher cumulative sniff time than females ($F_{1,13}=6.22$, $P=0.03$). However, the sex by genotype interaction was not significant ($F_{1,13}=2.94$, $P=0.11$). Notably, $P4h-tm^{-/-}$ mice were specifically interested in the stranger mouse odor since objects paired with the mouse's own odor ($F_{1,13}=0.91$, $P=0.36$), or banana odor ($F_{1,13}=1.17$, $P=0.30$), did not evoke more sniffing compared to WT mice (Fig. 5C,E). The results of the social behavior tests provide further evidence for abnormally daring and explorative behavior in $P4h-tm^{-/-}$ mice.

3.5 Decreased immobility time in behavioral despair tests in $P4h-tm^{-/-}$ mice

As anxiety and depressive-like behaviors display a high level of comorbidity (Yoshikawa et al. 2002; Meier et al. 2015), we next wanted to test if $P4h-tm^{-/-}$ mice display altered behavioral despair, a surrogate marker of depression (Castagne et al. 2011). This can be evaluated in tests where the animal is exposed to a desperate situation with no prospective escape. In the forced swim task (FST), the animals start to float when they surrender (*i.e.* become despaired) and in the tail suspension test (TST) they become immobile. Strikingly, the 5-month-old male $P4h-tm^{-/-}$ mice practically never

surrendered and struggled the whole 6-min test time in FST and TST, while WT mice spent long periods floating and immobile (Fig. 6 & Supplementary Videos 1 & 2. FST: WT, $n=9$, 88.4 ± 19.7 s; $P4h-tm^{-/-}$, $n=10$, 3.8 ± 2.7 s; $z=-3.10$, $P=0.0005$. TST: WT, $n=9$, 150.2 ± 22.5 s; $P4h-tm^{-/-}$, $n=10$, 7.7 ± 5.4 s; $z=-3.47$, $P<0.0001$). Additional FST trials performed in 2.5- and 14-month-old mice showed that the robustly decreased immobility time in $P4h-tm^{-/-}$ mice is consistent and does not depend on age (Fig. S1B).

3.6 Intact spatial learning and memory in $P4h-tm^{-/-}$ mice

Hyperactivity in $P4h-tm^{-/-}$ mice that was observed earlier could be detrimental for learning and memory. Therefore, we next tested 5-month-old male $P4h-tm^{-/-}$ and WT mice in the commonly used Morris swim task ('water maze') for spatial learning and memory (Tanila 2012). In this task, the mice were first trained to locate a hidden underwater platform in a large water pool based on room landmarks. This was done in several session during a 4-day acquisition phase. The acquisition phase was followed on day 5 by a probe trial in which the platform was removed to assess the search bias of the mice. $P4h-tm^{-/-}$ and WT mice learned equally fast to locate the hidden platform (test day main effect: $F_{3,14}=27.3$, $P<0.001$; genotype main effect: $F_{3,16}=0.004$, $P=0.95$; genotype-test day interaction: $F_{3,14}=1.8$, $P=0.19$) as assessed by the swim path length (Fig. 7A). They also displayed similar amount of thigmotaxis, *i.e.* swimming near the wall in search for an escape (Fig. 7B; genotype: $F_{1,16}=0.5$, $P=0.48$). However, the swimming speed was lower in $P4h-tm^{-/-}$ mice in the early acquisition phase but approached that of WT mice in later trials (Fig. 7C, genotype-test day interaction: $F_{3,14}=5.7$, $P=0.009$). In the probe test, there was no overall bias in the time spent between quadrants ($F_{3,14}=2.4$, $P=0.011$) and no quadrant by genotype interaction

($F_{3,14}=0.2$, $P=0.91$; Fig. 7D). However, when considering only crossings of the platform area in all four quadrants, the mice tended to make more crossings than by chance (25% of all crossings) in the target quadrant ($P<0.001$, one-sample t-test; Fig. 7E). However, there was no genotype difference in the number of crossings of the target platform area ($t_{16}=1.0$, $P=0.35$; Fig. 7E) or swim speed during the probe trial ($t_{16}=0.1$, $P=0.90$; Fig. 7F). We have also conducted novel object recognition (Fig. 4C,D) and contextual fear condition tests (data not shown), but the decreased fear and increased exploratory activity in *P4h-tm*^{-/-} mice precludes justified interpretation about learning and memory in these tests. Nevertheless, it seems that learning and memory is unaltered in *P4h-tm*^{-/-} mice.

3.7 Hyperactivity and suppressed behavioral despair observed in P4h-tm^{-/-} mice is absent in Hif-p4h-1^{-/-}, Hif-p4h-2^{gt/gt} and Hif-p4h-3^{-/-} mice

As P4H-TM and HIF-P4H-1, HIF-P4H-2 and HIF-P4H-3 all share a common biochemical function by stabilizing HIF (Koivunen et al. 2007; Leinonen et al. 2016), a question arises whether *Hif-p4h-1-3* deficient mice display a similar phenotype as *P4h-tm*^{-/-} mice. To this end, we utilized knock-out mouse lines of *Hif-p4h-1^{-/-}* and *Hif-p4h-3^{-/-}*, and a hypomorphic line of *Hif-p4h-2* (*Hif-p4h-2^{gt/gt}*) (knockout of this gene is embryonic lethal), and performed key behavioral tests that previously revealed the most distinct and consistent phenotype in *P4h-tm*^{-/-} mice. Locomotion in a novel open arena was unchanged in all the three mutant lines compared to their respective controls (Fig. 8A; *Hif-p4h-1^{-/-}*: $t_{18}=0.73$, $P=0.47$; *Hif-p4h-2^{gt/gt}*: $t_{23}=0.97$, $P=0.34$; *Hif-p4h-3^{-/-}*: $t_{22}=0.94$, $P=0.36$), as was also their performance in the tail suspension test (Fig. 8B; *Hif-p4h-1^{-/-}*: $t_{18}=0.76$, $P=0.75$; *Hif-p4h-2^{gt/gt}*: $t_{23}=1.53$, $P=0.14$; *Hif-p4h-3^{-/-}*: $t_{21}=0.54$, $P=0.60$). Overall,

we have so far observed no significant behavioral abnormalities in *Hif-p4h-1^{-/-}*, *Hif-p4h-2^{gt/gt}* or *Hif-p4h-3^{-/-}* mice, whereas the behavioral phenotype of *P4H-tm^{-/-}* mice consistently differs from their WT controls.

4. Discussion

We report here the first behavioral characterization of *P4h-tm^{-/-}* mice. We found that loss of P4H-TM function in mice causes a striking phenotype. *P4h-tm^{-/-}* mice appeared daring and distinctly explorative in all paradigms assessing fear, anxiety and social behavior. For instance, in a test where we triggered panic reaction by supplying 10 % CO₂ into breathing air, WT mice showed substantially increased freezing behavior, an innate fear reaction in prey animals, compared to baseline situation (same test in room air), whereas in *P4h-tm^{-/-}* this reaction was significantly less profound. A human correlate for this is a test where 5 % CO₂ in breathing air often causes panic attacks in panic disorder patients but rarely in controls (Rassovsky and Kushner 2003). Exploration in *P4h-tm^{-/-}* mice was especially increased towards novelty, and even more interestingly, towards fellow mice and their odors. In the object recognition task, *P4h-tm^{-/-}* mice were more interested in a novel than a familiar object while WT mice showed no preference. In the social approach test, *P4h-tm^{-/-}* showed increased exploration of the cage with a stranger mouse compared to WT mice, and in the odor recognition test they were sniffing stranger odors more than WT mice, while banana odor did not cause differential effect between the genotypes. Most staggeringly, *P4h-tm^{-/-}* mice practically never surrendered in behavioral despair tests and fought back the entire test time. FST and TST paradigms are commonly used in assessment of antidepressant drug efficacy (Castagne et al. 2011), but no antidepressant therapy so far has led to such a complete

loss of behavioral despair response in FST and TST as seen in *P4h-tm^{-/-}* mice (cf. (Cryan, Mombereau, and Vassout 2005; Castagne et al. 2011). The combination of hyperactivity/hyperexploration, increased disposition to initiate social contacts and robust absence of behavioral despair has seldom been reported in any genetically modified mouse line. To our knowledge, the only mouse line that resembles *P4h-tm^{-/-}* mice is a *Ncan^{-/-}* mouse that the authors propose a mouse model of mania (Miro et al. 2012). These mice deficient of neurocan showed mild hyperlocomotion, increased time spent on open arms of an elevated plus maze, and ~50% decrease in immobility time in the FST. However, in marble burying task *Ncan^{-/-}* mice buried significantly more marbles than their WT controls while *P4h-tm^{-/-}* left more marbles visible than their controls in our study.

Results of some of the anxiety and behavioral despair tests we used could be, however, affected by the hyperactivity found in *P4h-tm^{-/-}* mice. Specifically, the modestly increased exploration of open arms in the EPM test and decreased latency to start feeding in the NSF test could be solely consequences of hyperactivity rather than decreased anxiety. Instead, the differential panic reaction of WT and *P4h-tm^{-/-}* mice in elevated CO₂ environment cannot be explained by hyperactivity. Decreased marble burying and emphasized exploration towards novelty rather than familiarity in *P4h-tm^{-/-}* mice are also unlikely explained by hyperactivity alone. Conclusions from behavioral despair tests (FST and TST) are known to be constrained if the tested treatment causes hyperactivity (Porsolt, Bertin, and Jalfre 1977). However, the fact that the measured locomotor activity was increased by around 30 % unlikely alone explains the nearly complete loss of immobility in FST and TST in *P4h-tm^{-/-}* mice. For comparison, a

psychostimulant dexamphetamine that can increase locomotor activity by 300 % in WT mice has been shown to significantly decrease immobility time in these tests but not to lead to complete loss of immobility (Vaugeois et al. 1996). Nevertheless, further investigation using depression models and additional tests such as sucrose preference is needed to confirm the anti-depressive effects of P4H-TM loss of function.

The expression pattern of P4H-TM in the mouse brain shows remarkable regional differences. It is completely lacking in caudate-putamen while being high in amygdala, bed nucleus of stria terminalis (BNST), lateral septum, entire hypothalamus, and certain thalamic nuclei such as paraventricular nucleus and medial frontal/anterior cingulate cortex. Interestingly, these brain regions constitute the core neural circuit controlling anxiety responses (Anthony et al. 2014; Calhoon and Tye 2015). In addition, medial amygdala, BNST, lateral septum and ventromedial hypothalamus have been intimately linked with aggressive behavior in male rodents (Clarke and File 1982; Wong et al. 2016; Chen and Hong 2018). Thus, the striking fearless and relentless phenotype of *P4h-tm*^{-/-} mice observed in the present study is fully compatible with the protein expression pattern. Hyperlocomotion has been conventionally ascribed to treatments that increase the activity of the mesolimbic dopamine system, especially in nucleus accumbens (White, Whitaker, and White 2006). However, neither *n. accumbens* nor ventral tegmental area (VTA) showed much P4H-TM expression in the mouse. On the other hand, we saw substantial P4H-TM expression in the CA3 subregion of hippocampus throughout its septo-temporal axis, while ventral hippocampus is well known to modulate locomotor and exploratory activity through its connections with *n. accumbens* and VTA (White, Whitaker, and White 2006). In addition, histaminergic

neurons in the tuberomammillary nucleus of the hypothalamus modulate locomotor activity through their projections to *n. accumbens* (Bristow and Bennett 1988) and orexin neurons in the lateral hypothalamus modulate locomotor activity via their VTA projections (Jones et al. 2001). Thus, P4H-TM deficit in ventral hippocampus and medial and lateral hypothalamus may account for the motor hyperactivity of *P4h-tm*^{-/-} mice.

The increased activity and exploration in *P4h-tm*^{-/-} mice could refer to attention deficit. We therefore investigated spatial learning and memory in *P4h-tm*^{-/-} mice using Morris swim task. *P4h-tm*^{-/-} mice learned to locate the hidden platform equally fast as WT mice, displayed same degree of thigmotaxis during the learning phase, and finally showed a similar search bias as WT mice in the probe test. Therefore, it seems unlikely that hyperactivity in *P4h-tm*^{-/-} mice is detrimental in terms of attention. Notably, navigation in the water pool is visually guided (Leinonen and Tanila 2018), and we have earlier detected cone-driven visual dysfunction in *P4h-tm*^{-/-} mice by electroretinogram (ERG) responses (Leinonen et al. 2016). However, we also recorded ERG responses to high-contrast pattern stimuli and did not find impairments. Normal navigation of *P4h-tm*^{-/-} mice in the Morris swim task is compatible with this finding.

The courageousness and perseverance observed in *P4h-tm*^{-/-} mice are behavioral characteristics that are often the desired outcomes of psychiatric therapy, helping the affected patients cope with overt anxiety (often towards social situations) and depression/despair. This raises the intriguing idea of targeting P4H-TM with selective pharmacotherapy for treatment of anxiety and depression. Indeed, we found that the phenotype was specifically associated with P4H-TM dysfunction as mutations in

all other HIF-P4Hs in mice lacked the effect on locomotion and behavioral despair. HIF-P4H inhibitors have already been developed and some of them are undergoing phase 3 clinical trials for treatment of renal anemia (Koivunen and Kietzmann 2018). Since the amino acid sequence of P4H-TM is distinct from other HIF-P4Hs (Koivunen et al. 2007), development of selective P4H-TM inhibitors should be feasible. In the future, studies with conditional *P4h-tm*^{-/-} mice will be instrumental to investigate whether timed and localized inactivation of P4H-TM will result in equally robust behavioral changes. Further, major efforts need to be directed in studying if localized HIF stabilization caused by absence of P4H-TM can cause such changes in behavior, or alternatively if P4H-TM has another yet undetermined substrate.

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Figure legends

Figure 1. P4H-TM expression in different brain areas as revealed by β -galactosidase staining. [A] A thick (1 mm) coronal section at the level of caudal septum. Strong expression levels can be seen throughout the hypothalamus (HT), amygdala (Amg) and lateral septum (Ls). The neocortex (Ctx) shows layer-specific staining that is particularly dense in insular cortex (InC). [B] A thick section at the level of rostral hippocampus. Strong staining is evident in lateral hypothalamus (LH), Amg and Ctx. In addition, the thalamus is positive, especially the laterodorsal (ThLD) and mediodorsal (ThMD) nuclei. [C] A thin section at the level of mid-hippocampus, showing particularly strong staining of Ca3 pyramidal cells and cortical pyramidal layers (L2/3, L5). [D] A close-up at the caudal septal level. Besides Ls, the superficial layers of cingulate cortex (Cg), paraventricular thalamus (ThPV), and bed nucleus of stria terminalis (BNST) stain strongly positive. Note also the strong staining in the ventricular wall. [E] A section through the mid amygdala with staining of the central (AmgC), lateral (AmgL), basomedial (AmgBM) and medial (AmgM) nuclei. [F] A section through the pons. The deep cerebellar nuclei, fastigial (FN), interposed (IPN) and dentate (DN) nuclei all stain positive, as well as medial (MVe) and lateral (LVe) vestibular nuclei. 4V = fourth ventricle.

Figure 2. Increased exploratory activity in *P4h-tm*^{-/-} mice. [A-B] *P4h-tm*^{-/-} mice moved a much longer distance than their wild-type (WT) littermates in new test environments. [A] Mouse cohort 1 was tested in a 53 cm x 31 cm closed box with 16 cm translucent walls. [B] Mouse cohort 2 was tested twice in a 26 cm x 26 cm open-top box with 39 cm transparent walls. Sessions 1 and 2 were separated by two days. Both *P4h-tm*^{-/-} (grey) and WT mice (black) decreased their exploration from session 1 to session 2 as an indication of habituation to the test environment. However, *P4h-tm*^{-/-} mice remained significantly more active than WT mice. Unpaired t-test (Fig. A): ****P*<0.001. Bonferroni's post-hoc test (Fig. B): ***P*<0.01, ****P*<0.001. Group means+SEMs as well as individual values are shown.

Figure 3. Elevated plus-maze (EPM) test shows more exploration in open space in *P4h-tm*^{-/-} mice. Bonferroni's post hoc test: * $P < 0.05$. Group means+SEMs as well as individual values are shown.

Figure 4. Tests of panic reaction, fear and anxiety show dampened responses in *P4h-tm*^{-/-} mice. [A] In the panic test, freezing behavior (an innate reaction to threat in prey animals) was first tested in an open arena and room air. Ten days later, the test was repeated in room air supplemented with 10 % CO₂. When compared to test in room air, WT mice increased their freezing significantly more compared to *P4h-tm*^{-/-} mice (genotype x test day interaction: $F_{1,17}=68.1$, $P<0.0001$). [B] In the novelty suppressed feeding test, the mouse was placed in a novel cage with a food pellet after 20 h food deprivation. *P4h-tm*^{-/-} mice started to bite the pellet earlier than WT mice in a novel cage, but in the home cage they consumed less food in a 5-min trial. [C] In the marble burying test, novel objects, glass marbles, were introduced into the home cage. Anxious mice tend to bury the marbles under the cage bedding. *P4h-tm*^{-/-} mice left more marbles visible than WT mice, speaking for decreased anxiety toward novel objects. [D-E] In the novel object recognition test, the mouse was introduced to a familiar and a novel object pair on two consecutive days. [D] *P4h-tm*^{-/-} (grey) and WT mice (black) made the same number of exploratory visits toward the familiar object on both days. [E] *P4h-tm*^{-/-} showed more interest, or less fear, toward the novel object than WT mice on the first test day (interaction: $F_{1,19}=5.0$, $P=0.04$), while after familiarization (day 2) the number of exploratory visits did not differ between the genotypes. Unpaired t-test (Figs. B,C); * $P<0.05$, *** $P<0.001$. Bonferroni's post-hoc test (Fig. A,E); * $P<0.05$, *** $P<0.001$. Group means+SEMs as well as individual values are shown.

Figure 5. Increased social behavior in *P4h-tm*^{-/-} mice. [A] In the social approach test, the mouse could freely explore either an empty cage or a cage with an unknown mouse inside. *P4h-tm*^{-/-} mice spent significantly more time in exploring the cage with a stranger mouse. [B] In the social interaction test, where two mice were placed in a new large open arena, *P4h-tm*^{-/-} mice appeared to spend more time in contact with a stranger mouse than WT mice, although the effect only approached significance ($F_{1,13}=4.51$, $P=0.05$). [C-F] In the odor recognition test, the mouse was presented with a pair of odorized wooden balls in its home cage; on day 1 one's own odor vs. odor of an unknown mouse [C-D], and on day 2 banana extract vs. another unknown mouse [F]. *P4H-tm*^{-/-} mice sniffed the ball with a stranger mouse odor significantly more than WT mice on day 2. Statistical analysis was performed by 2-way ANOVA and *P*-values are presented for genotype main effect. Group means+SEMs as well as individual values are shown.

Figure 6. Decreased immobility time in behavioral despair tests in *P4h-tm*^{-/-} mice.

Almost all *P4h-tm*^{-/-} were constantly moving during the 6-min test while most WT mice showed long periods of immobility in forced swim and tail suspension tests. Mann-Whitney U-test: *** $P < 0.001$. Group means+SEMs as well as individual values are shown.

Figure 7. Unimpaired spatial learning and memory in *P4h-tm*^{-/-} mice as assessed by Morris swim task. [A] The swim path length decreased similarly across days in *P4h-tm*^{-/-} and WT mice (days 1-4, learning phase). [B] Thigmotaxis, the tendency to swim near the pool wall, also declined over days to the same extent in both genotypes. [C] WT mice swam faster on the first test day than during later trials, whereas *P4h-tm*^{-/-} mice maintain the same swimming speed throughout the test (ANOVA-RM: interaction; $F_{3,14}=5.7$, $P=0.009$). Bonferroni post-hoc test for day 1: $*P<0.01$. [D-F] During the probe test on day 5 to determine the spatial search bias, the escape platform was removed. [D] The mice did not show a clear search bias in terms of time between the quadrants (ANOVA-RM: ($F_{3,14}=2.4$, $P=0.011$)). [E] Mice crossed the platform location in the target quadrant more often than by chance, ($\# p<0.001$, one-sample t-test). However, there was no genotype difference in search bias by either measure (see results section for statistics). [F] Swimming speed did not differ between the genotypes during the probe test. Group means+SEMs are shown in [A-E], in [F] also individual values.

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917 **Fig. 8. The phenotype observed in key tests in *P4h-tm*^{-/-} mice is absent in *Hif-p4h-***
918 ***1*^{-/-}, *Hif-p4h-2*^{gt/gt} and *Hif-p4h-3*^{-/-} mice. [A] Locomotion in an open arena, and [B]**
919 **behavioral despair response in the tail-suspension test. Group means+SEMs as well as**
920 **individual values are shown.**

Table 1. Description of cohorts used in *P4h-tm^{-/-}* mouse experiments.

Behavior test	WT male, n	-/- male, n	WT female, n	-/- female, n	Age (months)	Cohort #	Data Fig.
Spontaneous activity (53 x 31 cm arena)	9	10	-	-	5	1	2A
Spontaneous activity (26 x 26 cm arena)	6	12	-	-	5	2	2B
Open field	6	11	-	-	5	2*	S1A
Open field	4	5	-	-	14	3**	S1A
Elevated plus maze	10	11	-	-	2.5	4	3
Elevated plus maze	6	11	-	-	5	2*	3
Elevated plus maze	4	6	-	-	14	3	3
Panic test (freezing behavior)	9	10	-	-	5	1	4A
Novelty suppressed feeding	9	10	-	-	5	1	4B
Marble burying	6	12	-	-	5	2	4C
Novel object recognition	10	11	-	-	2.5	4	4D-E
Social approach	4	5	4	4	5	5	5A
Social interaction	4	5	4	4	5	5	5B
Odor recognition	4	5	4	4	5	5	5C-F
Forced swim	9	10	-	-	5	1	6
Forced swim	10	11	-	-	2.5	4	S1B
Forced swim	4	3	-	-	14	3***	S1B
Tail suspension	9	10	-	-	5	1	6
Morris swim task	6	12	-	-	5	2	7A-F

*one *P4h-tm^{-/-}* mouse was removed from cohort 2 and **one to ***three mice from cohort 3 during the course of the experiments due health related reasons.

Null mutation in *P4h-tm* leads to increased social behavior, decreased anxiety and absence of despair**Highlights**

- P4H-TM is a transmembrane HIF prolyl 4-hydroxylase with an unknown function
- P4H-TM expression is high in brain regions related to emotional and social behavior
- P4h-tm deficient mice are social and show little anxiety or behavioral despair
- P4H-TM is a promising new target for anxiolytic and antidepressant drugs