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Melanocortin-4 receptor in the medial amygdala regulates emotional stress-induced anxiety-like behaviour, anorexia and corticosterone secretion

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Abstract

The central melanocortin system has been implicated in emotional stress-induced anxiety, anorexia and activation of the hypothalamo-pituitary-adrenal (HPA) axis. However, the underlying neural substrates have not been identified. The medial amygdala (MeA) is highly sensitive to emotional stress and expresses high levels of the melanocortin-4 receptor (MC4R). This study investigated the effects of activation and blockade of MC4R in the MeA on anxiety-like behaviour, food intake and corticosterone secretion. We demonstrate that MC4R-expressing neurons in the MeA were activated by acute restraint stress, as indicated by induction of c-*fos* mRNA expression. Infusion of a selective MC4R agonist into the MeA elicited anxiogenic-like effects in the elevated plus-maze test and decreased food intake. In contrast, local MeA infusion of SHU 9119, a MC4R antagonist, blocked restraint stress-induced anxiogenic and anorectic effects. Moreover, plasma corticosterone levels were increased by intra-MeA infusion of the MC4R agonist under non-stressed conditions and restraint stress-induced elevation of plasma corticosterone levels was attenuated by pretreatment with SHU 9119 in the MeA. Thus, stimulating MC4R in the MeA induces stress-like anxiogenic and anorectic effects as well as activation of the HPA axis, whereas antagonizing MC4R in this region blocks such effects induced by restraint stress. Together, our results implicate MC4R signalling in the MeA in behavioural and endocrine responses to stress.

Keywords

Anorexia; anxiety; corticosterone; medial amygdala; melanocortin-4 receptor; restraint stress

Introduction

The central melanocortin system, which consists of the endogenous melanocortin agonist, α-melanocyte-stimulating hormone (α-MSH) derived from proopiomelanocortin (POMC), the endogenous inverse agonist, agouti-related protein and the melanocortin-4 receptor (MC4R), has been well recognized for its function in the control of eating behaviour (Barsh & Schwartz, 2002; Cone, 2005; Farooqi & O'Rahilly, 2004). Accumulating evidence suggests a functional interaction between the central melanocortin system and the stress system. First, POMC neurons in the arcuate nucleus are rapidly activated by acute emotional stress (Liu *et al*. 2007). Second, levels of POMC mRNA and its derivative α-MSH increase after exposure

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to stress (Baubet *et al.* 1994; Harbuz & Lightman, 1989; Khorram *et al.* 1985; Sumpter *et al.* 1986; Yamano *et al.* 2004). Third, intracerebroventricular (i.c.v.) injection of melanocortin receptor agonists, particularly those selective for MC4R, induces stress-like endocrine and behavioural responses, such as activation of the hypothalamo-pituitary-adrenal (HPA) axis, increased anxiety and reduced food intake (de Barioglio *et al.* 1991; Gonzalez *et al.* 1996; Klenerova *et al.* 2008; Lu *et al.* 2003; Rao *et al.* 2003). Finally, i.c.v. injection of MC4R antagonists blocks stress-induced anxiogenic and anorectic effects (Chaki *et al.* 2003; Kokare *et al.* 2010; Liu *et al.* 2007; Vergoni *et al.* 1999). These findings suggest that activation of the central melanocortin system is involved in endocrine and behavioural responses to stress. However, the neural substrates involved remain to be elucidated.

The amygdala is one candidate brain area that may convey hyper-melanocortinergic tone during stress exposure. This structure is essential for the processing of emotions, including fear and anxiety (Aggleton, 1993; Davis, 1992; LeDoux, 2000). It consists of a group of anatomically and functionally distinct nuclei. Although various sub-regions of the amygdala have been reported to take part in stress responses, the medial amygdala (MeA) is highly sensitive to stressors with apredominantly emotional component, including restraint (Arnold *et al.* 1992; Cullinan *et al.* 1995; Dayas *et al.* 1999, 2001; Windle *et al.* 2004), immobilization (Imaki *et al.* 1993; Ma & Morilak, 2004; Roske *et al.* 2002), forced swim (Cullinan *et al.* 1995; Dayas *et al.* 2001), social defeat (Chung *et al.* 1999; Nikulina *et al.* 2004) and inescapable foot shock (Rosen *et al.* 1998). The MeA serves as one component of stress excitatory circuits (Dunn & Whitener, 1986; Matheson *et al.* 1971; Redgate & Fahringer, 1973). Direct stimulation of MeA increases corticosterone levels (Dunn & Whitener, 1986; Feldman *et al.* 1990), whereas lesions of the MeA attenuate the HPA axis response to stress (Dayas *et al.* 1999; Feldman *et al.* 1994). α-MSH/POMC projections within the amygdala arise from the arcuate nucleus and are particularly abundant in the MeA (Bagnol *et al.* 1999; O'Donohue *et al.* 1979 Watson *et al.* 1978b). The MeA also expresses high levels of MC4R (Kishi *et al.* 2003; Lu, 2001; Mountjoy *et al.* 1994). These findings led us to investigate whether MC4R-expressing neurons in the MeA are responsive to acute emotional stress and whether activation or blockade of MC4R in this region modulates emotional and feeding behaviours as well as activity of the HPA axis under basal and stress conditions. Because acute restraint stress activates melanocortinergic neurons (Liu *et al.* 2007) and induces anxiety and anorexia and activates the HPA axis (Chaki *et al.* 2003; Dayas *et al.* 1999; Rybkin *et al.* 1997; Thorsell *et al.* 1999), this stressor was used in this study to induce behavioural and endocrine changes.

Method

Animals

Adult male Sprague–Dawley rats (Charles River Laboratories Inc., USA), weighing 250–300 g, were housed in groups of two under conditions of constant temperature and humidity on a 12-h light/dark cycle (lights on 07:00 hours). Food and water were available *ad libitum*. Animals were allowed to acclimate to these housing conditions for 1 wk before experiments began. All animal procedures were conducted in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Surgery

Rats were anaesthetized with a cocktail (43 mg/kg ketamine, 9 mg/kg xylazine and 1.4 mg/kg acepromazine in saline) and fixed in a stereotaxic apparatus for cannula implantation. Using the stereotaxic coordinates, −2.6 mm posterior to Bregma, ±3.3 mm lateral to midline and −8.0 mm ventral to skull, a permanent 22-gauge stainless steel guide cannula (Plastics

One, USA) was implanted into the MeA. The guide cannula was secured in place using dental cement and three stainless steel mounting screws anchored to the skull. The guide cannula was 1 mm above the MeA to minimize tissue damage. A stainless-steel dummy cannula, extending 0.5 mm beyond the guide, was used to seal the guide cannula when not in use. After surgery, the animals were housed individually to avoid damage to guide and dummy cannulae.

In order to collect blood samples, the jugular vein catheterization procedure was performed. At 4 d after cannulation, a catheter was introduced into the jugular vein of deeply anaesthetized rats and sutured to the surrounding muscle to anchor it. After implantation into the vein, the catheter was tunnelled subcutaneously to exit the skin on the back of the rats. The catheter was filled with a sterile 50 U/ml heparin saline solution and plugged with a gold wire. Heparin saline flush solution was used to maintain patency of the indwelling intravenous (i.v.) catheters in blood sampling.

Microinjection

Animals were allowed to recover for 7 d after intra-MeA cannulation. During this period, they were handled daily to minimize stress caused by the microinjection procedure. Melanocortin 3/4 receptor antagonist SHU 9119 (Peninsula Laboratories, USA) and the highly selective MC4R agonist Cyclo (*β*-Ala-His-D-Phe-Arg-Trp-Glu)-NH2 (Bednarek *et al.* 2001) (Phoenix Pharmaceuticals, USA) were freshly dissolved in artificial cerebrospinal fluid (aCSF) before use. All intra-MeA microinjections were performed on conscious, unrestrained, freely moving rats in their home cages. Injections were performed over 1 min using a 28-gauge stainless-steel injector connected to a 5-*μ*l syringe (Hamilton Company, USA), which was operated by an infusion pump. The injector was inserted and extended 1 mm beyond the tip of the guide cannula. Drug solution or vehicle was infused in a volume of 0.5 *μ*l delivered over 1 min. An additional minute was allowed for diffusion and prevention of backflow through the needle track before the injector was withdrawn.

Experimental protocols

Animals were randomly assigned to either control or acute restraint stress groups. The stress procedure was performed in a separate procedure room. On the experimental day, animals were first moved to the procedure room for 2 h without any disturbance. All acute stress and microinjections were performed in the late light cycle.

Induction of c-fos mRNA in MC4R-expressing neurons in the MeA by acute restraint stress—For the exposure to acute restraint stress, rats were confined in white flexible plastic wrappers (19×25 cm) enclosed in a 20.5×7.5×7.5-cm clear Plexiglas open frame for 30 min. Holes at both ends of the open frame allowed free air circulation. At the end of restraint stress, animals were immediately killed by decapitation. The unstressed control animals were kept in the home cage without disturbance until decapitated. Brains were removed and quickly frozen in an isopentane-dry ice bath adjusted to −35 °C. Brain sections (20 *μ*m) were cut on a Leica cryostat (Leica Microsystems GmbH, Germany) through the amygdala, thaw mounted onto polylysine-coated slides and stored at −80 °C until processing for double-labelling fluorescence *in situ* hybridization to detect the co-localization of c-*fos* mRNA and MC4R mRNA in the MeA.

Effects of activation of MC4R in the MeA on food intake and anxiety-like behaviour—To examine the effect of activation of MC4R in the MeA on anxiety-like behaviour, the MC4R agonist (0, 0.1 and 1.0 nmol) was directly infused into the MeA 1 h before the elevated plus-maze test. The elevated plus-maze was made of black acrylic, with four arms (45-cm long and 12-cm wide) arranged in the shape of a ‘plus’ sign and elevated

to a height of 70 cm above the floor. Two arms opposite each other have no side or end walls (open arms) and the other two arms have side walls and end walls (45-cm high) but are open on top (closed arms). A central 12×12 cm square platform provides access to all arms. Rats were placed in the centre square facing the corner between a closed arm and an open arm and were allowed to explore the elevated plus-maze for 5 min. Their activity on the elevated plus-maze was recorded by a digital CCD camera and analysed using an EthoVision video tracking system (Noldus Information Technology Inc., USA). After each test, the maze was thoroughly cleaned with 20% alcohol to eliminate the odour and trace of the previously tested animal. The time spent on the open and closed arms and the number of entries made into each arm were measured. Entry was defined as all four paws being positioned within one arm. The degree of anxiety was assessed by calculating the percentage of open arm entries (entries into the open arms/total entries into all arms) and percentage of open arm time (time spent in the open arms/total time spent in all arms).

To investigate the effect of activation of MC4R in the MeA on food intake, rats were weighed and counterbalanced into different treatment groups prior to the experiment. The MC4R agonist (0, 0.1 and 1.0 nmol) was directly infused into the MeA 1 h before the dark cycle (18:00 hours). A pre-weighed chow hopper was placed in the home cage of each rat at the onset of the dark cycle (19:00 hours). Food intake was measured by weighing the remaining pellets and the spillage for 30 min, 120 min and 12 h. A red light was provided during the measurement of food consumption in the dark cycle. To minimize disruption of food accessibility, two sets of containers were used to provide pre-weighed food to each animal. Food intake was calculated by subtracting the weight of remaining food from the initial weight.

Effects of blockade of MC4R in the MeA on restraint stress-induced anxiety and anorexia—To determine the effects of blockade of MC4R in MeA on stress-induced anxiety-like behaviour, rats received an intra-MeA microinjection of a MC4R antagonist, SHU 9119 (0, 0.5 and 1 nmol). After intra-MeA injection (30 min later), rats were subjected to either no stress (control) or 30-min restraint stress. Rats were tested in the elevated plus-maze 30 min after the onset of restraint exposure. The elevated plus-maze test was performed as described above and elsewhere (Liu *et al.* 2007).

To examine the effect of blockade of MC4R in the MeA on stress-induced anorexia, rats were weighed and counterbalanced into different treatment groups prior to the experiment. The SHU 9119 (0, 0.5 and 1 nmol) was infused into the MeA 1 h before the dark cycle (18:00 hours). Animals were subjected to either no stress or 30-min restraint stress at 30 min after intra-MeA injection (18:30 hours). A pre-weighed chow hopper was placed in the home cage of each rat at the onset of the dark cycle (19:00 hours). Food intake was measured by weighing the remaining pellets and the spillage for 30 min, 120 min and 12 h, as described above.

Effects of activation or blockade of MC4R in the MeA on restraint stress-induced stimulation of the HPA axis—To investigate the effect of MC4R signalling in the MeA on HPA axis activity, the experiments were conducted in the early light cycle when basal corticosterone levels are low and the HPA axis is highly sensitive to stress and pharmacological stimulation (Dallman *et al.* 1994). To determine the effect of the MC4R agonist on corticosterone levels in the plasma, a baseline blood sample was drawn through the indwelling jugular vein catheter prior to intra-MeA injection of the MC4R agonist (1.0 nmol) and vehicle. Following microinjection, blood samples were collected every 15 min for 60 min.

To examine the effect of blockade of MC4R on corticosterone levels under basal and stressed conditions, rats received an intra-MeA injection of SHU 9119 (0.1 and 1.0 nmol) or vehicle after a baseline blood sample was collected. Fifteen minutes after the intra-MeA injection, animals were subjected to non-stressed control condition or restraint stress for 30 min. Blood samples were taken through the jugular vein catheter at 0, 15, 30, 45 and 60 min after microinjection. Animals were released from the restrainer at the end of 30-min restraint stress.

Histological verification of cannula placement

Histological verification of the intra-MeA cannula was performed at the end of the experiments. Rats were injected with 0.5 μl India ink via an injector under anaesthesia and decapitated. Brains were then removed and frozen in a dry ice-isopentane bath (−35 °C). Brain sections at 20 μm were cut in the coronal plane with a cryostat. The sections were mounted on poly-lysine-coated slides and stained with Toluidine Blue. The cannula placement was determined according to the rat brain atlas (Paxinos & Watson, 1998) (Fig. 8).

Determination of corticosterone concentrations in plasma

Plasma corticosterone levels were determined using a highly specific corticosterone antibody (Chemicon International, USA). Briefly, 10 μl duplicate samples of plasma were heated at 70 °C for 30 min to denature corticosterone-binding protein and incubated overnight with corticosterone antibody and [^{3}H]corticosterone (PerkinElmer, USA). Free and bound corticosterone were separated by incubation with charcoal for 15 min.

Double-labelling fluorescence *in situ* hybridization

To examine the co-localization of c-*fos* mRNA with MC4R mRNA in the MeA, double-labelling fluorescence *in situ* hybridization was performed. Antisense and sense cRNA probes for c-*fos* mRNA and MC4R mRNA were labelled by fluorescein-12-UTP or digoxigenin-11-UTP (Roche Diagnostics, USA) using a standard transcription method. Brain sections were hybridized with a mixture of c-*fos* and MC4R cRNA probes for 18 h at 55 °C. The following day, brain sections were washed with sodium citrate buffer (SSC) and treated with RNase a (200 μg/ml) for 1 h at 37 °C. After the final wash in 0.1×SSC at 68 °C for 1 h, sections were then transferred to the 0.05 M phosphate-buffered saline (PBS) and processed to visualize fluorescein or digoxigenin-labelled mRNA using the TSA Plus Fluorescence System kit (PerkinElmer). Briefly, brain sections were incubated in 2% H_2O_2 in PBS for 30 min. After rinsing in Tris buffered saline, the sections were treated with a blocking buffer (PerkinElmer) for 1 h and then incubated with an anti-fluorescein antibody conjugated to horseradish peroxidase (HRP; Roche Applied Science) for 1 h. After three washes, the sections were incubated with the fluorescein tyramide amplification reagent (PerkinElmer) for 15 min to reveal c-*fos* signalling. To visualize MC4R mRNA, the sections were incubated in 2% H_2O_2 for 30 min, followed by sheep anti-digoxigenin antibody (Roche Diagnostics) overnight. After three washes, the sections were then incubated with anti-sheep antibody conjugated to HRP (Sigma, USA). The sections were washed and incubated with the cyanine 3 tyramide amplification reagent (PerkinElmer) for 15 min for visualization of MC4R mRNA. After rinsing, the slides were coverslipped using ProLong® Gold antifade reagent (Invitrogen, USA). The MeA neurons that were positive for c-*fos* mRNA and MC4R mRNA were identified with an Olympus BX52 fluorescent microscope (Olympus Corporation, Japan).

Data analysis

Results are expressed as means±S.E.M. Statistical analyses were performed by using one-way analysis of variance (ANOVA) on the elevated plus-maze test, one-way ANOVA with repeated measures on food intake and corticosterone secretion and two-way ANOVA with repeated measures on the effects of SHU 9119 and restraint stress on corticosterone levels, followed by a *post-hoc* Bonferroni/Dunn or Tukey/Kramer (for unequal *n*) test.

Results

Stress-induced c-fos mRNA expression in MC4R-expressing neurons in the MeA

Within the amygdala, MC4R mRNA displayed moderate expression in the MeA with a high-density of signal in the anteroventral MeA (Kishi *et al.* 2003; Lu, 2001). c-*fos* is widely used as a marker of neuronal activation in response to stress. To determine if restraint stress activates MC4R-expressing neurons in the MeA, double-labelling fluorescent *in situ* hybridization was used to reveal the co-localization of c-*fos* mRNA and MC4R mRNA in the MeA. As shown in Fig. 1, while non-stressed control rats exhibited negligible c-*fos* mRNA expression in MeA, 30-min restraint stress induced robust c-*fos* mRNA expression, suggesting that the MeA is highly sensitive to emotional stress. This is consistent with previous findings reported by us and others (Cullinan *et al.* 1995; Dayas *et al.* 2001; Liu *et al.* 2007). Moreover, we found that a majority (>60%) of *c-fos* mRNA expressing neurons were also positive for MC4R mRNA (Fig. 1c). No signal was detected using sense probes for *c-fos* and MC4R mRNA (Fig. 1b*).* These results suggest that MC4R-expressing neurons in the MeA are responsive to stress.

Effects of activation of MC4R in the MeA on anxiety levels and food intake in non-stressed rats

The effects of activation of MC4R in the MeA by local infusion of the MC4R agonist on anxiety-like behaviour were evaluated in the elevated plus-maze test. This test has been widely used to evaluate anxiety levels in rodents and is sensitive to anxiolytic and anxiogenic drugs (Griebel *et al.* 1996; Pellow *et al.* 1985). The percentage of open arm entries and time spent in the open arms in the elevated plus-maze test has been validated as measures of anxiety (Pellow *et al.* 1985). Rats received an intra-MeA microinjection of the MC4R agonist (0, 0.1, or 1 nmol) 1 h before testing in the elevated plus-maze. ANOVA revealed a significant main effect of drug treatment. The MC4R agonist decreased the percentage of open arm entries *(*$F_{2,21}$ =5.508, $p < 0.05$) and the percentage of time spent in the open arms ($F_{2,21}$ =4.157, $p<0.05$), but had no significant effect on total arm entries ($F_{2,21}$ = 0.816, $p = 0.4$*6). Post-hoc* analysis indicated that MC4R agonist at the dose of 1.0 nmol significantly reduced the percentage of open arm entries and the time spent in the open arm compared to vehicle treatment ($p<0.05$) (Fig. 2a, b).

The effects of activation of MC4R in MeA on spontaneous food intake were determined in non-fasted rats after intra-MeA infusion of two doses (0.1 and 1.0 nmol) of the MC4R agonist. Intra-MeA infusion was performed 1 h before the dark cycle. Food was provided at the onset of the dark cycle and food intake was measured 30 min, 120 min and 12 h after food was provided. One-way ANOVA with repeated measures revealed a main effect of treatment ($F_{2,18}$=5.983, $p<0.01$). *Post-hoc* analysis showed that MC4R agonist at the dose of 1.0 nmol significantly decreased spontaneous food intake within 30 and 120 min after food was provided when compared to the vehicle-treated group ($p<0.01$ for both time points) (Fig. 3). The lower dose of the MC4R agonist (0.1 nmol) did not significantly affect food intake measured at either 30 or 120 min (Fig. 3). There was no difference in 12 h food intake between three treatment groups (aCSF: 6.83±0.45 g; 0.1 nmol MC4R agonist: 6.59±0.42 g; 1.0 nmol MC4R agonist: 7.14±0.30 g; food intake was adjusted by 100 g body weight).

Effects of blockade of MC4R in the MeA on acute stress-induced anxiety-like behaviour and anorexia

Acute restraint stress induces anxiogenic effects (Ebner *et al*. 2004; Liu *et al*. 2007 Moller *et al*. 1997; Thorsell *et al*. 1999). To determine whether activation of MC4R contributes to restraint stress-induced anxiety-like behaviour, rats received an intra-MeA infusion of the MC4R antagonist SHU 9119 (0, 0.5 or 1.0 nmol), followed by exposure to 30-min restraint stress. Animals were tested in the elevated plus-maze right after restraint stress. ANOVA revealed significant effects of treatment on the percentage of open arm entries ($F_{4,36}$=2.523, p=0.05) and an approaching significant effect of time spent on the open arms ($F_{4,36}$=1.802, p=0.10) but not on total arm entries ($F_{4,36}$=0.658, p=0.63). *Post-hoc* analysis showed that intra-MeA injection of 1 nmol SHU 9119 significantly reversed the anxiogenic effect of restraint stress measured on the elevated-plus maze (Fig. 4, p<0.05).

We and others have previously shown that acute restraint stress results in short-term anorexia (Chaki *et al*. 2003; Liu *et al*. 2007; Rybkin *et al*. 1997). To examine the role of MC4R signalling in the MeA in stress-induced anorexia, the MC4R antagonist SHU 9119 (0, 0.5 or 1.0 nmol) was infused into the MeA 1 h before the onset of the dark cycle, followed by exposure to 30-min restraint stress in non-fasted rats. Food was provided at the onset of the dark cycle. ANOVA with repeated measures revealed a significant main effect of treatment on spontaneous food intake ($F_{4,96}$=5.779, p<0.01). Food intake was significantly reduced at 30 min (Fig. 5a, p<0.001) and 120 min (Fig. 5b, p<0.01) after restraint stress. The cumulative food intake at 12 h after exposure to restraint stress exhibited no difference from the non-stressed control condition (Fig. 5c, p=0.12). The intra-MeA injection of 1 nmol SHU 9119 alone had no significant effect on food intake (Fig. 5), but reversed restraint stress-induced reduction of food intake at 30 and 20 min after stress exposure (30-min food intake: p<0.001; 120-min food intake: p<0.01). The lower dose of SHU 9110 (0.5 nmol) did not elicit a significant effect on stress-induced suppression of food intake at any time point measured (Fig. 5).

Effects of activation of MC4R in the MeA on plasma corticosterone levels

To determine the effects of activation of MC4R in the MeA on the HPA axis, we measured plasma corticosterone levels in response to an intra-MeA injection of MC4R agonist. Blood samples were taken through an indwelling jugular vein catheter immediately before the intra-MeA injection (baseline) and at 15, 30, 45 and 60 min after the intra-MeA injection of 1.0 nmol MC4R agonist. ANOVA analysis with repeated measures revealed a significant effect of treatment on plasma corticosterone levels ($F_{1,52}$=23.556; p<0.001). *Post-hoc* analyses indicated that MC4R agonist at the dose of 1.0 nmol significantly increased plasma corticosterone levels at 15, 30, 45 and 60 min post-injection compared to vehicle treatment (Fig. 6). These results indicate that activation of MC4R in the MeA is sufficient to stimulate HPA activity under non-stressed conditions.

Effects of blockade of MC4R in the MeA on restraint stress-induced elevation of corticosterone levels

To examine the role of MC4R signalling in the MeA in stress-induced stimulation of the HPA axis, rats were pretreated with SHU 9119 (1.0 nmol) through the implanted intra-MeA cannula, followed by 30-min restraint stress. Blood samples were taken through the jugular vein catheter at 0, 15, 30, 45, 60 and 75 min after the intra-MeA microinjection. Two-way ANOVA with repeated measures revealed a main effect of stress ($F_{1,92}$=8.216; p<0.01) but not treatment ($F_{1,92}$=0.816; p<0.38). There was a significant interaction between stress and treatment ($F_{1,92}$=4.357; p<0.05). Corticosterone levels rapidly increased after the onset of restraint stress and declined to basal levels after the termination of restraint stress. SHU 9119 treatment alone had no effect on plasma corticosterone levels, but significantly

attenuated restraint stress-induced corticosterone secretion ($F_{1,68}$=6.316, p<0.01). *Post-hoc* analysis indicated that SHU 9119 (1.0 nmol) decreased restraint stress-induced corticosterone secretion at 30 min after the onset of restraint stress (p<0.01) and 15 min after the termination of restraint stress (p<0.05) (Fig. 7). These findings suggest that MC4R signalling in the MeA is involved in stress-induced HPA activation.

Discussion

The present study demonstrated that MC4R-expressing neurons in MeA are activated by acute restraint stress, accompanied by anxiety-like behaviour, reduction of food intake and stimulation of the HPA axis. Infusion of a MC4R agonist to the MeA induces stress-like anxiogenic and anorectic effects and elevation of plasma corticosterone levels. Blockade of MC4R in this brain region attenuated behavioural and endocrine responses to restraint stress. These results provide evidence that the MeA is an important neural substrate involved in mediating the effects of MC4R signalling in stress responses.

Our previous studies have shown that POMC neurons in the arcuate nucleus are activated by restraint stress (Liu *et al*. 2007). POMC neurons in the arcuate nucleus provide melanocortinergic input to the amygdala (Bagnol *et al*. 1999; O'Donohue *et al*. 1979 Watson *et al*. 1978b). Arcuate lesions almost completely eliminate α-MSH immunoreactivity in the amygdala (O'Donohue *et al*. 1979). Among the different amygdaloid nuclei, the MeA contains the most abundant α-MSH immunoreactive fibres (Bagnol *et al*. 1999; Jacobowitz & O'Donohue, 1978; O'Donohue *et al*. 1979 Watson *et al*. 1978a, b). This area also expresses high levels of MC4R (Kishi *et al*. 2003; Lu, 2001; Mountjoy *et al*. 1994). A number of studies have reported that neurons in the MeA are highly sensitive to emotional stress, including restraint stress (Arnold *et al*. 1992; Cullinan *et al*. 1995; Dayas *et al*. 1999, 2001; Windle *et al*. 2004). The present study confirmed neuronal activation in the MeA following 30-min restraint stress. Furthermore, using double-labelling fluorescent *in situ* hybridization, we demonstrated that restraint stress induced activation of MC4R-expressing neurons, as indicated by c-*fos* mRNA induction. This suggests that melanocortin target neurons in this area are one component of stress excitatory circuits. Given the findings of rapid activation of arcuate POMC neurons by acute stress (Liu *et al*. 2007) and the arcuate-MeA melanocortin projections (O'Donohue *et al*. 1979), enhanced arcuate melanocortinergic input into the MeA during stress may contribute to neuronal activation in the MeA.

We have previously shown that acute restraint stress-induced activation of POMC neurons is accompanied by anxiogenic-like behaviours and this was attenuated by blockade of melanocortin receptors via i.c.v. injection of SHU 9119, a MC4R antagonist (Liu *et al*. 2007). The neural circuits mediating the central effects of melanocortin signalling in stress-induced anxiety behaviours are poorly understood. Several lines of evidence support a role for the MeA in modulating anxiety-related behaviours. Various pharmacological manipulations in this region have been shown to modulate anxiety states (Duxon *et al*. 1997; Ebner *et al*. 2004; Forestiero *et al*. 2006; Kokare *et al*. 2005). Additionally, electrical stimulation of the MeA produces anxiogenic-like effects (Adamec & Shallow, 2000; Morgan *et al*. 1999), whereas lesions of the MeA caused anxiolytic-like effects (Blanchard & Blanchard, 1972; Luiten *et al*. 1985). In the present study, we showed that direct infusion of a MC4R agonist into the MeA caused anxiogenic effects, as indicated by decreased entries into open arms and time spent on the open arms in the elevated plus-maze test. In contrast, an intra-MeA injection of SHU 9110 blocked restraint stress-induced anxiogenic effects. These findings, together with the observation that MC4R neurons are activated by restraint stress, imply that enhanced MC4R signalling in the MeA may represent an underlying mechanism by which restraint stress exerts its anxiogenic effects.

The role of the central melanocortin system in controlling food intake and body weight is well documented (Barsh & Schwartz, 2002; Cone, 2005; Farooqi & O'Rahilly, 2004). A number of studies have largely focused on feeding effects of melanocortin signalling under basal, non-stressed conditions. We have shown that melanocortin signalling participates in emotional stress-induced anorectic effects in rodents (Liu *et al.* 2007). An i.c.v. injection of SHU 9119 blocks the reduction of food intake induced by restraint stress (Liu *et al.* 2007). It has been shown that electrical stimulation of the MeA suppresses food intake in food-deprived rats (White & Fisher, 1969). Conversely, lesions in the MeA induce excessive weight gain (Coscina *et al.* 2000; King *et al.* 2003). These findings support that the MeA exerts a regulatory effect on food intake. In the present study, we found that activation of M4R in the MeA decreased spontaneous food intake for up to 2 h and blockade of MC4R in this area attenuated restraint stress-induced anorectic effects, suggesting that MC4R signalling in this region regulates feeding. Of note, intra-MeA injection of SHU 9119 alone had no effect on food intake, which is in contrast to the orexigenic effects of SHU 9119 elicited by i.c.v. injection or intra-paraventricular nucleus (PVN) injection (Blevins *et al.* 2009; Garza *et al.* 2008; Giraudo *et al.* 1998; Liu *et al.* 2007; Seeley *et al.* 1997). These results suggest that MC4R signals in the MeA have a minimal effect on normal feeding but convey anorectic signals during stress.

Both anxiety and anorexia have been linked to abnormal HPA axis function (Licinio *et al.* 1996; Pego *et al.* 2010). Previous work has demonstrated that activation of MC4R via intra-PVN injection or i.c.v. administration of α-MSH and its analogues stimulates ACTH secretion and elevates plasma corticosterone levels (Dhillo *et al.* 2002; Lu *et al.* 2003; Ludwig *et al.* 1998; von Frijtag *et al.* 1998). In this study, we found that infusion of the MC4R agonist into the MeA increased corticosterone levels, suggesting that MC4R activation in the MeA is sufficient to stimulate the HPA axis. Blockade of MC4R by intra-MeA injection of SHU 9119 had no effect on plasma corticosterone levels under non-stressed conditions. However, SHU 9119 in the MeA attenuated restraint stress-induced increase in corticosterone levels. The significant effects of SHU 9119 were observed after 30-min exposure to restraint stress and continued for 15 min after the cessation of stress exposure. This could imply that blockade of melanocortin signalling in the MeA has little effect on the peak effect of acute restraint stress on the HPA axis, but rather accelerates the termination of an activated HPA axis.

One of the important questions that remain to be answered is what could be the downstream substrates of MC4R that mediates its behavioural and endocrine effects in the MeA. Several lines of evidence suggest that the corticotrophin-releasing hormone (CRH) is one of the candidates that relay central MC4R signals. CRH plays a critical role in mediating anxiety, feeding and HPA activation in response to stress (Bale *et al.* 2002; Vale *et al.* 1981), via activation of two receptor subtypes, CRHR1 and CRHR2 (Chen *et al.* 1993; Lovenberg *et al.* 1995; Perrin *et al.* 1995). Central melanocortin signalling has been shown to functionally interact with the CRH/CRH receptor system in modulating anxiety, feeding and HPA responses to stress. First, central administration of α-MSH up-regulates CRH mRNA levels and increases CRH release from hypothalamic explants (Fekete *et al.* 2000; Lu *et al.* 2003). Second, the anorectic effects and HPA stimulation induced by MC4R agonists can be blocked by pretreatment with α-helical-CRH9-41, a non-selective CRHR antagonist (Kamisoyama *et al.* 2009; Lu *et al.* 2003). Moreover, i.c.v. injection of CRH antiserum attenuates the anxiogenic effect induced by α-MSH (Vecsernyes *et al.* 2000). The next question that arose is how MC4R in the MeA interacts with the CRH/CRHR system. Because of the absence of CRH neurons in the MeA, MC4R signals in the MeA must be conveyed to CRH neurons via the efferents of the MeA. One of the target regions of the MeA efferents is to the bed nucleus of stria terminalis (BNST), which contains CRH neurons (Canteras *et al.* 1995; Coolen & Wood, 1998; Roozendaal *et al.* 2009). A number of

studies have suggested that the BNST is involved in stress-elicited emotional responses and HPA activation (Choi *et al.* 2007; Forray & Gysling, 2004; Hammack *et al.* 2007; Herman & Cullinan, 1997; Herman *et al.* 2005; Lee & Davis, 1997; Walker *et al.* 2003, 2009). Stress activates CRH neurons in the BNST (Chappell *et al.* 1986; Stout *et al.* 2000), which can, in turn, excite CRH neurons in the PVN via a monosynaptic pathway (Dong *et al.* 2001). Moreover, activation of CRH receptors in the BNST induces anxiety-like behaviour (Lee & Davis, 1997; Sahuque *et al.* 2006) and blockade of CRH receptors in this area suppresses stress-induced anxiety (Cooper & Huhman, 2005). In addition, stress-induced inhibition of feeding behaviour has been linked to the BNST (Ciccocioppo *et al.* 2003; Ohata & Shibasaki, 2011). These findings suggest that CRH neurons in the BNST may function as a downstream neural substrate of MeA MC4R signals in the regulation of behavioural and endocrine responses to stress. The MeA also sends efferent projections to the immediate area surrounding the PVN, an area containing GABAergic interneurons (Herman *et al.* 1996). Given the direct synaptic connection between GABAergic inputs and CRH neurons in the PVN (Cullinan, 2000; Miklos & Kovacs, 2002), MC4R signalling in the MeA may influence the HPA output via interneuronal projections to CRH neurons in the PVN. CRH neurons in the PVN are not only essential to regulate the HPA axis function, but also affect food intake (Bale *et al.* 2002; Brady *et al.* 1990; Menzaghi *et al.* 1993; Suemaru *et al.* 1986; Vale *et al.* 1981).

However, MC4R signals in the brain sites other than MeA may also participate in stress responses. Indeed, we noted that the effective dosage of intra-MeA injection of SHU 9119 for attenuating stress-induced anorectic and anxiogenic effects is higher than that of i.c.v. administration used in our previous work (Liu *et al.* 2007). The i.c.v. injections allow the drugs to access their receptors in multiple brain regions. MC4R in these brain sites may act in concert to regulate stress responses, thus leading to a greater effect than a single brain site. Alternatively, we cannot exclude the possibility that diffusion of SHU 9110 from the MeA injection site into other areas may contribute to the observed effects. Also, the quality of the peptide compounds may vary between different batches or with time. Further studies are required to identify the exact neural circuits and downstream mechanisms underlying MC4R-mediated behavioural and endocrine responses to stress.

Clinical studies have shown that anxiety disorders are common in people with eating disorders, including anorexia nervosa (Bulik *et al.* 1997; Deep *et al.* 1995; Godart *et al.* 2000). Dysregulation of the HPA axis is a common pathological feature of anxiety and eating disorders (Licinio *et al.* 1996; Pego *et al.* 2010). Stress can result in an overactive HPA axis and trigger disordered eating and affective disorders such as anxiety. The present study provides evidence that melanocortin signalling in the MeA is involved in the regulation of emotional and feeding behaviours and endocrine responses to stress and suggests that abnormal melanocortinergic activity in the MeA may contribute to the pathophysiology and pathogenesis of anxiety and anorexia nervosa.

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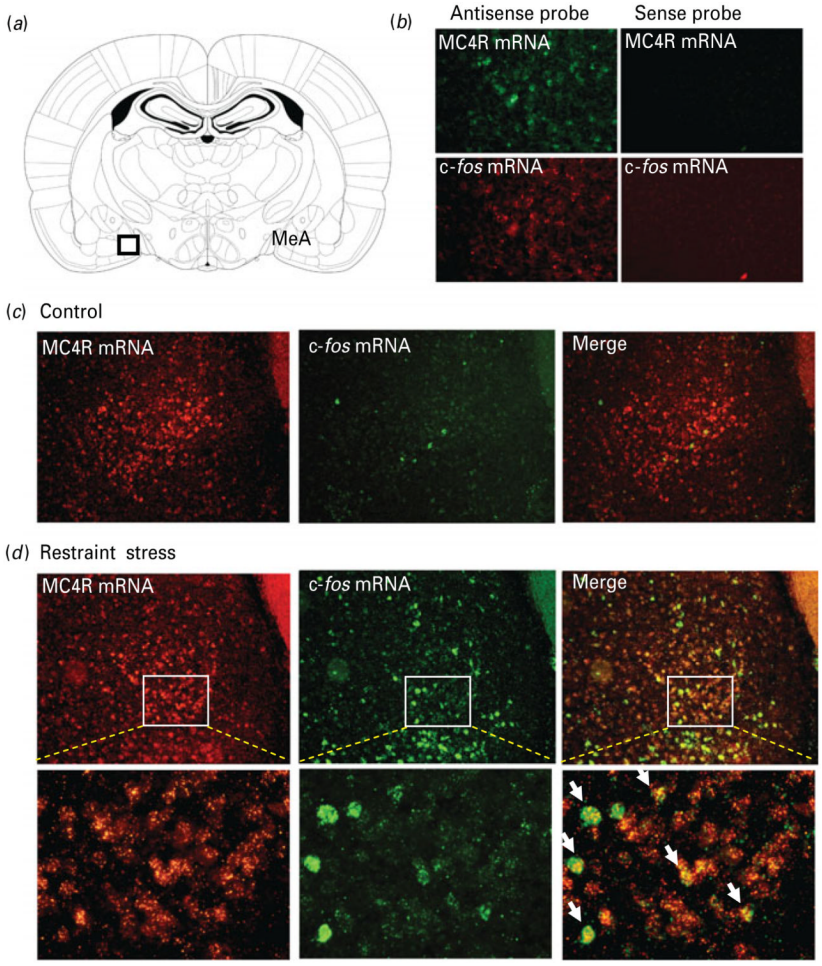

Fig. 1. Acute restraint stress activates melanocortin-4 receptor (MC4R)-expressing neurons in the medial amygdala

Double-labelling fluorescent *in situ* hybridization showing the co-localization of c-*fos* mRNA (green) with MC4R mRNA (red) in the medial amygdala (MeA). (*a*) Schematic diagram of coronal brain section through the amygdala (Paxinos & Watson, 1998); (*b*) double-labelling fluorescent *in situ* hybridization using sense and anti-sense cRNA probes to detect expression of MC4R and c-*fos* mRNA in rat MeA; (*c*) representative images showing c-*fos* mRNA and MC4R mRNA expression in the MeA from a naive rat; (*d*) representative images showing the co-localization of c-*fos* mRNA and MC4R mRNA expression in the MeA from a rat exposed to 30-min restraint stress. The white arrowheads indicate cells double-labelled for c-*fos* mRNA and MC4R mRNA.

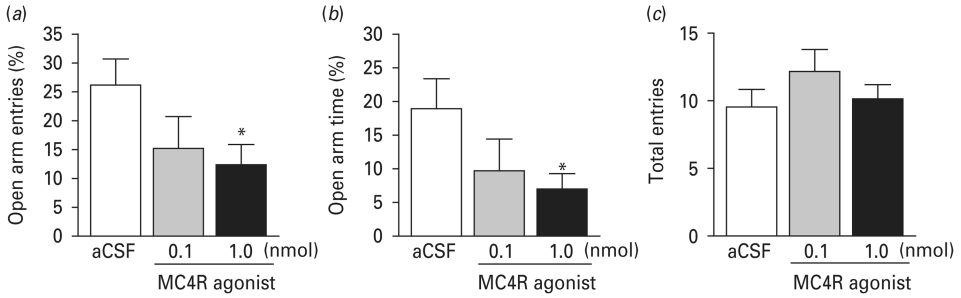

Fig. 2.

Activation of melanocortin-4 receptor (MC4R) in the medial amygdala induces anxiogenic-like effects. Rats received intra-MeA microinjection of the MC4R agonist (0, 0.1 or 1 nmol) 1 h before the elevated plus-maze test. The number of entries made into the open arms and closed arms and time spent on the open arms and closed arms were measured. (*a*) Percentage of open arm entries = number of entries made onto open arms/total number of entries made onto both open and closed arms ×100; (*b*) percentage of open arm time = time spent on open arms/total time spent on both open and closed arms ×100; (*c*) total number of entries made onto both open and closed arms. Vehicle [artificial cerebrospinal fluid (aCSF)] treatment, n =11; 0.1 nmol MC4R agonist, n=6; 1.0 nmol MC4R agonist, n=7. Data are expressed as means±S.E.M. * p<0.05 compared to aCSF controls.

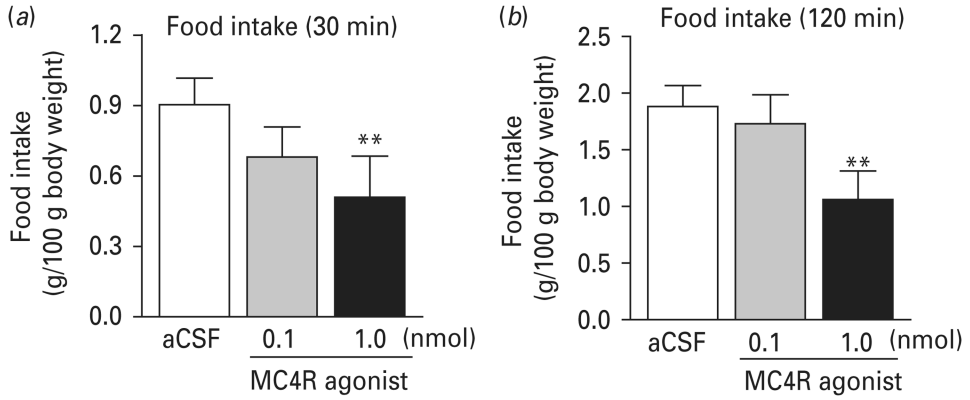

Fig. 3.

Activation of melanocortin-4 receptor (MC4R) in the medial amygdala induces anorectic effects. The MC4R agonist (0, 0.1 or 1 nmol) was infused into the medial amygdala 1 h before the dark cycle. Food was provided at the onset of dark cycle. Food consumption was measured 30 min (*a*) and 120 min (*b*) after the onset of dark cycle. Data are expressed as mean±S.E.M. Vehicle [artificial cerebrospinal fluid (aCSF)] treatment, n=8; 0.1 nmol MC4R agonist, n=6; 1.0 nmol MC4R agonist, n=7. **p<0.01 compared to aCSF controls.

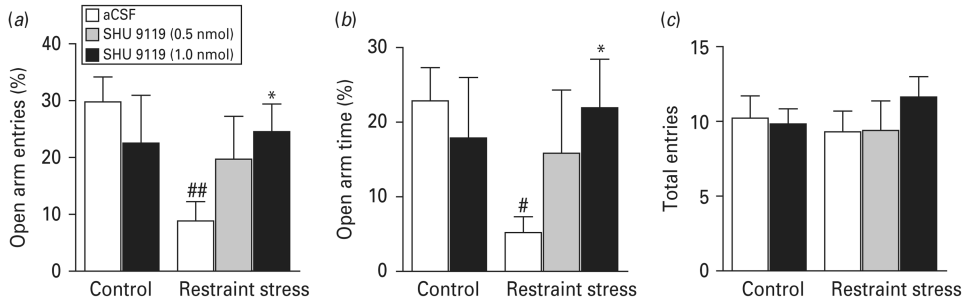

Fig. 4.

Blockade of melanocortin-4 receptor (MC4R) in the medial amygdala attenuates restraint stress-induced anxiogenic effects. SHU 9110 (0, 0.5 or 1.0 nmol) was infused into the medial amygdala 30 min prior to exposure to restraint stress. The elevated plus-maze test was performed following 30-min restraint stress. *(a)* Percentage of open arm entries = number of entries made onto open arms/total number of entries made onto both open and closed arms × 100; (*b*) percentage of open arm time = time spent on open arms/total time spent on both open and closed arms × 100; (c) total number of entries made onto both open and closed arms. Vehicle treatment without stress, n=9; 1.0 nmol SHU 9110 without stress, n=6; vehicle treatment with stress, n=10. 0.5 nmol SHU 9110 with stress, n=5; 1.0 nmol SHU 9110 with stress, n=11. Data are expressed as means ± S.E.M. # p<0.05, ## p<0.01 compared to vehicle-treated controls without stress. * p<0.05 compared to vehicle-treated controls with stress.

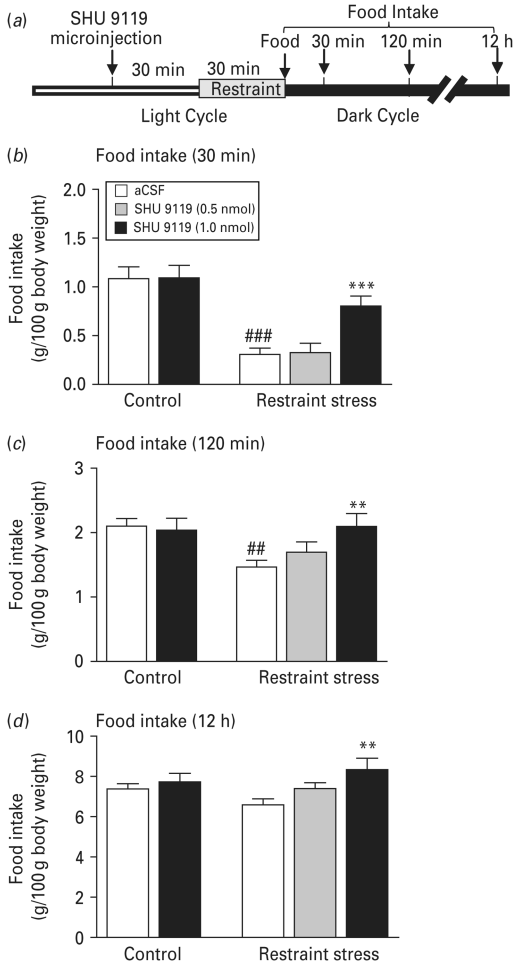

Fig. 5.

Blockade of melanocortin-4 receptor (MC4R) in the medial amygdala attenuates restraint stress-induced anorectic effects. (*a*) Timeline of the experimental procedure. SHU 9110 (0, 0.5 or 1.0 nmol) was infused into the medial amygdala 1 h before the dark cycle. Restraint stress was applied to animals 30 min after microinjection. Food was provided at the onset of dark cycle. Food consumption was measured 30 min (*b*), 120 min (*c*) and 12 h (*d*) after food was provided. Vehicle treatment without stress, n=12; 1.0 nmol SHU 9110 without stress, n=10; vehicle treatment with stress, n=14; 0.5 nmol SHU 9110 with stress, n=6; 1.0 nmol SHU 9110 with stress, n=11. Data are expressed as means±S.E.M. ## p<0.01, ### p<0.001 compared to vehicle-treated, non-stressed controls. ** p<0.01, *** p<0.001 compared to vehicle-treated controls with the same stress exposure.

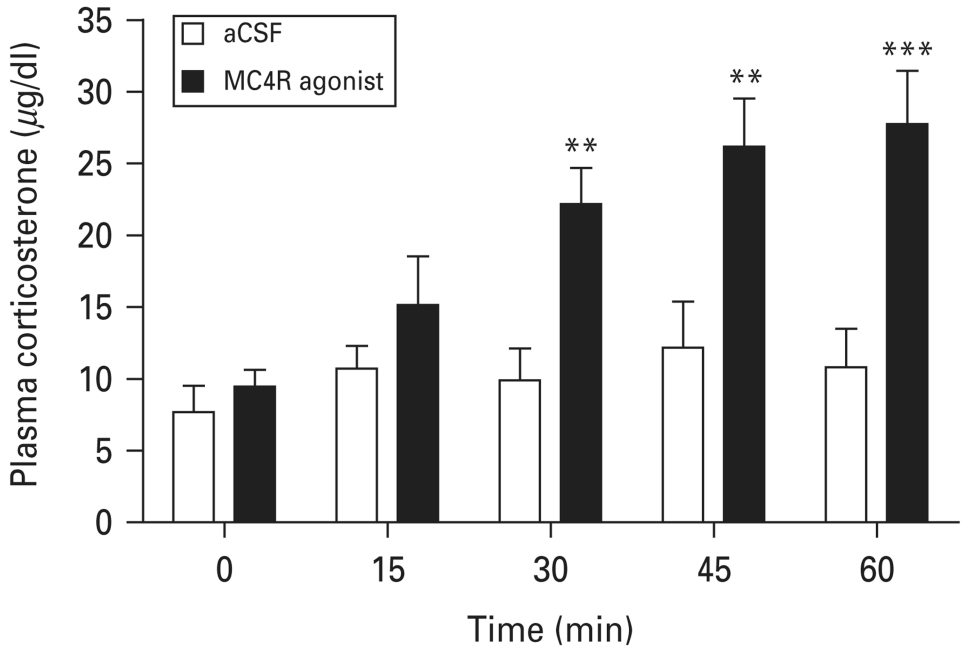

Fig. 6.

Effects of activation of melanocortin-4 receptor (MC4R) in the medial amygdala on the time course of plasma corticosterone levels. Blood samples were collected from the jugular vein before (baseline) and after infusion of the MC4R agonist (1.0 nmol) or vehicle [artificial cerebrospinal fluid (aCSF)] into the medial amygdalda at 15-min intervals. Vehicle, n=8; MC4R agonist, n=7. ** p<0.01, *** p<0.001 compared to vehicle controls.

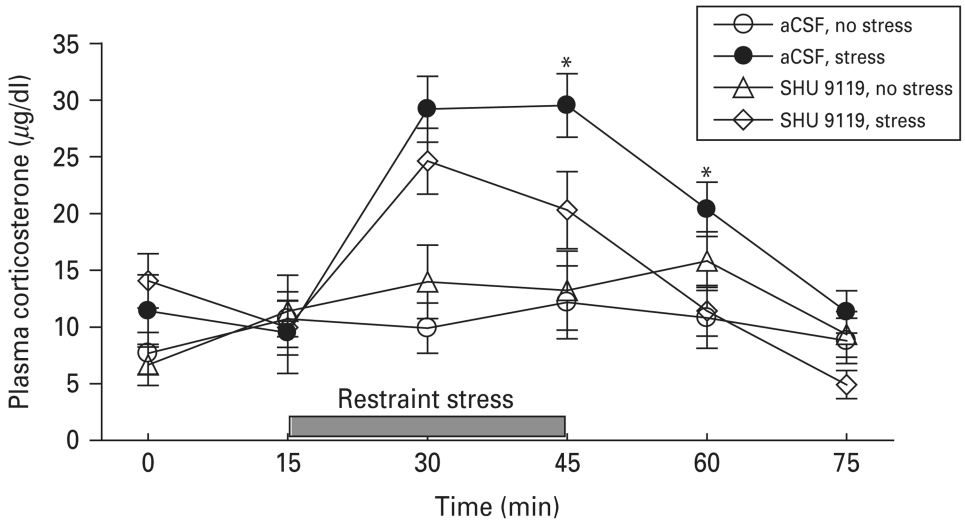

Fig. 7.

Effects of blockade of melanocortin-4 receptor (MC4R) in the medial amygdala on the time course of restraint stress-induced increase in plasma corticosterone levels. Blood samples were collected from the jugular vein before and after infusion of SHU 9110 or vehicle into the medial amygdala. Rats were subjected to 30-min restraint stress and blood samples were collected immediately before and at 15 min intervals after the initiation of restraint stress. Animals that received microinjection of vehicle or SHU 9110 (1.0 nmol) into the medial amygdala without exposure to restraint stress served as non-stressed controls. Vehicle treatment without stress, n=8; 1.0 nmol SHU 9110 without stress, n=4; vehicle treatment with stress, n=7; 1.0 nmol SHU 9110 with stress, n=9. Data are expressed as mean±S.E.M. * p<0.05 compared to SHU 9110 treatment with restraint stress.

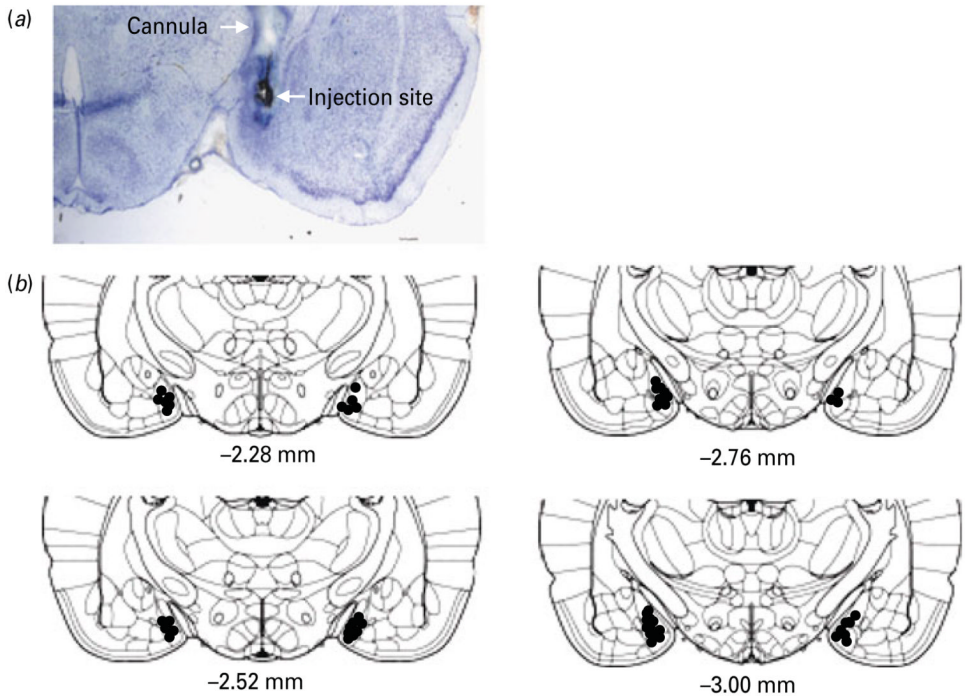

Fig. 8.

(*a*) Representative image showing dye diffusion at the injection site; (*b*) schematic drawings of coronal brain sections through the amygdala showing placements of microinjection sites in the medial amygdala. The drawings of coronal sections were derived from the atlas of Paxinos & Watson (1998).