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Melanin-Concentrating Hormone-1 Receptor Modulates Neuroendocrine, Behavioral, and Corticolimbic Neurochemical Stress Responses in Mice

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Repeated exposure to stressful conditions is linked to the etiology of affective disorders. The melanin-concentrating hormone-1 receptor (MCHR1) may be a novel mechanism that is involved in the modulation of stress responses and affective states. The role of MCHR1 in neuroendocrine, behavioral, and neurochemical stress, and anxiety-related responses was examined by monitoring the effects of melanin-concentrating hormone (MCH) and the selective MCHR1 antagonist, GW3430, in inbred C57Bl/6NTac and MCHR1-knockout (KO) and wild-type (WT) mice. Intracerebroventricular injection of MCH increased plasma corticosterone, and produced anxiety-related responses in the elevated plus maze. The selective MCHR1 antagonist, GW3430, blocked the neuroendocrine and behavioral effects of MCH and produced anxiolytic-like effects by itself in animal models of anxiety. Moreover, KO mice had an anxiolytic-like phenotype in behavioral models of anxiety, and GW3430 had anxiolytic-like effects in WT, but not KO mice. Lastly, stressor-evoked acetylcholine release within the prefrontal cortex of inbred and WT mice, but not KO mice, was blocked by GW3430. We show that MCH elicits anxiety-like responses and that the effects of a selective MCHR1 antagonist and the phenotype of KO mice are consistent with anxiolytic-like action. Distinct behavioral, physiological, and neurochemical stress, and anxiety-related responses were selectively modulated by the MCHR1, and these actions may involve corticolimbic regulation of stress responsivity and anxiety.
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INTRODUCTION

Stress evokes a complex array of neurobiological and behavioral responses. The inappropriate regulation of stress responses is associated with the development of anxiety disorders, depression, and schizophrenia (Gispen-de Wied and Jansen, 2002; Kathol *et al*, 1989; Rasmusson *et al*, 2001; van Praag, 2004). Investigating the CNS mechanisms that regulate responses to stressful stimuli and anxiety-related responses may lead to advanced neuropsychiatric therapies.

The lateral hypothalamic neuropeptide, melanin-concentrating hormone (MCH), is structurally identical in rats, mice, and humans (Breton *et al*, 1993). The effects of MCH in humans are mediated via two G-protein-coupled receptors, MCHR1 (Chambers *et al*, 1999; Saito *et al*, 1999) and MCHR2 (An *et al*, 2001; Sailer *et al*, 2001). In rodents, only MCHR1 is functional (Tan *et al*, 2002). MCH acting through the MCHR1 has a proven role in energy homeostasis (Forray, 2003; Pissios and Maratos-Flier, 2003).

The MCH system may also be important for controlling stress responsivity and anxiety. MCH-containing neurons (Bittencourt *et al*, 1992) and MCHR1 (Hervieu *et al*, 2000) are widely distributed in the brain, and significant populations can be found within limbic brain regions that regulate stress and affect. Frontal cortical regions, the amygdala, and hippocampus are key modulators of stress responses and affective states (Herman *et al*, 1996). Recently, it was shown that the prefrontal cortex (PFC) directly regulates neurobiological and behavioral sequelae of psychological stress (Amat *et al*, 2005). MCH innervation of corticolimbic regions may provide a neural basis for MCH to regulate stress and anxiety-related responses.

There is a limited and conflicting literature on the role of MCH in stress and anxiety. MCH injected directly into the lateral ventricle or the paraventricular nucleus (PVN) of the hypothalamus stimulated the hypothalamic-pituitary-adrenocortical (HPA) axis in some experiments (Jezova *et al*, 1992; Kennedy *et al*, 2003), but blocked stress-induced

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activation of the HPA axis in other studies (Bluet-Pajot *et al*, 1995; Ludwig *et al*, 1998). In the elevated plus maze (EPM), intracerebroventricular (i.c.v.) infusion of MCH was reported to have either anxiogenic- (Gonzalez *et al*, 1996) or anxiolytic-like (Kela *et al*, 2003; Monzon and De Barioglio, 1999; Monzon *et al*, 2001) effects. Finally, novel MCHR1 antagonists blocked MCH-induced release of corticotrophin-releasing factor (Kennedy *et al*, 2003), and resembled known anxiolytics and antidepressants in animal models (Borowsky *et al*, 2002; Chaki *et al*, 2005).

The purpose of the present study was to examine the effects of MCH and pharmacological blockade of the MCHR1 on distinct behavioral and neuroendocrine indices of stress and anxiety under the same experimental conditions, and to characterize the phenotype of MCHR1 knockout (KO) and -wild-type (WT) mice in behavioral and neurochemical models of anxiety- and stress-related responses. The hypothesis was that MCH would activate the HPA axis and produce anxiogenic behavior via the MCHR1. We further hypothesized that MCHR1 blockade with a selective MCHR1 antagonist or gene deletion in KO mice would have anxiolytic-like effects on behavioral, hypothalamic and corticolimbic stress, and anxiety-related responses. The specific aims were to (1) measure plasma ACTH and corticosterone levels and anxiety-related behavior after central MCH injection, (2) determine the role of the MCHR1 in anxiety-related responses using established naturalistic and physiological animal models, and (3) address the central antistress activity of the MCHR1 by measuring stressor-evoked acetylcholine (ACh) release within the PFC.

MATERIALS AND METHODS

Animals

All experiments were performed according to the policies of the Animal Care and Use Committee of Eli Lilly and Company, in conjunction with the American Association for the Accreditation of Laboratory Animal Care-approved guidelines. Animals were individually housed in an environmentally controlled facility where food and water were available *ad libitum*. Mice were maintained on a 12 h light/dark cycle (0600/1800 hours), and all procedures were performed during the light period. Adult, male MCHR1-KO and -WT mice were derived by heterozygous intercross and had a mixed 129SvJ × C57Bl/6 background (Chen *et al*, 2002). Genotyping was performed by PCR analysis on mouse-tail DNA. KO, WT, and C57Bl/6NTac (C57) mice were 3–5 months old and weighed 25–35 g during testing. Animals were used only once.

Drugs and MCH

MCH (American Peptide, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid (aCSF). The potent and selective MCHR1 antagonist, GW3430 (Handlon *et al*, 2004), was synthesized at Lilly Research Laboratories (Indianapolis, IN). GW3430 and Alprazolam (RBI, Natick, MA) were suspended in 1% acetic acid/0.5% carboxymethylcellulose/0.125% Tween-80 in ddH_2O. Chlordiazepoxide (CDP) HCl (Sigma-Aldrich, St Louis, MO) was dissolved in saline.

I.c.v. Cannulation Surgery

Mice were anesthetized with a ketamine/xylazine mixture, placed in a stereotaxic apparatus and unilaterally implanted with a permanent 22-G stainless-steel hypodermic guide cannula (Plastics One, Roanoke, VA) aimed at the lateral ventricle (coordinates in millimeter from bregma: AP, −1.0; ML, +1.0; DV, −2.0 to 2.5) (Franklin and Paxinos, 1997). Guide cannulae were anchored to the skull using three stainless-steel screws (BAS, Lafayette, IN), cyanoacrylate glue, and cranioplastic cement. A dummy cannula was inserted into the guide and animals were left to recover for 1–2 weeks.

Elevated Plus Maze

EPM (Hamilton-Kinder, Poway, CA) consisted of two open arms (37.5 × 5 × 0.3 cm) and two closed arms (37.5 × 5 × 15 cm), which extended from a 5 × 5 cm central platform. The maze was elevated 68 cm above the floor, and illumination throughout the test room was approximately 500 lx. EPM testing was conducted between 0700 and 1200 hours. Mice were transferred in their home cages to the test room and allowed to acclimate for 1–2 h. Animals were individually placed on the center platform of the EPM facing an open arm, and allowed to freely explore the EPM for 5 min. The EPM was cleaned with methanol after testing each animal. Light beam breaks were automatically recorded using Motor Monitor software (Hamilton-Kinder). Locomotor activity (ie total number of beam breaks), time spent, and distance traveled within open and closed arms, and the number of entries and pokes into each arm were measured.

MCH (0.1, 1, or 3 μg, i.c.v.) or aCSF in a total volume of 3 μl was infused over 1 min via a stainless-steel injector (Plastics One) projecting 0.5 mm beyond the tip of the guide cannula ($n = 16$–19 mice per group). The injector was left in place for 1 min after the infusion and a dummy cannula was reinserted into the guide. Mice were returned to their home cages for 30 min before being placed on the EPM. A 30-min pretreatment time was chosen because MCH elicited significant elevations in plasma corticosterone 30 min after i.c.v. infusion. Where indicated, mice ($n = 11$–14 per group) were injected (intraperitoneally (i.p.); 0.1 ml/10 g body weight) with vehicle or GW3430 (30 mg/kg) 2 h prior to the MCH infusion.

A separate set of mice ($n = 8$–11) was injected with GW3430 (10, 30, or 100 mg/kg, i.p.) and tested on the EPM 2 h later in the absence of exogenously administered MCH. MCHR1-KO and WT mice ($n = 8$–13) were injected with vehicle or GW3430 (60 mg/kg, i.p.) 2 h prior to EPM testing.

Plasma ACTH and Corticosterone

At 1 h into the light phase of the animal's light/dark cycle, mice were transferred to a test room and allowed to acclimate for 1 h. Randomly assigned mice ($n = 7$–10 per group) were gently restrained by hand and injected with aCSF or MCH (3 μg in a total volume of 3 μl) via direct, vertical, free-hand i.c.v. injection to a depth of 3.5 mm below bregma with a cuffed 27-G needle attached to a 25 μl Hamilton syringe (Rupniak and Williams, 1994). A 3-μg

dose of MCH was used because it elicited an anxiogenic response in a pilot study in the EPM. Mice were killed 15, 30, or 60 min after MCH injection. Mice were acclimated to the injection procedure by being gently restrained once per day for 3 days prior to testing. The free-hand injection procedure itself did not elicit greater plasma ACTH or corticosterone release when compared with injection via chronically implanted cannulae.

In a separate experiment, mice ($n = 8$–12 per group) were injected (0.1 ml/10 g body weight, i.p.) with vehicle or GW3430 (1, 3, 10, or 30 mg/kg) and returned to their home cages. After 2 h, mice were injected (i.c.v.) with aCSF or MCH (3 μg) and killed by decapitation after 30 min. Trunk blood was collected into polyethylene EDTA-coated microcentrifuge vials (Fisher Scientific, Somerville, NJ) and stored on ice until the end of the experiment. Plasma was separated from the samples by centrifugation (15 min × 3000 r.p.m., 4°C) and frozen at −80°C until assaying for plasma ACTH and corticosterone using ^{125}I radioimmunoassay kits (MP Biomedicals, Irvine, CA). Standards and plasma samples from each experiment were run in duplicate in single assays.

Stress-Induced Hyperthermia (SIH)

Once per week for 5 weeks, mice ($n = 20$) were transported to a procedure room, weighed, and administered a randomly assigned dose, in mixed order, of either vehicle, 10, 60, or 100 mg/kg GW3430, or 3 mg/kg alprazolam (i.p.; 0.1 ml/10 g body weight) in a within-subjects design. Immediately after dosing, the experimenter turned off the lights and left the room. After 2 h, mice were moved individually into an adjacent room with normal lighting conditions and the baseline core body temperature (T_1, in °C) was measured using a Physitemp BAT-12 Microprobe Thermometer and Rectal Probe for Mouse (Physitemp Instruments Inc., Clifton, NJ). The rectal probe was dipped in mineral oil to facilitate insertion to approximately 2 cm depth. After 12 min, core body temperature was measured again (T_2) to determine the stress-induced rise in core body temperature. The difference between the two temperatures ($\Delta T = T_2 - T_1$) constituted the measure of stress-SIH.

A separate group of C57 mice ($n = 50$) was used to confirm the anxiolytic activity of GW3430 in a between-subjects design SIH experiment. Mice were randomly assigned to one of five dose groups: vehicle, 10, 30, or 100 mg/kg GW3430, or 1 mg/kg alprazolam ($n = 10$ per group). As the magnitude of SIH reduction was similar when GW3430 was injected in doses of 60 or 100 mg/kg, we adjusted the doses tested in the second SIH experiment (ie 10, 30, and 100) to derive an approximate minimum effective dose. All other procedures for this experiment were identical to those described above.

In the third SIH experiment, KO ($n = 16$) and WT ($n = 14$) mice were transported from the colony room to the procedure room, weighed, and placed into their home cages. The lights were turned off and mice remained undisturbed for 1 h. Baseline core body temperature and SIH was measured as described above.

PFC ACh Response to Predator Odor Stressor (POS)

A: Characterization of psychogenic stressor. KO and WT mice ($n = 6$–7 per genotype) were transported in their home cages to a test room at approximately 0800 hours and were allowed to acclimate for 1 h. Mice were randomly assigned to a POS-exposed group or a no-stressor control group. Mice exposed to the POS were gently picked up by the base of the tail and transferred from their home cages to a cylindrical Plexiglas chamber (diameter, 10 cm; height, 25 cm) lined with soiled rat bedding. Mice in the control group were gently picked up by the base of the tail and returned to their home cages. Mice were killed by decapitation 10 min after the onset of the POS exposure or control treatment. Trunk blood samples were collected and processed for ACTH and corticosterone measurements as described above.

B: Microdialysis probe implantation. Mice were anesthetized with a ketamine/xylazine mixture, placed in a stereotaxic apparatus, and unilaterally implanted with a 2 mm microdialysis probe (CMA, North Chelmsford, MA) aimed at the PFC (coordinates in millimeter from bregma: AP, +2.0; ML, −0.25; DV, −3). The PFC cortex was defined as the area including the cingulate, prelimbic, and infralimbic cortical regions (Franklin and Paxinos, 1997). Visible Light Cured Restorative (Henry Schein, Melville, NY) anchored by two stainless-steel screws (BAS, Lafayette, IN) was used to affix the probe to the skull. Animals were left to recover for 36–40 h.

C: In vivo *PFC ACh microdialysis.* Mice were transferred to a separate environmentally controlled room between 0700 and 0800 hours, connected to a liquid swivel perfusion system for use with freely moving animals (BAS, Lafayette, IN), and aCSF (composition in mM: NaCl, 150; KCl, 3; $CaCl_2$, 1.7; $MgCl_2$, 0.9; pH = 6.0) was perfused through the probe with a syringe pump (BAS) at a rate of 1.5 μl/min for a 2.5–3 h acclimation period. Immediately after the acclimation period, four to five consecutive 30-min samples were collected to establish baseline dialysate concentrations of ACh. Next, mice were injected (i.p.; 0.1 ml/10 g body weight) with saline, CDP (10 mg/kg), vehicle, or GW3430 (60 mg/kg). A 60-mg/kg dose of GW3430 was chosen because it blocked the effects of MCH on the HPA axis, as described in the Results section. After a pretreatment time of 30 min (saline and CDP groups) or 2 h (vehicle and GW3430 groups), mice were exposed to the POS (ie cylindrical Plexiglas chambers (diameter, 10 cm; height, 25 cm) lined with soiled rat bedding were placed into the microdialysis test chambers and mice were gently transferred into a cylinder for 10 min). Some mice that were injected with CDP or GW3430 were not exposed to the stressor and were used to determine the effects of CDP and GW3430 on basal ACh efflux. Four 30-min samples were collected after the onset of the POS exposure period. Mice remained attached to the perfusion system and sample collection continued uninterrupted throughout the experiment.

Microdialysis in KO and WT mice was performed according to the procedure outlined above. KO and WT mice were injected with either vehicle or GW3430

(60 mg/kg) and exposed to the stressor after a 2-h pretreatment interval.

Dialysates were collected into refrigerated fraction collectors (BAS) and analyzed the same day or stored at −20°C until analysis. ACh levels in the dialysate were quantified using HPLC coupled to electrochemical detection. ACh was separated on a 1 × 150 mm BetaBasic C18 column (ThermoHypersil, USA) (mobile phase: 100 mM Na_2HPO_4, 2.0 mM sodium octasulfonic acid, 0.5 mM tetramethylammonium chloride, 100 µl of Microbicide/21 (Reagent MB ESA, USA); pH to 8.0 with phosphoric acid) coupled to an enzyme reactor (BAS) that converted ACh, resulting in the production of H_2O_2 for detection on a enzyme-coated glassy carbon electrode with the potential set at 100 mV. A precolumn enzyme reactor was present to degrade choline (BAS). At the end of the experiment, animals were killed by CO_2 inhalation and cresyl violet dye was perfused through the probes to mark the location. Brains were removed and sliced into 30 µm coronal sections for histological verification of probe placement (Figure 1). Animals with probes located outside of the PFC were excluded from analysis.

Basal dialysate concentrations of ACh expressed in fmol/15 µl were calculated for each animal from the average of the baseline samples. To account for variations in probe recovery, ACh levels were converted to the percent of baseline values for analyses of drug effects and genotype on stress-evoked PFC ACh dialysate levels.

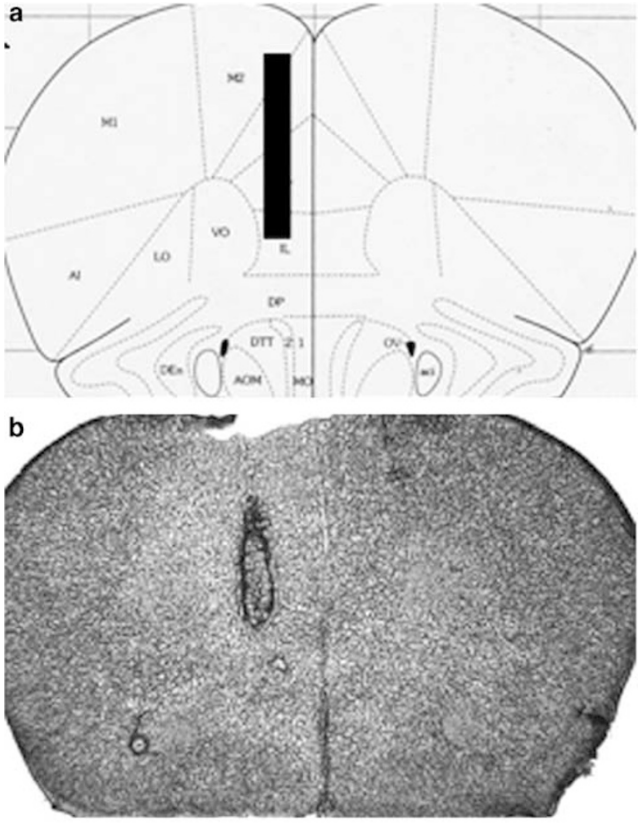

Figure 1 Representative location of microdialysis probes within the mouse PFC. (a) Black bar indicates the target for the 2 mm active dialysis membrane. (b) Representative coronal mouse brain section with probe localization identified by perfusion with cresyl violet dye (approximately bregma + 2.0 mm, according to the stereotaxic Atlas of Franklin and Paxinos, 1997).

RESULTS

MCH is Anxiogenic in the EPM

Analysis of variance (ANOVA) with *post hoc* Duncan's test revealed that MCH (3 µg, i.c.v.) administered 30 min prior to testing significantly increased anxiogenic-like behavior in the EPM. Mice injected with 3 µg MCH spent significantly less time within the open arms ($F_{4,75} = 4.4$, $p < 0.01$), entered the open arms less frequently ($F_{4,75} = 6.9$, $p < 0.01$), and traveled less distance (data not shown) in the open arms ($F_{4,75} = 5.5$, $p < 0.01$), as compared to mice injected with 0, 0.1, or 1 µg of MCH (Figure 2). The amount of time spent within the closed arms of the EPM was significantly elevated in mice injected with 3 µg MCH, as compared to mice injected with 0 or 1 µg MCH ($F_{4,75} = 6.7$, $p < 0.01$). MCH had no significant effects on the total number of ambulations within the EPM.

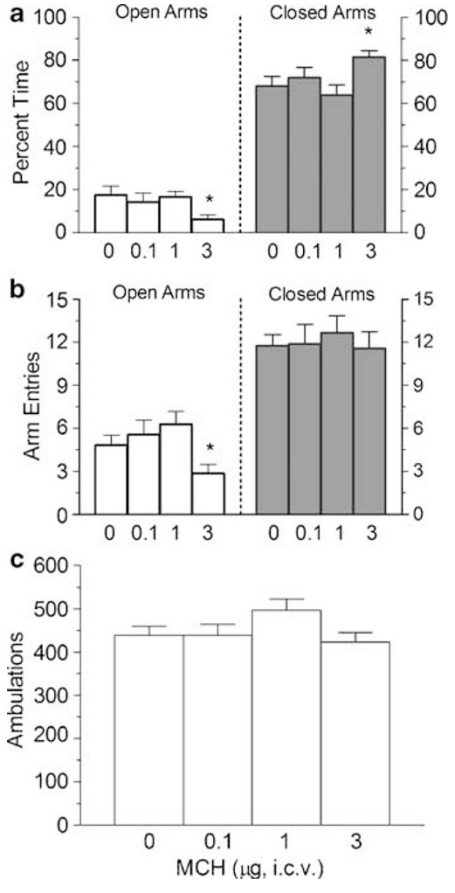

Figure 2 Intracerebroventricular injection of MCH (3 µg) reduced (a) time spent within the open arms and (b) open arm entries in the automated elevated plus maze in C57 mice ($n = 8$–13 per dose group). White and dark gray bars represent behaviors within the open arms and closed arms, respectively. (c) Total locomotor activity during the 5-min test session was not affected by MCH. Data are shown as mean ± SEM. *$P < 0.05$ *vs* vehicle-treated animals.

Effects of an MCHR1 Antagonist in the EPM

One-way ANOVA with *post hoc* Duncan's test revealed that 30 and 100 mg/kg doses of GW3430, in the absence of MCH, significantly increased open arm time ($F_{3,35} = 3.5$, $p = 0.03$), entries ($F_{3,35} = 5.7$, $p < 0.01$), and distance t raveled ($F_{3,35} = 5.4$, $p < 0.01$; data not shown) within the open arms, as compared to vehicle-injected mice (Figure 3). No significant effects of 10 mg/kg GW3430 were observed in the EPM. GW3430 (10, 30, or 100 mg/kg) did not significantly affect the total number of ambulations in the EPM.

ANOVA revealed that mice pretreated (i.p.) with vehicle and injected (i.c.v.) with 3 μg MCH (vehicle + MCH) 2 h later spent significantly less time within the open arms and significantly more time within the closed arms of the EPM, as compared to mice injected with vehicle + aCSF (Figure 4a). Pretreatment with GW3430 (30 mg/kg, i.p.) 2 h before the infusion of MCH significantly reversed the effects of MCH on time spent in the open and closed arms. No statistically significant differences in open or closed arm entries were observed (Figure 4b). Total ambulations within the EPM were significantly higher in vehicle + MCH mice when compared to vehicle + vehicle mice (Figure 4c).

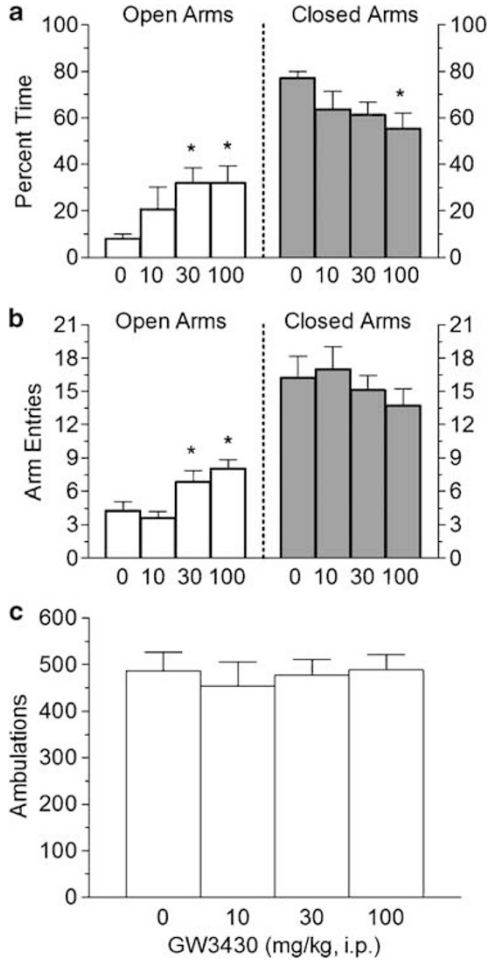

Figure 3 The MCHR1 antagonist, GW3430 (30 and 100 mg/kg, i.p.), increased (a) the percent of open arm time and (b) open arm entries on the automated elevated plus maze. (c) Overall locomotor activity during the 5-min test session was not affected by the administration of GW3430. Open and black bars represent behaviors within the open arms and closed arms, respectively. Data represent the mean ± SEM in eight of the eleven C57Bl/6 mice per dose group. *$P < 0.05$ *vs* vehicle-treated animals.

MCHR1-KO and WT Mice in the EPM

GW3430 (60 mg/kg, i.p.) injected 2 h prior to testing significantly increased open arm time (Figure 5a) and open arm distance (data not shown) in WT mice, but had no significant effects in KO mice. Two-way ANOVAs on open arm time and open arm distance revealed nonsignificant main effects of dose and genotype, whereas the dose × genotype interactions were significant ($F_{1,39} = 4.4$, $p < 0.05$, and $F_{1,39} = 4.1$, $p < 0.05$, respectively). *Post hoc* Duncan's test indicated that GW3430 significantly elevated open arm time and distance in WT mice, as compared to KO mice. There were no significant differences in basal EPM behaviors between the genotypes. However, a trend ($P < 0.1$) toward increased open arm time and entries in KO, as compared to WT mice, was noted (time: KO, 11 ± 5%; WT, 3.5 ± 2%, entries: KO, 3.9 ± 1; WT, 1.7 ± 0.7).

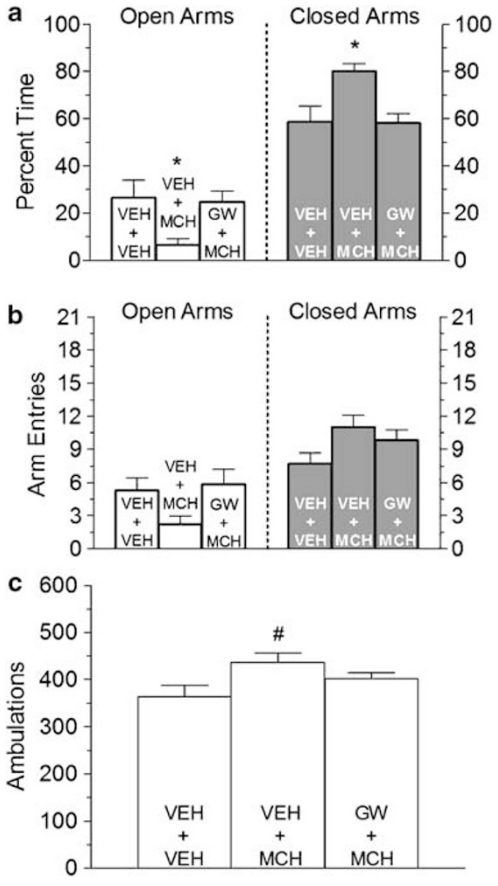

Figure 4 Pretreatment with GW3430 (30 mg/kg, i.p.) significantly reversed MCH (3 μg, i.c.v.)-induced suppression of open arm time in the automated elevated plus maze. (a) Percent time spent within the open and closed arms, (b) open and closed arm entries, and (c) overall locomotor activity during the 5-min test session. White and gray bars represent behaviors within the open arms and closed arms, respectively. Data are shown as mean ± SEM ($n = 12$–14 per group). *$P < 0.05$ *vs* VEH + VEH and GW + MCH; #$P < 0.05$ *vs* VEH + VEH.

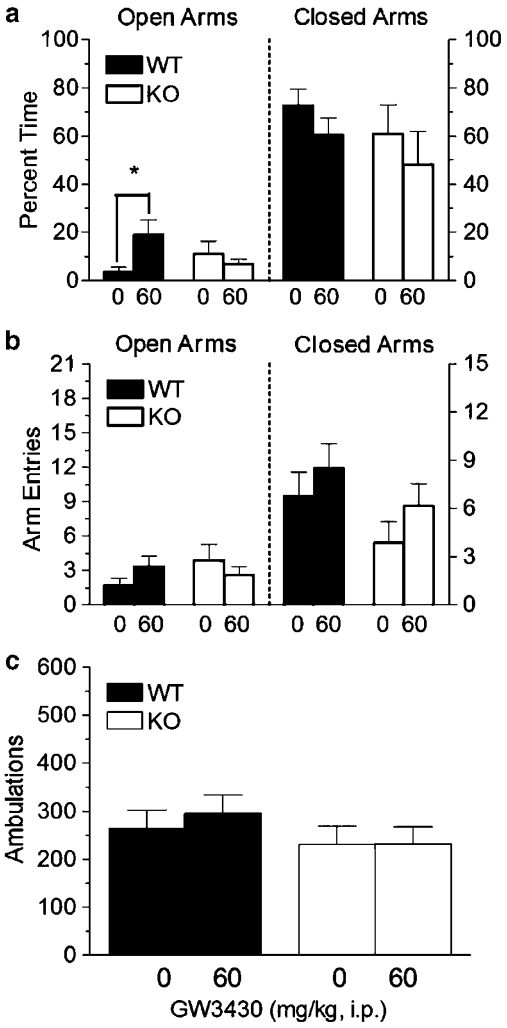

Figure 5 The MCHR1 antagonist, GW3430 (60 mg/kg, i.p.), increased (a) percent time spent within the open arms of the automated elevated plus maze (EPM) in WT, but not KO, mice. Basal anxiety-related behaviors on the EPM did not significantly differ between KO ($n = 8$–10) and WT ($n = 12$–13) mice. (b) GW3430 had no significant effects on open arm entries. (c) Overall locomotor activity during the 5-min test session was not different between the genotypes. Open and shaded bars represent KO and WT mice, respectively. Data are shown as mean ± SEM. *$P < 0.05$ *vs* vehicle-treated WT mice.

MCH Stimulates Neuroendocrine Stress Responses

MCH (3 μg, i.c.v.) significantly elevated plasma levels of ACTH and corticosterone (Figure 6). ANOVA with *post hoc* Bonferonni's multiple comparison test revealed that ACTH was significantly ($p < 0.05$) elevated 15 min after the injection of MCH, whereas corticosterone was significantly higher 30 and 60 min following MCH injection.

Pretreatment with GW3430 reversed the increase in corticosterone levels observed 30 min after MCH infusion (Figure 7). ANOVA with *post hoc* Bonferonni's test indicated that corticosterone levels in mice pretreated with 10 or 30 mg/kg (i.p.) of GW3430 prior to MCH infusion were significantly lower when compared to mice pretreated with vehicle. MCH did not significantly affect ACTH levels after 30 min, and no significant changes in ACTH were seen in mice pretreated with GW3430.

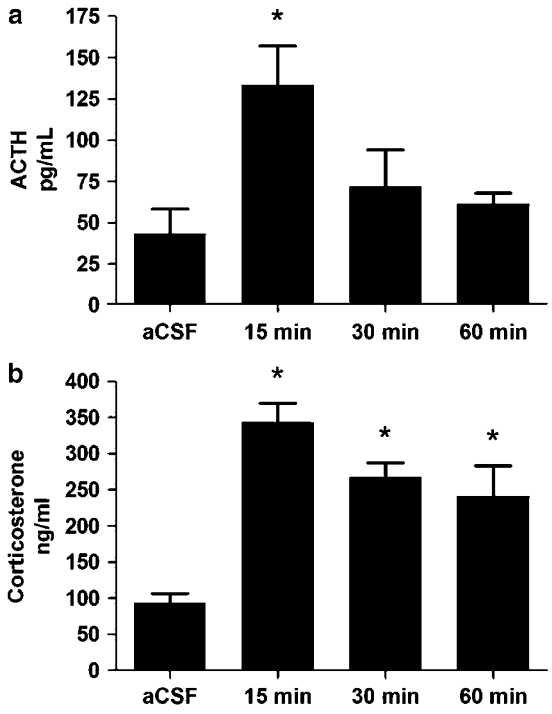

Figure 6 Plasma concentrations of (a) ACTH and (b) corticosterone 30 min after the i.c.v. injection of aCSF, or 15, 30, or 60 min (min) after the injection of MCH (3 μg). Data represent the mean plasma concentration ± SEM of ACTH and corticosterone in four of the five mice per group. *$P < 0.05$ *vs* aCSF.

Stress-Induced Hyperthermia

In the SIH paradigm, the measure for anxiety is the increase in temperature (ΔT) over a 15-min window in response to the mild stress of measuring rectal temperature. In the first experiment, the mean core body temperatures (in °C ± SEM) measured at baseline (T_1) were as follows: 36.61 ± 0.08 (vehicle), 36.85 ± 0.14 (10 mg/kg GW3430), 36.85 ± 0.14 (60 mg/kg), 36.03 ± 0.14 (100 mg/kg), and 35.61 ± 0.14 (3 mg/kg alprazolam). Repeated measures ANOVA revealed a significant ($p < 0.05$) difference between basal body temperatures ($F_{4,72} = 16.85$, $p < 0.001$). *Post hoc* tests confirmed that both the MCHR1 antagonist GW3430 at 100 mg/kg and the benzodiazepine alprazolam at 3 mg/kg significantly decreased core body temperature relative to vehicle controls. Figure 8a depicts the stress-related increase in core body temperature relative to baseline for all mice in this experiment. Repeated measures ANOVA conducted on the difference scores revealed a significant difference between groups ($F_{4,72} = 35.73$, $p < 0.001$). *Post hoc* analyses confirmed that both the 60 and 100 mg/kg doses of GW3430 significantly attenuated the stress-related increase in core body temperature, indicating anxiolytic-like effects. Alprazolam (3 mg/kg) also significantly attenuated SIH in this experiment.

In the second experiment, no differences in core body temperature were observed between groups during the baseline assessment at the end of the 2-h pretreatment period ($p > 0.05$; data not shown). Figure 8b depicts the stress-related increase in core body temperature measured in this experiment. A one-way ANOVA conducted on the

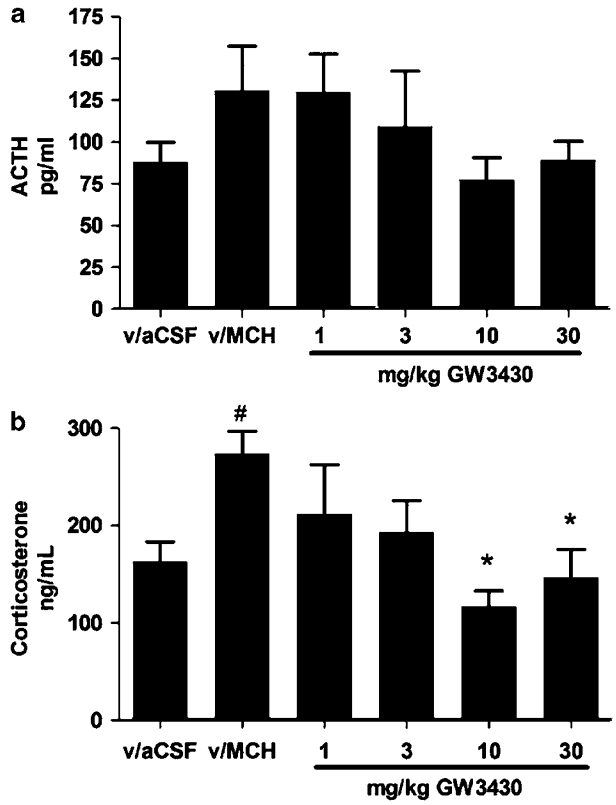

Figure 7 Plasma levels of (a) ACTH and (b) corticosterone 30 min after the injection of aCSF or MCH (3 μg, i.c.v.). GW3430 (1, 3 10 and 30 mg/kg, i.p.), or vehicle (v) was injected 2 h before the infusion of MCH. These data represent the mean plasma concentration ± SEM of ACTH and corticosterone in six of the seven mice per group. $^{\#}P<0.05$ *vs* v/aCSF; $^{*}P<0.05$ *vs* v/MCH.

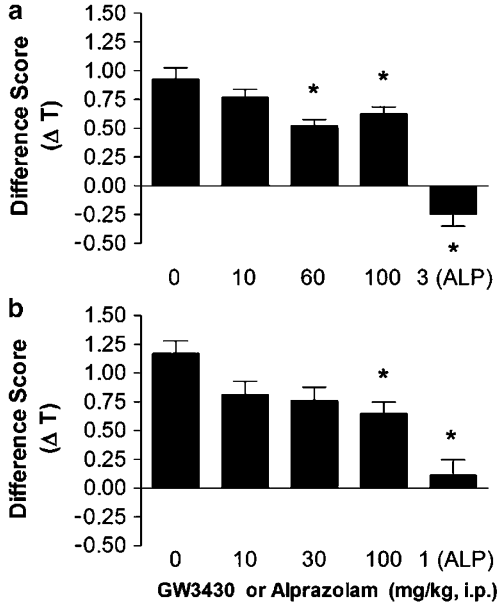

Figure 8 Effects of the MCHR1 antagonist, GW3430, and alprazolam in the stress-induced hyperthermia paradigm in C57 mice. (a) Within-subjects design ($n=20$) and (b) between-subjects design ($n=10$ per dose group). The first rectal temperature measurement (T_1) was followed 12 min later by the second rectal temperature measurement (T_2). The vehicle or drug was administered 2 h before T_1. The change in body temperature from T_1 to T_2 are shown as mean ± SEM. $^{*}P<0.05$ *vs* vehicle-treated mice.

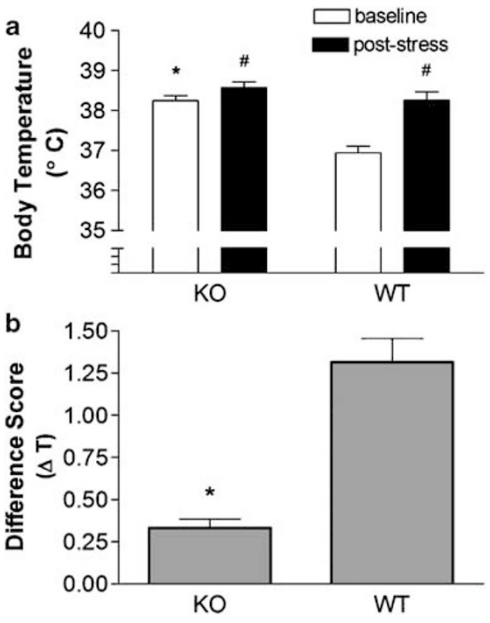

Figure 9 KO mice have an elevated body temperature but show a significant stress-induced hyperthermia response that is attenuated when compared to WT mice ($n=15$ per genotype). (a) Basal rectal temperature (T_1) and rectal temperature 12 min later (T_2) in KO and WT mice. (b) The difference in body temperature (ΔT) between T_1 and T_2 measurements. Data are shown as mean ± SEM. $^{*}P<0.05$ KO *vs* WT mice, $^{\#}P<0.05$ T_2 *vs* respective T_1.

difference scores revealed a significant difference between treatment groups ($F_{4,49}=9.85$, $p<0.001$). *Post hoc* analyses indicated that both 100 mg/kg GW3430 and 1 mg/kg alprazolam significantly attenuated SIH, relative to the vehicle control group ($p<0.02$ and $p<0.001$, respectively). A trend ($p=0.07$) toward significance was observed in mice injected with 30 mg/kg GW3430 (Figure 8b).

SIH was also assessed in KO and WT mice (Figure 9a and b). An independent samples *t*-test indicated that the baseline core body temperature was significantly higher in KO than WT mice ($t_{28}=6.08$, $p<0.001$). Regardless, paired-samples *t*-tests indicated that both KO and WT mice demonstrated significant SIH ($t_{15}=5.99$, $p<0.001$, $t_{13}=9.03$, $p<0.001$, respectively), and that the hyperthermic response was significantly reduced in KO mice, compared to WT mice ($t_{28}=6.63$, $p<0.001$).

Anxiolytic-Like Effect of GW3430 on PFC Stress Response

The POS elicited a 300–400% increase in plasma ACTH and corticosterone in KO and WT mice (data not shown). ACTH levels were 40–60 pg/ml in KO and WT mice that were not exposed to the POS, and 170–180 pg/ml in KO and WT mice exposed to the POS. Corticosterone levels were 30–50 ng/ml in no-POS mice and 120–170 ng/ml in POS-exposed KO and WT mice. Two-way ANOVA revealed a significant main effect of treatment (stressor *vs* no stressor; $F_{1,26}=42.5$, $p<0.001$), a nonsignificant main effect of genotype, and a nonsignificant treatment × genotype interaction on ACTH levels. A separate ANOVA indicated a significant main effect of treatment ($F_{1,26}=40.7$, $p<0.001$), a significant

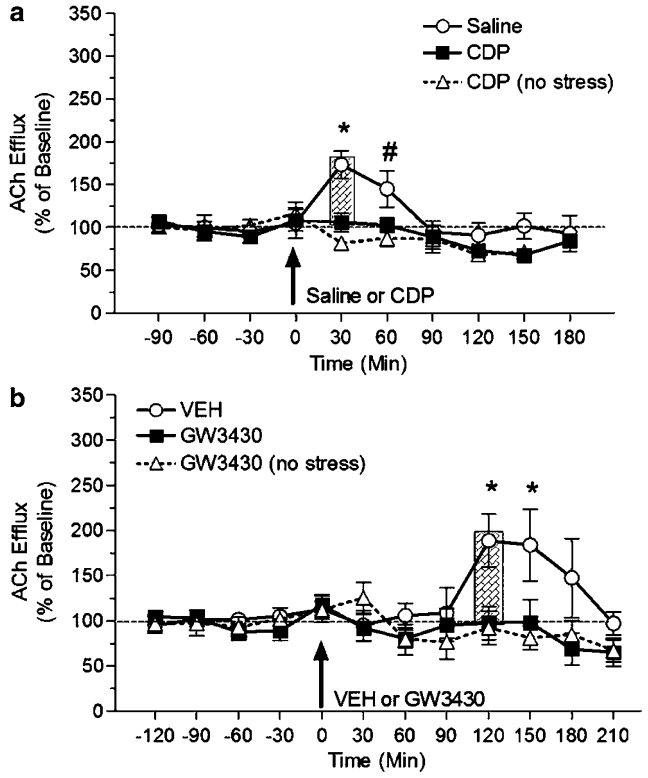

Figure 10 Chlordiazepoxide and the MCHR1 antagonist, GW3430, block POS-evoked ACh release within the PFC. (a) Saline or CDP (10 mg/kg, i.p.) was injected (arrow) 30 min prior to POS exposure (shaded bar). (b) VEH or GW3430 (60 mg/kg, i.p.) was injected 120 min before POS. Data represent the mean ± SEM and are expressed as the percent of baseline ($n = 6$–9 C57 mice per dose group). Baseline ACh dialysate content was derived from the average of three to four samples preceding drug injection. (a) $^{*}P < 0.05$ SAL *vs* CDP and CDP-(no stress); $^{\#}P < 0.05$ saline *vs* CDP-(no stress), and $P = 0.056$ *vs* CDP. (b) $^{*}P < 0.05$ VEH *vs* GW3430 and GW3430-(no stress).

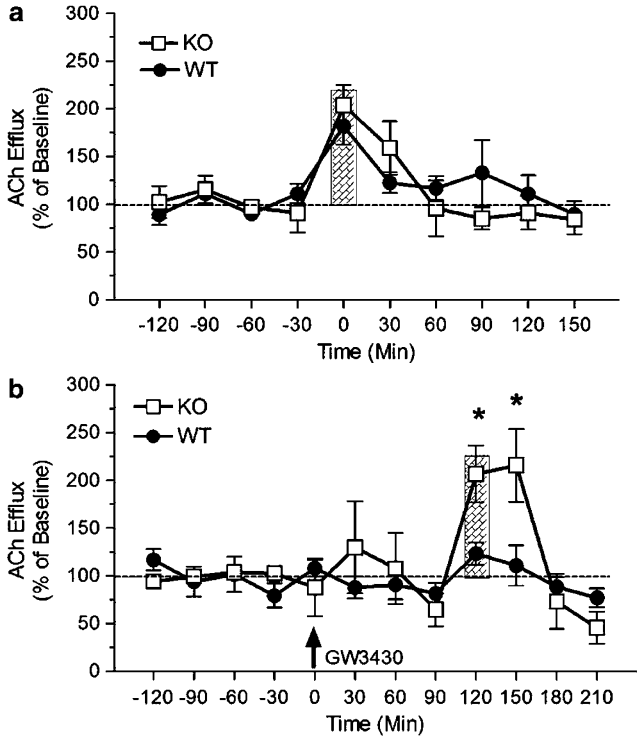

Figure 11 ACh release within the PFC in KO and WT mice. (a) Time course of PFC ACh efflux before and after 10-min exposure to POS (shaded bar) in KO and WT mice ($n = 6$–7 mice per genotype). (b) ACh efflux within the PFC of KO and WT mice ($n = 4$–6 mice per genotype) injected (arrow) with 60 mg/kg GW3430 and exposed to POS. These data represent the mean ± SEM and are expressed as the percent of baseline. Baseline dialysate content was derived from the average of three to four samples preceding the zero time point. $^{*}P < 0.05$ KO *vs* WT mice.

main effect of genotype ($F_{1,26} = 6.0$, $p < 0.05$), and a non-significant treatment × genotype interaction on corticosterone levels.

Using ANOVA, we found no significant differences between C57, KO, and WT mice in basal dialysate levels of PFC extracellular ACh. The average basal efflux of ACh in PFC dialysates was 24 ± 5 fmol/15 µl in C57 mice ($n = 26$), 27 ± 7 in KO mice ($n = 13$), and 21 ± 3 in WT ($n = 15$) mice.

ACh release was significantly ($P < 0.05$) elevated (+ 175%) in mice injected with saline and exposed to the POS (SAL + POS), but not in mice injected with CDP and exposed to the POS (CDP + POS) (Figure 10). ANOVA with time as a repeated measure revealed significant main effects of group (SAL + POS, CDP + POS, CDP-no stressor) ($F_{2,17} = 3.6$, $p < 0.05$) and time ($F_{8,136} = 6.4$, $p < 0.001$), and a significant group × time interaction ($F_{16,136} = 1.8$, $p < 0.05$). *Post hoc* analyses (Duncan's test) revealed that PFC ACh release was significantly higher during the 30 min after the POS in SAL + POS mice compared to CDP + POS and CDP-no stressor mice. From 30 to 60 min after the POS, ACh levels in SAL + POS mice were significantly higher than levels in CDP-no stressor mice, and a trend ($p = 0.06$) toward higher levels in SAL + POS *vs* CDP + POS mice was observed.

POS-evoked PFC ACh release was also attenuated by pretreatment with the MCHR1 antagonist, GW3430 (60 mg/kg, i.p.), but not by the injection of vehicle (Figure 10). ANOVA with repeated measures on time revealed a significant main effect of group (vehicle + POS, GW3430 + POS, and GW3430-no stressor; $F_{2,20} = 3.7$, $p < 0.05$), a significant main effect of time ($F_{11,220} = 2.9$, $p < 0.01$), and a significant group × time interaction ($F_{22,220} = 1.9$, $p = 0.01$). *Post hoc* Duncan's test revealed that PFC ACh release was significantly elevated for 1 h after POS exposure in the vehicle + POS group of mice, as compared to the GW3430 + POS and GW3430-no stressor groups.

Basal PFC ACh dialysate levels were significantly elevated in KO (+ 190%) and WT (+ 160%) mice during the 30 min after POS exposure (Figure 11). POS-induced PFC ACh release was attenuated by pretreatment with GW3430 (60 mg/kg, i.p.) in WT, but not KO mice (Figure 11). Two-way ANOVA with repeated measures on time revealed a significant main effect of time ($F_{11,88} = 6.2$, $p < 0.01$), a nonsignificant main effect of genotype, and a significant time × genotype interaction ($F_{11,88} = 3.0$, $p < 0.01$). *Post hoc* comparisons revealed that the increase in PFC ACh release during the first two 30-min dialysate samples collected after POS exposure were significantly elevated in KO mice, as compared to WT mice. Prior to POS exposure, GW3430 had no significant ($P = 0.7$) effects on basal levels of PFC ACh efflux in KO or WT mice.

DISCUSSION

Our results are consistent with an anxiogenic effect of MCH and an antistress, anxiolytic-like effect of MCHR1 blockade or gene deletion. We demonstrate that centrally administered MCH increased anxiety-related behavior in the EPM and increased plasma ACTH and corticosterone levels. Pretreatment with a selective MCHR1 antagonist, GW3430, reversed the anxiogenic effects of MCH in the EPM and restored plasma corticosterone to control group levels. GW3430 had anxiolytic-like effects in the absence of exogenously administered MCH in the EPM and SIH assays, and was anxiolytic-like in C57 and WT mice, but not MCHR1-KO mice, in the EPM. MCHR1-KO mice had an anxiolytic-like profile when compared to WT mice in the SIH model. Finally, for the first time we show that the role of the MCHR1 in regulating stress responsivity may involve a limbic ACh circuit that includes the PFC.

The EPM is a commonly used model of anxiety-related behavior in rodents. Anxiogenic compounds, including the $GABA_A$ receptor inverse agonist FG-7142, reduce time spent in the open arms and entries into the open arms, whereas clinically efficacious anxiolytic drugs produce the inverse effects (ie more time and entries in the open arms) (Lister, 1987; Pellow *et al*, 1985). In the present study, centrally administered MCH significantly reduced open arm time and entries without affecting overall locomotor activity within the maze. GW3430 blocked the anxiogenic effects of MCH and was anxiolytic-like when administered in the absence of exogenous MCH. It is unlikely that the effects of GW3430 in the EPM are due to nonspecific effects on locomotor activity because ambulations during the test were not different among treatment groups.

Stressful and anxiogenic stimuli elevate plasma ACTH and corticosterone concentrations by activating the HPA axis. Central to the HPA axis stress–response system is the PVN of the hypothalamus (Herman *et al*, 1996). MCH neurons project into the PVN (Bittencourt *et al*, 1992) and, when stimulated, may activate the HPA stress system and trigger the release of ACTH and corticosterone. MCH increased ACTH and corticosterone levels, and the effects of MCH on corticosterone were reversed by GW3430. The effect of GW3430 on MCH-evoked ACTH levels is unclear because the time point chosen for blood sampling (ie 30 min after MCH injection) may be inappropriate for detecting stressor-evoked elevations in plasma ACTH. As shown in Figure 6, the effects of MCH on ACTH were significant at 15, but not 30 or 60 min after injection.

It was previously reported that MCH had divergent effects on stress and anxiety-related biological indices. MCH injected directly into the lateral ventricle or the PVN stimulated the HPA axis (Jezova *et al*, 1992; Kennedy *et al*, 2003), and MCHR1 antagonists resembled known antidepressant and anxiolytic drugs (Borowsky *et al*, 2002; Chaki *et al*, 2005). Our data support these findings and expand on them by demonstrating a direct relationship between the anxiogenic effects of MCH and the reversal of these effects by selective MCHR1 blockade. In contrast, it was reported that MCH reduced peak circadian levels of ACTH (Bluet-Pajot *et al*, 1995; Ludwig *et al*, 1998) and blocked stress-induced secretion of ACTH and corticosterone (Ludwig *et al*, 1998). MCH was also reported to have anxiolytic-like activity in the EPM in rats (Monzon and De Barioglio, 1999; Monzon *et al*, 2001). It is likely that species differences and wide circadian variation in basal HPA axis activity account for the divergent results. Alternatively, MCH administration combined with exposure to stressors may evoke pronounced negative feedback onto the HPA axis and result in the decreased stress hormone levels reported in some prior studies.

Further support for an anxiolytic-like role of MCHR1 blockade was obtained by measuring SIH in mice. Autonomic hyperactivity is a representative symptom of generalized anxiety disorder as described in the DSM-IV (American Psychological Association, 1994), and SIH is an integral component of the autonomic response to anxiogenic or stressful stimuli (Spooren *et al*, 2002; Van der Heyden *et al*, 1997). In the present study, the known anxiolytic, alprazolam, and GW3430 significantly attenuated SIH. Moreover, SIH was significantly reduced in MCHR1-KO mice when compared to WT mice, although KO mice had a higher core body temperature than WT mice. The highest tested doses of alprazolam and GW3430 (3 and 100 mg/kg, respectively) significantly reduced core body temperature. However, this nonspecific effect of GW3430 was not reproduced in a second experiment, whereas the blockade of SIH by GW3430 was replicated.

MCH-containing neurons (Bittencourt *et al*, 1992) and MCHR1 (Hervieu *et al*, 2000) densely populate limbic brain regions (eg PFC) associated with depression, anxiety, and stress responses. The MCHR1 modulates neurotransmission within limbic brain regions (Smith *et al*, 2005), and may have clinically relevant effects linked to its mesolimbic localization (Georgescu *et al*, 2005). Corticolimbic areas, including the PFC, are central to processing complex aspects of stress in humans (Shin *et al*, 2005) and rats (Amat *et al*, 2005; Jinks and McGregor, 1997). Several groups have reported that various psychogenic stressors elicit PFC ACh release (Laplante *et al*, 2004; Mark *et al*, 1996; Rosenblad and Nilsson, 1993; Thiel *et al*, 1998). Thus, we hypothesized that MCHR1 blockade would attenuate the effects of a POS on PFC ACh release. Exposure to POS elevated PFC ACh efflux to similar levels as other stressful stimuli (Laplante *et al*, 2004; Mark *et al*, 1996), and pretreatment with CDP or GW3430 reversed this effect. The specificity of the mechanism of action underlying these results is the MCHR1 because GW3430 had no effect on POS-evoked PFC ACh release in MCHR1-KO mice. Thus, it appears that the MCHR1 may be important in regulating corticolimbic stress responsivity.

In response to stressful stimuli, the PFC may inhibit key subcortical stress centers, such as the PVN. Lesions of the PFC potentiated stress-evoked ACTH and corticosterone release (Diorio *et al*, 1993), and inactivation of the PFC increased stress-evoked c-fos expression within the dorsomedial hypothalamus and amygdaloid nuclei, both of which regulate activity of the PVN (McDougall *et al*, 2004). Spencer *et al* (2005) proposed that the PFC might regulate the PVN via a circuit involving the bed nucleus of the stria terminalis (BNST). The MCHR1 is moderately to extensively expressed within the frontal cortex, PVN, BNST, and several closely associated amygdaloid nuclei (Hervieu *et al*, 2000), and may function within these brain regions to modulate neuroendocrine responses to stressful and anxiogenic

stimuli. Examining the effects of site-specific infusions of MCH and MCHR1 antagonists may identify critical regions underlying the role of MCH in stress and anxiety.

In conclusion, distinct behavioral, physiological, and neurochemical stress, and anxiety-related responses were modulated by the MCHR1 in the present study. We propose that the MCHR1 may regulate stress responsivity and anxiety by acting within higher-order brain regions, such as the PFC, to regulate primary neural stress centers (eg the PVN). Overall, the MCHR1 may be an important component in the neuronal circuit that regulates stress, anxiety, and affective responses.

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