

Genetic Inactivation of Melanin-Concentrating Hormone Receptor Subtype 1 (MCHR1) in Mice Exerts Anxiolytic-Like Behavioral Effects

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The biological effects of the melanin-concentrating hormone (MCH) are mediated by the melanin concentrating hormone receptor 1 (MCHR1) in mice. This receptor is enriched in brain areas that are involved in the modulation of mood and affect, suggesting that MCH-dependent signaling may influence neurobiological mechanisms underlying fear and anxiety processes. To test this, we have generated mice lacking functional MCHR1 and characterized phenotypic traits using a number of behavioral tests. Mice carrying a null mutation of the MCHR1 gene display anxiolytic-like behavior across a battery different behavioral paradigms commonly used to assess fear and anxiety responses in rodents: open field, elevated plus maze, social interaction, and stress-induced hyperthermia. The brain serotonin (5-HT) system is central to the control of mood- and anxiety-related processes. To examine the impact of MCHR1 receptor deletion on 5-HT neurotransmission, we used *in vivo* microdialysis in freely moving knockout and wild-type mice. Baseline dialysate 5-HT levels were significantly lower in MCHR1 knockout mice as compared with wild-type controls (9.53 ± 0.24 fmol for wild types vs 6.91 ± 0.36 fmol for knockouts) in the prefrontal cortex (PFC), one of the main target structures of the serotonergic system and one that is highly associated with the control of emotional processes. Moreover, forced swim increased 5-HT efflux in the PFC of wild-type but not MCHR1 knockout mice. In summary, we show that MCHR1 can modulate stress- and anxiety-like behaviors and suggest that this may be due to changes in serotonergic transmission in forebrain regions.

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INTRODUCTION

Melanin-concentrating hormone (MCH) is a cyclic neuropeptide, first isolated from the pituitary gland of salmon (Kawauchi *et al*, 1983), known to play a role in the control of food intake and energy balance in mammals. MCH-producing neurons in the rodent and primate brains are located in the lateral hypothalamus and zona incerta and extensively project to many brain areas (Bittencourt *et al*, 1992; Bittencourt *et al*, 1998), suggesting possible neuro-modulatory functions for MCH (reviewed in Griffond and Baker, 2002). The biological effects of MCH are mediated by two G-protein-coupled receptors, melanin-concentrating hormone receptor subtype 1 (MCHR1) (SLC-1/GPR24) and melanin-concentrating hormone receptor subtype 2 (MCHR2), both of which belong to the rhodopsin-like

G-protein-coupled receptor family of proteins. The MCHR1 subtype is expressed in most species examined, including rodents and humans, whereas functional MCHR2 receptors are expressed in humans and non-human primates and in a few other species, but not in rodents (Tan *et al*, 2002).

MCH signaling in the CNS has been most often described in the regulation of nutritional and energy homeostasis. Direct central administration of MCH stimulates food intake in rodents (Qu *et al*, 1996), while the targeted disruption of the prepro-MCH gene in mice results in a lean phenotype, as a result of hypophagia and increased metabolic rate (Shimada *et al*, 1998). Likewise, receptor knockout mice are lean and are protected against diet-induced obesity (Chen *et al*, 2002; Marsh *et al*, 2002). Although the widespread innervations of MCH-containing fibers and the expression of MCHR1 in brain areas such as the hippocampus, basolateral amygdala, and prefrontal cortex (PFC) (Saito *et al*, 2001) suggests potential involvement in the control of emotion and cognition, only a few reports have directly addressed this issue. Intracranial MCH administration has been shown to regulate response to novelty (Monzon *et al*, 2001; Kela *et al*, 2003) and learning and memory (Monzon *et al*, 1999). In the most suggestive

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study to date, acute systemic administration of a selective MCHR1 receptor antagonist to rodents elicited both anxiolytic- and antidepressant-like effects (Borowsky *et al*, 2002; Chaki *et al*, 2005).

Our study provides genetic evidence that the MCHR1 receptor subtype can mediate anxiety responses in rodents. Specifically, we have generated mice with a targeted null mutation of the MCHR1 receptor gene and tested them on a series of anxiety-related behavioral paradigms. We show that the lack of functional MCHR1 receptors in knockout mice results in an anxiolytic-like phenotype, observed consistently across a validated set of behavioral paradigms. In addition, we show that basal extracellular levels of serotonin (5-HT) are significantly lower in the PFC of MCHR1 knockout mice, a region of the brain critical for the regulation of emotion. Moreover, acute forced swim stress significantly increases 5-HT levels in the PFC of MCHR1 wild-type but not knockout mice. Taken together, our data show that functional inactivation of MCHR1 in mice results in an anxiolytic-like phenotype and induces changes in prefrontal cortical 5-HT transmission. These data suggest that the development of MCHR1 receptor antagonists may constitute a novel alternative strategy for the treatment of anxiety disorders.

MATERIALS AND METHODS

Animals

Generation of MCHR1-deficient mice. MCHR1-deficient mice were generated at Lexicon Genetics Inc. (The Woodlands, TX). Both exons 1 and 2 from the MCHR1 gene were substituted for a 5.3 kb neo cassette in the MCHR1 targeting vector, along with a total of approximately 8.5 kb of homology: ~3.5 kb 5' arm and ~5 kb 3' arm (Figure 1a). The targeting vector was introduced into embryonic stem (ES) cells derived from the 129SvEvBrd mouse strain. The integrity of the targeting events on the 5' end was confirmed by Southern blot analysis of ES cells genomic DNA after digestion with *Bgl*III, using a probe located upstream of the arms of homology. The integrity of the targeting events on the 3' end was confirmed after digestion of genomic DNA with *Kpn*I, using a probe located downstream of the arms of homology. Targeted ES cell clones were injected into host blastocysts and resulting chimeric mice were bred to C57BL/6J albino mice to generate F1 heterozygotes. F1 heterozygotes were backcrossed to C57BL/6J mice for one generation. The resulting N1F1 heterozygotes were intercrossed to generate F2 homozygous mice.

The genotypes of wild-type and mutant mice were identified by triplex PCR analysis of genomic DNA from tail biopsies (Figure 1b). The wild-type-specific primer pair (a/b) produced a product of 490 bp and the knockout-specific primer (c/b) pair produced a product of 643 bp. Primers used were as follows: a-5'GACCTTTCGAAAACGCTTGG; b-5'CTCACAGTAGAGCCCTAGC; c-5'GTGTGGCGGACCGCTATCA. To confirm the absence of MCHR1 mRNA in homozygous mutant mice, RT-PCR was performed on RNA prepared from whole brains of knockout and wild-type mice (Figure 1c). The following exon-spanning primer pair was utilized to generate a 1012 bp

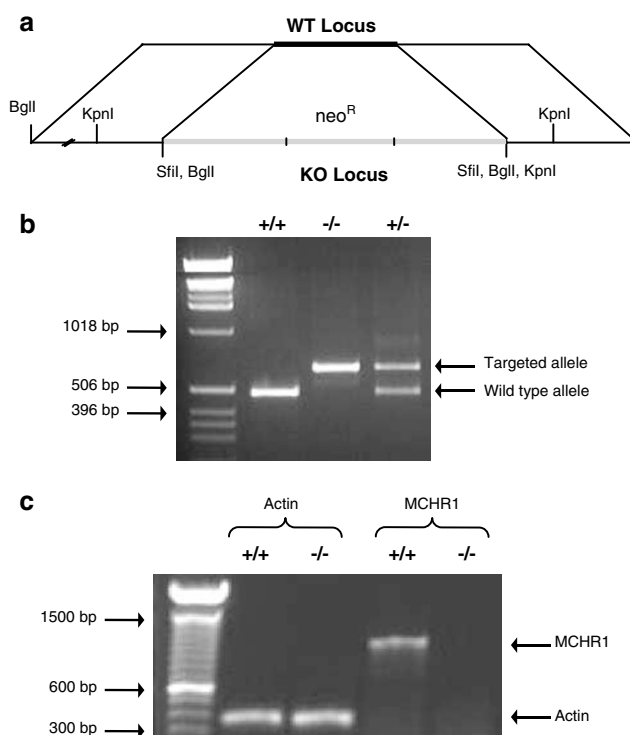


Figure 1 Generation of MCHR1-deficient mice. (a) Schematic map of the MCHR1 wild-type locus and the inactivated MCHR1 allele. Black box, open reading frame of the MCHR1 gene; gray box, replacement cassette in mutated locus with *neo^R* (neomycin resistance) gene cassette. (b) Genotyping of animals. Mice were screened using PCR-based analysis of tail genomic DNA to identify wild-type, homozygous mutant, or heterozygous mutant animals. (c) Confirmation of MCHR1 expression. RT-PCR analysis of brain RNA from wild-type and knockout animals was performed to confirm the absence of MCHR1 message in homozygous mutant mice. β -Actin was used to control for RNA degradation and fidelity of PCR.

product: 5'CAACATCTCCGATGGCCAGGATAA (forward) and 5'TGCCTTTCGCTTCTGTCCCTCTCCTC (reverse).

Housing

Subjects were 3- to 10-month-old sexually naïve mice bred in our facility. The animal housing rooms were on a 12-h light/dark cycle (lights on at 0600) with the temperature kept at 23–25°C, and the humidity maintained at 40–60%. Animals were housed in groups of 3–5, with *ad libitum* access to food and water and provided with environmental enrichment in the form of cotton nesting pads.

Behavioral Phenotyping

Testing. Knockout and wild-type littermates (also on a mixed background) were tested. Testing was performed in rooms dedicated to sensitive behavioral experiments and animals were brought to the rooms approximately 1 h before the testing began to allow for acclimation. All behavior testing was conducted in the second half of the light part of the light/dark cycle. There was a minimum of 72 h between behavioral tests, and the tests were carried out in the order listed below. All experiments were in compliance with approved protocols with oversight from

the Institutional Animal Care and Use Committee. All behavioral paradigms were pharmacologically validated prior to testing mutant mice (data not shown).

Open field test. The open field test was conducted as described previously (Holmes *et al*, 2003). The open field consisted of a square arena (40 cm length \times 40 cm width \times 38 cm depth) with clear Plexiglas floor and walls. Mice were placed in one corner of the open field and allowed to explore freely the arena during a 15-min test session. Both central and peripheral activities were measured using a computer-assisted activity system with appropriate software to facilitate data collection and analysis (San Diego Instruments, San Diego, CA). A total of 16 photobeams in each direction were positioned 2.5 cm apart, 2 cm above the floor, and center time was defined as time spent in the central 25 \times 25 cm area of the open field. Enhanced exploration of the unprotected central portion of a novel open field and reduced thigmotaxis (wall hugging) are behaviors most often correlated with reduced anxiety levels (Treit and Fundytus, 1988; Simon *et al*, 1994). In any given session, six test animals were run simultaneously in independent test chambers, with a pseudorandomization of placement of mutant and wild-type animals.

Elevated plus maze. The elevated plus maze was conducted as described previously (Lister, 1987; Holmes *et al*, 2003) and has been accordingly modified for use in our laboratory. Briefly, the apparatus was constructed of black Plexiglas and consisted of four arms (30 cm length \times 5 cm width): two closed arms with high, black walls (30 cm high) and two open arms with a small raised lip (0.5 cm). All four arms were connected by a center platform (5 \times 5 cm). The maze was elevated to a height of 30 cm above the ground. Each mouse was placed on the center platform facing an open arm to initiate the test session of 5 min. A desk lamp placed above the apparatus provided even lighting. Increased exploration of the relatively aversive open arms is indicative of reduced anxiety-like behavior in this paradigm. Open/closed arm entries and time spent in the open/closed arms were scored by two trained observers. Arm entries were scored upon entry of the two front paws into the arm.

Social interaction test in a novel environment. To investigate another measure of anxiety, we tested individual mice in a social interaction paradigm, as described (Miyakawa *et al*, 2003) with modifications appropriate for our facility and laboratory setting. Group-housed male mice were tested individually in this test. The test subject was placed in a novel arena (40 \times 40 cm) with cage bedding following which a group-housed C57Bl/6 male (intruder) was introduced into the arena. The two animals were allowed to explore freely for 10 min and the amount of time the test subject spent engaged in social investigation/physical interaction (approaching, sniffing, mounting, grooming, fighting) was monitored. Cage bedding was mixed between animals so as to provide no guidance cues for the next test pair. No intruders were used more than once during any testing session. The number of contacts, total contact time, and mean duration time per contact were

measured by two independent observers. Prior to the experiment, the pair of observers carried out a series of comparative validations, to ensure that there would be inter-rater reliability in the measures scored.

Stress-induced hyperthermia. The handling stress-induced paradigm was carried out as described previously (Van der Heyden *et al*, 1997; Bouwknecht and Paylor, 2002) with modifications for use in our facility. Animals were group housed and on the test day, the probe (Harvard Apparatus, Holliston, MA) was inserted into the rectum for about 2 cm and the temperature (T_1 = basal temperature) was recorded. The mild stress of handling and temperature probe insertion into the rectum causes a hyperthermic response, which was recorded 10 min later (T_2 = stressed temperature). The stress-induced hyperthermia was computed to be $\Delta T = T_2 - T_1$.

In Vivo Microdialysis

Surgery. Prior to surgery, mice were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and placed in a stereotaxic frame. A microdialysis guide cannula with a dummy (CMA/7 model; CMA, Chelmsford, MA) was implanted into the right PFC at the following coordinates relative to bregma: AP + 2 mm; ML -0.5 mm; DV -1.5 mm (Paxinos and Franklin, 2001).

Microdialysis. At 48 h after the surgical procedure, a CMA/7 microdialysis probe (2 mm membrane length) was inserted into the guide cannula and perfused at a constant flow rate of 1 μ l/min with artificial cerebrospinal fluid (aCSF) containing 1 μ M citalopram, using a microperfusion pump (Harvard Apparatus, Holliston, MA). After a 2-h stabilization period, five microdialysis samples (fractions 1-5) were collected every 10 min for estimation of basal extracellular 5-HT levels. Mice were then individually placed in a transparent 21 glass cylinder (19 cm tall) filled with water at room temperature ($25 \pm 2^\circ\text{C}$) to a depth of 13 cm. The mice were subjected to 10 min forced swim stress with concurrent collection of the microdialysis samples (fraction 6). Following the swim stress, three more microdialysate samples were collected (fractions 7-9). At the end of the microdialysis experiment, animals were euthanized and their brains were removed for histological verification of correct probe placement. Only animals with correct probe placement into the right PFC were included in the study.

HPLC. All equipment and materials for the HPLC analysis were obtained from ESA (ESA, Chelmsford, MA). PFC microdialysis samples were analyzed using an HPLC coupled with an electrochemical detector. The mobile phase consisted of a mixture of acetonitrile, phosphate buffer, and an ion-pairing agent. The mobile phase was delivered by a pump (Model 582, Solvent Delivery Module) at a flow rate of 0.25 ml/min through an MD-150 narrowbore column. 5-HT was detected using a coulometric detector (Coulochem III) coupled to a single-channel enhanced amperometric cell (Model 5041). The potential applied to the electrode was +150 mV; under these conditions, the limit of detection for

5-HT was approximately 2–3 fmol/sample. Data were analyzed using a computer-assisted software system. For microdialysis data, basal 5-HT levels were defined as the average level of the first five samples following stabilization.

Statistical Analyses

Statistical analyses were conducted using GraphPad Prism version 4.01 for Windows software (San Diego, CA). The effect of genotype was analyzed using unpaired two-tailed *t*-tests, analysis of variance (ANOVA), or repeated-measures ANOVA, followed by Newman–Keuls *post hoc* tests where appropriate. Specifically, open field activity and SIH temperature readings were analyzed by a two-way ANOVA (genotype \times time) with repeated measures for the factor of time. Extracellular levels of 5-HT in microdialysis samples collected every 10 min were analyzed by one- and two-way ANOVA, with repeated measures for the factor of time. Values in graphs are represented as mean \pm SEM. Sample sizes are indicated in figure legends and results were considered statistically significant if $p < 0.05$.

RESULTS

Generation of MCHR1 Knockout Mice

The entire coding sequence for MCHR1, consisting of two exons, was targeted and replaced with a neomycin phosphotransferase (*neo*^R) cassette (Figure 1a). Blastocyst injections of targeted ES cells resulted in chimeric mice, which were further bred with C57Bl/6 mice. Heterozygote matings produced homozygous wild-type, homozygous mutant, and heterozygous mice in the expected Mendelian ratios. The genetic inactivation of MCHR1 did not impact embryonic viability and the animals appeared grossly normal. Genomic DNA analyzed from tail biopsies confirmed genotypes (Figure 1b) and RT-PCR analysis verified that the null mutation resulted in the absence of MCHR1 mRNA in adult mutant animals (Figure 1c).

MCHR1 Knockout Mice Display Reduced Anxiety-Like Behavior

Open field activity. Knockout and wild-type mice were tested in the open field, a paradigm of exploratory behavior in a novel environment, and the results are presented in Figure 2. MCHR1 knockout animals spent a significantly greater fraction of time in the central portion of the novel open field. There was a significant effect of genotype ($p < 0.005$) and time ($p < 0.05$) when the data were analyzed in time intervals, but there were no significant genotype \times time interactions (Figure 2a). When the data were collapsed across the 15-min testing period, there was a significant effect of genotype on the distance covered in the central portion of the open field ($p < 0.05$; Figure 2b) as well as the % activity in the central portion of the open field (data not shown). The greater time spent and distance covered in the center was not a result of increased ambulation, as there were no significant genotypic differences in overall locomotion, as measured by total distance covered during the testing period (Figure 2c) and average speed (data not shown).

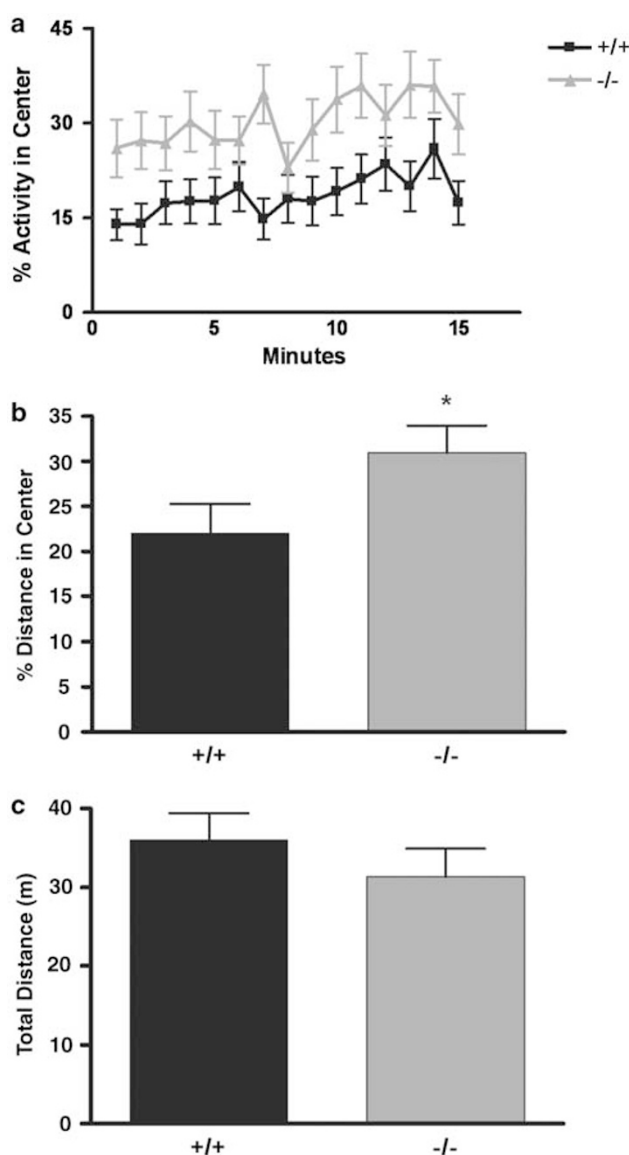


Figure 2 MCHR1 knockout mice show reduced anxiety-like behavior in a novel open field test. MCHR1 knockout (-/-) and wild-type (+/+) mice were allowed to explore freely an open field for a period of 15 min. (a) MCHR1 knockout mice spent a significantly greater fraction of time in the central portion of the open field during the 15-min exposure, indicative of reduced anxiety-like behavior in these mice. When analyzed in time-bin intervals, using a two-way ANOVA with time as the repeated measure, there was a significant effect of genotype (** $p < 0.005$) and time (* $p < 0.05$) but no genotype \times time interaction ($p = 0.85$). (b) When % distance covered in the center is examined, collapsed across the 15-min test session, there is a significant effect of genotype (* $p < 0.05$), as assessed by an unpaired *t*-test. (c) There were no significant effects of genotype on the total distance covered by each animal during the trial. Data are presented as mean (\pm SEM) and $n = 25$ –35 per genotype.

Elevated plus maze. In order to further explore the absence of functional MCHR1 as it relates to anxiety, a test based on the natural aversion of rodents to open or unprotected areas was conducted: the elevated plus maze. Knockout mice spent significantly more time exploring the open arms of the maze ($p < 0.01$) (Figure 3a). The % of entries into the open arms was also greater for MCHR1 knockout animals

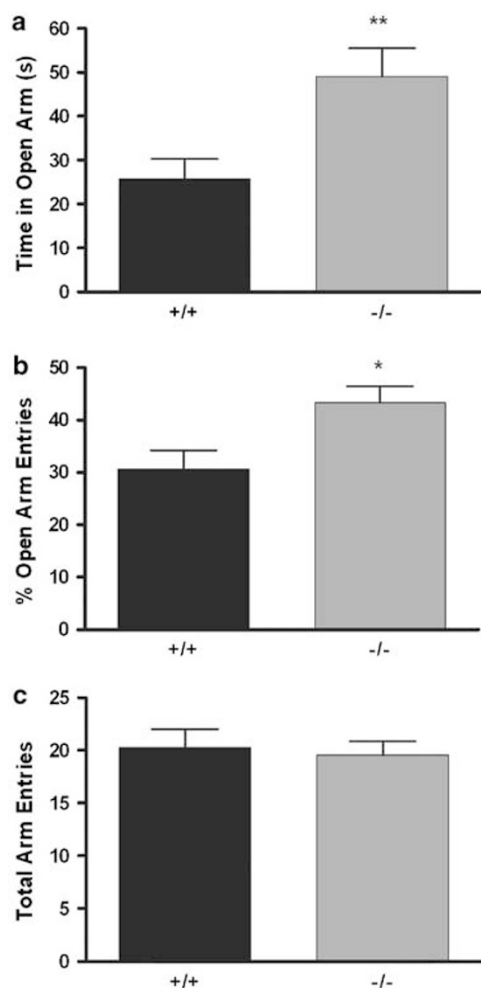


Figure 3 MCHRI knockout mice show reduced anxiety-like behavior on the elevated plus maze. MCHRI knockout (-/-) and wild-type (+/+) mice were subjected to the elevated plus maze for a period of 5 min. (a) MCHRI knockout mice spent a significantly greater amount of time exploring the aversive open arms of the maze (** $p < 0.01$), and (b) MCHRI knockout mice displayed a greater % of open arm entries (* $p < 0.05$). (c) No significant differences were detected in total arm entries between MCHRI knockout and wild-type mice, indicative of no differences in locomotor activity. All data were analyzed using an unpaired *t*-test and are presented as mean (\pm SEM); $n = 11$ per genotype.

($p < 0.05$) (Figure 3b). There were no overall differences in activity between genotypes, as measured by the total number of arm entries (Figure 3c).

Stress-induced hyperthermia. In mice, the stress-induced hyperthermia procedure has been used to measure anti-stress or anxiolytic effects of psychoactive drugs. Because the end point being monitored is independent of locomotion, this test is a necessary addition to the standard battery of paradigms used to probe for anxiety-related behaviors. We examined the effects of handling stress-induced increases in core body temperature in knockout and wild-type mice. We found that the basal resting temperature of the MCHRI knockout mice was significantly higher than that of controls ($p < 0.003$), but was not increased in response to handling stress. In contrast, a significant increase in body

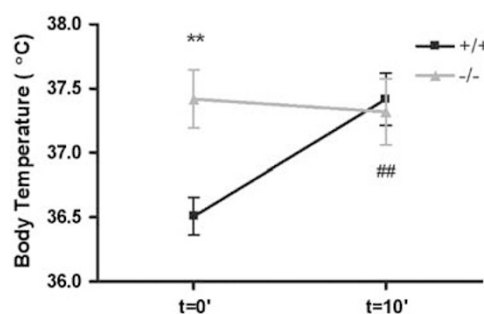


Figure 4 MCHRI knockout mice exhibit higher basal body temperature and are protected against stress-induced hyperthermia. Basal body temperatures were measured in MCHRI knockout (-/-) and wild-type (+/+) mice followed by a second reading 10 min later. A two-way ANOVA with time as the repeated measure indicated a significant effect of time (* $p < 0.05$) as well as a significant genotype \times time interaction (** $p < 0.005$). Knockout mice exhibit a higher basal body core temperature as compared to wild-type controls and differences were assessed by *t*-tests: $36.5^{\circ}\text{C} \pm 0.15$ for wild-type and $37.4^{\circ}\text{C} \pm 0.23$ for knockout mice (** $p < 0.003$). At 10 min following handling stress, wild-type, but not knockout, mice responded to the stress with a significant increase in core body temperature: $\Delta T = +0.91^{\circ}\text{C} \pm 0.25$ for wild-type and $-0.10^{\circ}\text{C} \pm 0.35$ for knockout mice. Data are presented as mean (\pm SEM) and $n = 11$ per genotype.

temperature following handling stress was observed in wild-type control animals ($p < 0.05$) (Figure 4).

Social interaction. It has been suggested that increased social contact is correlated with anxiolytic-like behavior (File and Hyde, 1978). We examined the behavior of MCHRI knockout and wild-type mice in a social interaction test in a novel environment. During a 10-min test period, knockout mice exhibited increased time spent per contact with an intruder-interactor mouse ($p < 0.05$) (Figure 5), when compared with wild-type controls. Contact is defined as sniffing, grooming, mounting, and following behaviors and has been presented in an aggregate manner. MCHRI knockout mice did not show increased signs of aggressive behavior in this test (data not shown).

MCHRI Knockout Mice have Altered Serotonin Neurotransmission in the Prefrontal Cortex

A great body of experimental and clinical evidence implicates central 5-HT transmission in the control of mood and anxiety. *In vivo* microdialysis, in freely moving mice, was used to assess whether MCHRI knockout mice had different basal 5-HT levels in the PFC, a region of the brain tightly associated with the regulation of emotion and cognition. Dialysate concentrations of a neurotransmitter can provide an estimate of extracellular concentrations. We found that MCHRI knockout mice have significantly lower extracellular levels of 5-HT in the PFC, as compared to wild-type animals (9.53 ± 0.24 fmol/10 μl sample for wild type vs 6.91 ± 0.36 fmol/10 μl sample for knockout; $p < 0.0005$) (Figure 6a). This effect appears to be specific to 5-HT, since no significant differences in extracellular dopamine (DA) and norepinephrine (NE) levels were detected in MCHRI knockout mice (data not shown). An acute forced swim stress of 10 min duration caused a marked and

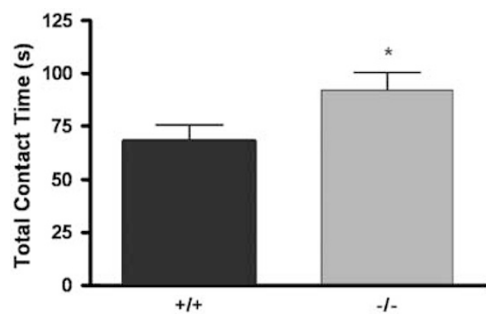


Figure 5 MCHRI knockout mice spent a greater amount of time engaged in social contact. MCHRI knockout mice (–/–), when compared to wild-type mice (+/+), spent a significantly greater amount of total contact time with a novel intruder-interactor mouse in a novel test environment, during a 10-min trial period (* $p < 0.05$), as assessed by an unpaired *t*-test, indicative of reduced fear and anxiety in the face of novelty. Contact is defined as sniffing, following, mounting, and grooming behavior. Data are presented as mean (\pm SEM) and $n = 15$ per genotype.

significant increase in 5-HT efflux from the PFC of wild-type mice ($p < 0.005$), while no significant effects were detected in knockout animals (Figure 6b).

DISCUSSION

Recent studies in rodents have provided intriguing evidence about the involvement of MCH in the mediation of emotional processes, but its exact role remains to be elucidated. Here we report the generation and behavioral analysis of mice carrying a null mutation of the MCHRI, the only functional MCH receptor present in mice. The absence of genomic DNA and mRNA was confirmed by PCR-based genotyping and RT-PCR, respectively. While the animals appear grossly normal, we present experimental evidence that genetic inactivation of MCHRI in mice results in a reduction in anxiety-like behaviors and in changes in serotonergic transmission in the PFC.

A behavioral characterization of the MCH receptor knockout mouse is a critical step in exploring MCH-dependent signaling in anxiety-related behaviors. Recruitment of the MCH system in the modulation of anxiety-like behavior is anatomically consistent with the widespread innervation by MCH-immunoreactive fibers of brain areas involved in the modulation of mood and affect (Zamir *et al*, 1986; Saito *et al*, 2001). While other groups have shown MCHRI knockout mice to have altered nutritional homeostasis and energy expenditure (Chen *et al*, 2002; Marsh *et al*, 2002), changes in autonomic regulation (Astrand *et al*, 2004), reduced bone mass (Bohlooly *et al*, 2004), and supersensitive mesolimbic DA transmission (Smith *et al*, 2005), our study is the first to demonstrate reduced anxiety-like responses in these mice. We show that MCHRI knockout mice display anxiolytic-like behavior in four different behavioral paradigms that are widely used to assess such responses in rodents.

We observed enhanced exploration of the unprotected central portion of a novel open field and reduced thigmotaxis, behaviors most often correlated with reduced anxiety levels (Treit and Fundytus, 1988; Simon *et al*, 1994).

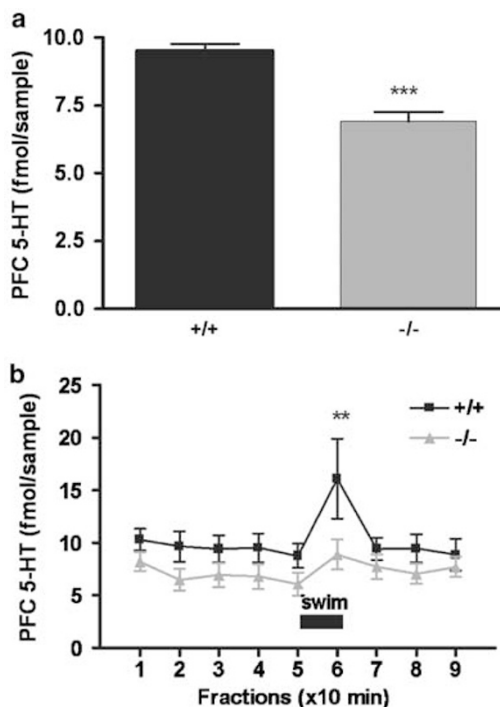


Figure 6 MCHRI knockout mice have reduced extracellular 5-HT levels in the PFC. Basal extracellular 5-HT levels and the effect of a 10-min forced swim on 5-HT efflux in the PFC of knockout (–/–) and wild-type animals (+/+), as assessed by *in vivo* microdialysis followed by HPLC analysis, are shown. (a) MCHRI knockout mice have significantly lower basal extracellular levels of 5-HT in the PFC: 9.52 ± 0.24 fmol for wild-type vs 6.91 ± 0.36 fmol for knockout mice. Basal levels of PFC 5-HT were calculated by averaging the first five microdialysis samples (collected every 10 min), and values are expressed as mean (\pm SEM) fmol of 5-HT in a $10 \mu\text{l}$ microdialysis sample. A *t*-test analysis indicated significant differences between the weighted means of baseline samples between knockout and wild-type animals (** $p < 0.0005$; $n = 9$ per genotype). (b) Effect of forced swim stress on PFC 5-HT levels. Forced swim caused a significant increase in 5-HT release from the PFC of wild-type but not knockout mice. One-way ANOVA with time as the repeated measure indicated a significant change in 5-HT efflux over time in the wild-type group (** $p < 0.005$), but not in the knockout group. *Post hoc* comparisons of individual samples revealed a significantly higher 5-HT efflux following the forced swim only in the wild-type animals. Two-way ANOVA with time as the repeated measure indicated a significant effect of time (** $p < 0.0005$) but not of genotype ($p < 0.08$), and there was no genotype \times time interaction.

In the elevated plus maze, knockout mice spent a greater amount of time exploring the aversive open arms of the maze, as compared to wild-type controls, consistent with a reduced anxiety-like response in this test. It is important to note that decreases in measures of anxiety in the open field and the elevated plus maze did not result from increased locomotor activity, since no significant differences in the total distance traveled during the open field session and the number of total arm entries were observed between knockout and wild-type animals on these tests, respectively.

MCHRI knockout mice also displayed anxiolytic-like behavior when tested in a novel-environment social interaction test. Knockout mice spent a significantly greater amount of time investigating an intruder male mouse, when compared to wild-type controls. No signs of increased aggression were detected, further suggesting reduced fear

and anxiety in the face of novelty. An anxiolytic-like response was also observed in a model of stress-induced hyperthermia. In this paradigm, MCHR1 knockout mice appeared to be protected against handling stress-induced increases in core body temperature, as compared to wild-type controls. We observe here, however, that MCHR1 knockout mice have basal body temperature 1.0°C higher than wild-type controls (also observed by Astrand *et al* (2004), but only during the dark phase). While the lack of effect of stress-induced hyperthermia in knockout mice may have been due to their higher basal body temperature, other groups have shown stress-induced hyperthermia to exceed a 2°C change in body temperature in mice with similar C57Bl/6 genetic backgrounds (Butterweck *et al*, 2003). Therefore, a physiological limit may not have been reached in the MCHR1 knockouts, thus further suggesting an anxiolytic-like phenotype in these mutant mice.

The data obtained studying this MCHR1 knockout mouse model are consistent with published findings that pharmacological inactivation of MCHR1 receptors results in a reduction in measures of anxiety. Reduced anxiety-like behavior following acute treatment with SNAP-7941 (Borowsky *et al*, 2002) and ATC0065/0175 (Chaki *et al*, 2005) was observed in rats and mice using a variety of behavioral paradigms, much like the present study. Modulation of anxiety-like behavioral responses has also been observed following i.c.v. injections of the MCH ligand (Gonzalez *et al*, 1996; Monzon and De Barioglio, 1999; Kela *et al*, 2003).

Both the expression of MCHR1 and the innervation of MCH-containing fibers have been described in the rodent dorsal raphe (Hervieu *et al*, 2000; Saito *et al*, 2001), the region of the brain where the cell bodies for serotonergic neurons are located. It has been known for several decades that serotonergic transmission plays a key role in the modulation of mood, anxiety and stress (extensively reviewed in Gordon and Hen, 2004). Specific changes in the 5-HT system of patients with affective disorders have been found primarily in the PFC, a brain region tightly associated with the control of emotion and cognition (Stockmeier, 1997; Dean *et al*, 1999; Miller, 1999). Moreover, 5-HT transmission in the PFC has been implicated specifically in modulating anxiety and fear. For example, 5-HT efflux is increased in the PFC in the elevated plus maze (Rex *et al*, 1993), a test of anxiety, as well as during conditioned fear (Hashimoto *et al*, 1999). Acute stressful events such as forced swim can increase 5-HT transmission in the brains of freely moving mice (Kirby *et al*, 1997; Yoshitake *et al*, 2004). Further existence of a link between fear/anxiety processes and 5-HT is demonstrated by the fact that several clinically used anti-anxiety medications (eg SSRIs, 5-HT_{1A} agonists) are believed to exert their therapeutic effects through a selective modulation of serotonergic neurotransmission. To address whether the absence of functional MCHR1 receptors alters 5-HT transmission in the PFC, we performed *in vivo* microdialysis studies in freely moving mice. We found that MCHR1 knockout mice have reduced basal extracellular 5-HT levels in the PFC, but no differences in DA and NE, and that while acute swim stress increased 5-HT efflux in the PFC of wild-type mice, no significant effects were detected in knockout animals. This suggests that genetic inactivation

of MCHR1 blocks the neurochemical effects of acute stress exposure in a brain area largely implicated in behavioral responses to fear and anxiety.

Since several lines of evidence indicate that altered serotonergic transmission in the PFC can modulate fear- and anxiety-related behaviors, one could speculate that the lower basal 5-HT levels and the blunted 5-HT response following acute stress may underlie the decrease in anxiety states in MCHR1 knockout mice. In this regard, it is interesting to note that 5HT_{1A} receptor knockout mice have increased 5-HT levels in forebrain areas, including the PFC (Parsons *et al*, 2001), and display concomitant anxious behavior (Heisler *et al*, 1998; Parks *et al*, 1998; Ramboz *et al*, 1998). This suggests that disinhibition of 5-HT neurotransmission could lead to increases in anxiety-like behaviors. On the other hand, our data show that genetic inactivation of MCHR1 reduces PFC 5-HT transmission, possibly contributing to the observed anxiolytic-like phenotype. Several possibilities could explain the reduced 5-HT transmission in the PFC of MCHR1 knockout mice. For example, MCH-mediated signaling through MCHR1 may usually provide a tonic excitatory drive of raphe serotonergic neurons. Thus, in the knockout mice, the lack of functional MCHR1 receptors normally expressed in the raphe (Hervieu *et al*, 2000; Saito *et al*, 2001) may lead to reduced 5-HT levels in projection fields like the PFC and ultimately modulate anxiety-like behavior and response to acute stress. Alternatively, the lack of functional MCHR1 could lead to changes in 5HT_{1A} inhibitory autoreceptors or in 5-HT transporter expression/activity. In addition, one explanation that must also be considered is that MCH signaling through its cognate receptor may act in a trophic manner to promote development of the serotonergic circuitry necessary for normal anxiety-like behaviors. However, in light of studies concluding that acute administration of a specific MCHR1 antagonist reduces anxiety-like behavior (Borowsky *et al*, 2002; Chaki *et al*, 2005), the trophic hypothesis of MCH signaling-induced effects on serotonergic transmission cannot solely explain the observed reduction in anxiety-like behaviors.

To complement the studies presented here and to further examine effects of MCHR1 downregulation on neurochemistry and associated behaviors, studies utilizing conditional knockouts and selective MCHR1 receptor antagonists are warranted. In conclusion, our findings show that genetic inactivation of the MCHR1 results both in reduced anxiety-like behavior across a battery of tests in mice and in altered prefrontal cortical serotonergic transmission. These data shed light on the role of the MCH signaling system in the regulation of fear and anxiety and provide further evidence that pharmacological manipulation of this system may represent an alternative strategy for the clinical management of anxiety disorders.

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