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Effects of swim stress and α-MSH acute pre-treatment on brain 5-HT transporter and corticosterone receptor

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Abstract

The forced swim test (FST) can lead to stress-related diseases such as depression, through activation of hypothalamic-pituitary-adrenal axis (HPAA) and corticosteroid disregulation. Among the proopiomelanocortin (POMC)-derived peptides, α-melanocyte-stimulating hormone (α-MSH) has been shown to regulate long-lasting behavioral responses. Moreover, serotonergic pathways in various brain areas are activated by stressors, a feature that suggests a role for serotonin in both stress-induced HPAA disregulation and depressive physiopathology. Taking all together these data, we investigated the effects of the FST exposure and the effects of pre-treatment with α-MSH on cortical synaptosomal serotonin transporter (SERT) activity, corticosterone (CORT) plasma levels and on glucocorticoid receptor (GR) occupancy and expression in rat hippocampus. Young male rats were divided into three groups treated with saline or with α-MSH at doses of 1 or 4 μg/rat, 15 min prior to FST. Our data show that FST increased CORT secretion; GR levels in hippocampus decreased in density after stress without variations in affinity; GR redistributed from the cytosolic to the nuclear tissue fraction; finally, SERT activity strongly increased. All these effects were blocked by pre-treatment with α-MSH at the higher dose.

Keywords: Forced swim test; Serotonin; α-MSH; Corticosteroid; Glucocorticoid receptors; Stress

1. Introduction

FST, as originally proposed by Porsolt et al. (1978), is one of the few models of stress for studying depression (FST is also called the 'despair' test) and for evaluating the efficacy of antidepressant drugs in rats (Connor et al., 2000; Kelliher et al., 2003; Roche et al., 2003). Furthermore FST induces relevant behavioral and physical effects such as a strong activation of HPAA, that leads to high CORT plasma levels. Then released corticosteroids, by acting on their specific nuclear receptors, activate a long-loop negative feedback on HPAA and the stress-induced corticosteroid release disregulation which may lead to various stress-related diseases such as depression (Carrasco and Van de Kar, 2003; Chaouloff, 2000; Holsboer, 2000; McEwen, 2000; Porter et al., 2004; Van Praag, 2004).

POMC-derived neuropeptides, adrenocorticotrophic hormone (ACTH), α-MSH and β-endorphin (β-EP) are responsible for most of the stress response cascade. Among them, α-MSH showed wide physiological and behavioral activities (Cangemi et al., 1995; De Wied, 1999; Wikberg et al., 2000) and proved to regulate the long-lasting behavioral adaptation responses (O'Donohue et al., 1981; O'Donohue and Dorsa, 1982).

Furthermore several stressors induced a strong activation of the serotonergic system in different brain areas, including hypothalamus, amygdala, frontal cortex and raphe nuclei (Carrasco and Van de Kar, 2003; Chaouloff, 2000; Kawahara et al., 1993; Maswood et al., 1998; Shimizu et

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al., 1992), which suggests a role of serotonin (5-HT) in both stress-induced HPAA regulation (Carrasco and Van de Kar, 2003; Van Praag, 2004) and depressive physiopathology (Van Praag, 2004). This role is supported by the anti-depressant efficacy of the selective serotonin re-uptake inhibitors (SSRIs) that block the serotonin transporters (SERTs) (Connor et al., 2000).

The aim of our research was to investigate the effects of the FST exposure on rat cortex SERT activity, CORT plasma levels and on GR occupancy and expression in rat hippocampus and if a peripheral pre-treatment with α-MSH could modify stress-induced effects.

2. Materials and methods

2.1. Animals

Young male Sprague–Dawley rats (Charles River Italia, Calco, Lecco) weighing 200–250 g were used for the experiment, placed three per cage, and housed in an animal room (22–24 °C, 60% humidity) with food and water ad libitum and a dark/light cycle of 12/12 h, and handled daily during the week prior to the experiment. Ethical permission for the studies was granted by the Turin University bioethical committee; all animal procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Italian law regulating experiments on animals. Furthermore, all efforts were made to minimize animal suffering and to reduce the number of animals used. Rats were divided in the following groups: control group (C), non-stressed animals treated subcutaneously (s.c.) with 100 μl saline vehicle (V), 1 μg/rat (100 μl) α-MSH (T1), 4 μg/rat (100 μl) α-MSH (T2), and stressed rats (S) treated 15 min before FST according to the same treatment schedule (VS: vehicle + stress, T_1S and T_2S: α-MSH + stress). All animals were killed 30 min after injection by rapid decapitation; trunk blood was collected and centrifuged to obtain plasma samples for measurement of CORT levels; samples were stored at -80 °C until used. The brain was rapidly removed and dissected on an ice-cold plate; the hippocampus (for assessments of GR binding and cellular distribution), and cortex (for evaluation of serotonin re-uptake), were collected, immediately frozen in liquid N_2 and stored at −80 °C until used.

2.2. Chemicals

The CORT radioimmunoassay kit (Biotrack), [^{3}H]-dexamethasone (S.A. 23 Ci/mmol), nitrocellulose membrane (Hybond™ ECL™), ECL plus and [^{3}H]-5-HT (S.A. 17.8 Ci/mmol) were purchased from Amersham Italia, Cologno Monzese, Milano; the Bradford method from Biorad, Segrate, Milano; Polyclonal rabbit antibody from Santa Cruz Biotechnology, D.B.A Italia, Segrate, Milano; α-MSH and 5-HT from Sigma-Aldrich Srl, Milano.

2.3. Forced swimming test

The stress procedure we followed is the same reported by Deak et al. (2003), a slight modification of the FST previously described by Porsolt et al. (1978), Duncan et al. (1998) and Kelliher et al. (2003). Briefly, the rats were placed in a cylinder 40 cm high and with a diameter of 18 cm, water was filled to 30 cm high at a temperature of 25 °C. The animals were forced to swim for 15 min, after which they were killed by decapitation, and the tissues collected and stored as described. As a standard in the literature, after every session of FST the cylinder was freshly cleaned and disinfected prior to performing the FST on another rat.

2.4. Measurement of serotonin re-uptake

The measurement of 5-HT uptake was performed as described previously by Bonanno and Raiteri (1987), with some adjustments. Cortex samples, stored as described, were homogenized with a glass/teflon Potter in a sodium-phosphate buffer (Na_2HPO_4 80 mM, NaH_2PO_4 20 mM, pH 7.2) with 0.32 M sucrose, and centrifuged at 1000×*g* for 5 min at 4 °C. Supernatant fraction was then ultracentrifuged at 12,000×*g* for 20 min at 4 °C, and the obtained pellet (containing synaptosomes) was re-suspended with a Krebs buffer (125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM $NaHCO_3$, 1 mM NaH_2PO_4) with 10 mM glucose. The protein assay was performed according to the Lowry method (1951). Samples obtained were incubated (100 μl, with a concentration of 0.6–0.8 mg of proteins/tube) with 50 μl [^{3}H]-5-HT (0.0625 μM to 0.5 μM) and 50 μl Krebs buffer, with tubes in 37 °C water (total re-uptake) or in ice (non-specific re-uptake). After 5 min, the reaction was stopped with 15 ml of ice-cold Krebs buffer and subsequent filtration using Whatman GF/B filters. Filters were put in vials with 4 ml Biofluor (Packard) and cpm read with a Packcard liquid scintillation counter. V_{max} and K_m were obtained by Lineweaver–Burk linearization.

2.5. Plasma corticosterone levels and GR binding assay

Circulating levels of CORT were determined utilizing a rat CORT radioimmunoassay Kit from Amersham Pharmacia Biotech. All plasma samples were run in duplicate in a single assay.

Glucocorticoid receptor binding levels in cytosolic tissue preparations were measured using the procedure previously described by Miller et al. (1990), with partial modifications. Briefly, tissues from three animals of each group were homogenized with a motor-driven pestle in a buffer solution (10 mM Tris, 1 mM EDTA, 20 mM $MoNa_2O_4$, 10% glycerol, 5 mM dithiothreitol, pH 7,4) at 4 °C. The homogenate was then ultracentrifuged (105,000×*g*) for 60 min at 4 °C. The supernatant (cytosol) was incubated for 18–24 h in the presence of [1,2,4,6,7-^{3}H]-dexamethasone (0.6–10 nM) at 4 °C. Binding in the presence of excess of

corticosterone (2.5 μM) was used for determining non-specific binding. After incubation, the unbound hormone was removed by treatment with dextran-coated charcoal. Proteins were determined with the Bradford method (1976) with bovine serum albumin as a protein reference. The results obtained by Scatchard analysis were expressed as femtomoles of hormone specifically bound per milligram of cytosol protein.

2.6. GR protein cellular distribution

GR protein cellular distribution was determined by Western blotting. For Western blotting the tissues were prepared as described by Spencer et al. (2000). For the cytosolic and nuclear extract preparations, hippocampus tissues from four animals of the same group were homogenized with a hand-held dounce glass-on-glass tissue grinder in a 50 mM Tris buffer (pH 7.2) containing 6 mM $MgCl_2$, 1 mM EDTA, 10% sucrose, 1 mM phenylmethyl–sulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin. The homogenates were centrifuged for 5 min at 2000×*g* at 4 °C. The resulting supernatant and pellet were further processed to generate cytosol and nuclear extract, respectively. For the cytosol preparation, the supernatant was ultracentrifuged (105,000×*g*) for 1 h at 4 °C and the final supernatant was used as the cytosolic tissue fraction. For the nuclear extract preparation, the pellet was washed twice by re-suspension in 0.5 ml of homogenization buffer. The washed pellet was then re-suspended in a 0.25 ml homogenization buffer containing 0.5 M NaCl. This suspension was then incubated for 1 h in an ice bath with frequent vortexing. Subsequently, the tissue suspension was centrifuged (8000×*g*) for 10 min at 4 °C. The final supernatant was used as the tissue nuclear extract. Protein concentrations for each cytosolic and nuclear sample were determined according to the Bradford method (1976). For the Western blotting procedure, samples (30 μg) were mixed with a Laemmli's sample buffer and boiled for 5 min. Samples were loaded into 8% bis-acrylamide gels and separated by SDS polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred from gels to nitrocellulose membranes. Blots were blocked for 1 h in a PBS-buffered saline solution with 0.1% Tween-20 and 5% low fat milk. Membranes were incubated (4 °C) overnight with the polyclonal rabbit antibody GR (P-20), (1:500 Santa Cruz Biotechnology). Immunopositive bands were visualized by a chemiluminescent method (ECL, Amersham), captured by the Kodak Image Station 440CF system and analysed for their optical density with the Kodak 1D Image Analysis Software.

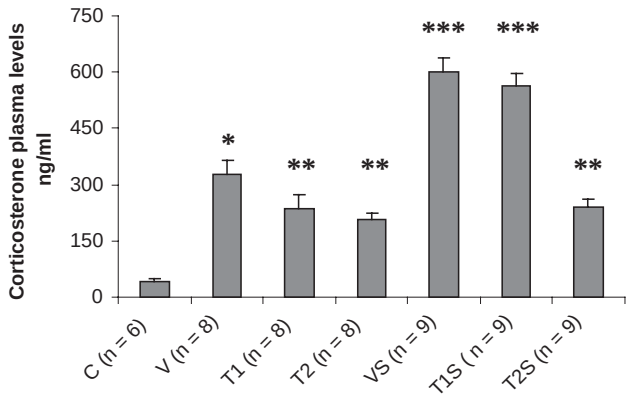

Fig. 1. Circulating levels of corticosterone in plasma samples. C=control group, V=saline vehicle, T1=1 μg/rat α-MSH, T2=4 μg/rat α-MSH, VS=vehicle+stress, T1S=1 μg/rat α-MSH+stress, T2S=4 μg/rat α-MSH+stress. Anova $p<0.001$; Neuman–Keuls $p<0.05$: *vs. C, T1, T2; **vs. C, ***vs. all groups.

Table 1
[^{3}H]-5-HT re-uptake on synaptosomal preparation from cortical areas

	V_{max} (pmol/mg prot/min)	K_m (μM)
C	2.803±0.18	1.043±0.49
V	4.071±0.55	1.098±0.72
T1	3.866±0.73	1.095±0.43
T2	2.931±0.61	0.516±0.11
VS	6.124±1.43*	1.693±0.41*
T1S	4.459±0.75**	1.177±0.82
T2S	3.454±0.42	0.875±0.24

C=control group, V=saline vehicle, T1=1 μg/rat α-MSH, T2=4 μg/rat α-MSH, VS=vehicle+stress, T1S=1 μg/rat α-MSH+stress, T2S=4 μg/rat α-MSH+stress.
V_{max}: Anova $p<0.001$; Newman–Keuls $p<0.05$: *vs. all groups; **vs. C, V, T2.
K_m: Anova $p<0.005$; Newman–Keuls $p<0.05$: *vs. T2.

2.7. Statistical analysis

The data were analysed by ANOVA followed by Newman–Keuls post hoc analysis. All data in text and figures were expressed as mean and standard deviation.

3. Results

3.1. Plasma corticosterone levels

The CORT plasma level evaluation revealed that, although the rats had been handled daily for a week, the

Table 2
GR levels on cytosolic preparations from hippocampus

	GR (B_{max}) (fmol/mg protein)	GR affinity (K_d) (nM)
C, $n=6$	89±22	0.60±0.17
V, $n=6$	77±26	1.17±0.70
T1, $n=6$	74±12	1.13±0.68
T2, $n=6$	80±16	1.05±0.59
VS, $n=6$	42±14*	1.35±0.60
T1S, $n=6$	40±18*	0.57±0.17
T2S, $n=6$	70±12	1.40±0.60

C=control group, V=saline vehicle, T1=1 μg/rat α-MSH, T2=4 μg/rat α-MSH, VS=vehicle+stress, T1S=1 μg/rat α-MSH+stress, T2S=4 μg/rat α-MSH+stress.
Anova $p<0.001$; Newman Keuls $p<0.05$: *vs. C, V, T1, T2, T2S.

saline subcutaneous (s.c.) injection significantly increased hormone levels (Fig. 1). The s.c. administration of α-MSH 1 μg/rat (T_1) or 4 μg/rat (T_2) both reduced the corticosterone plasma level increase caused by injection only. The FST strongly increased plasma CORT. The low dose of α-MSH slightly reduced while the high dose blocked FST-induced plasma CORT increases (Fig. 1).

3.2. Measurement of serotonin re-uptake

The evaluation of [^{3}H]-5-HT re-uptake on synaptosomal preparations from cortical areas revealed a stimulation of SERT activity (V_{max}) after saline treatment (Table 1). In the saline-treated rats exposed to FST (VS), a strong activation of SERT-mediated re-uptake with reduced affinity (K_m) was observed. α-MSH administration at 1 μg/rat (T_1) did not inhibit the s.c. injection procedure-inducing SERT activation, but partially reversed the FST-activated re-uptake (T_1S), while at the dose of 4 μg/rat (T_2) fully counteracted both s.c. injection stress stimulation and FST-activated re-uptake (T_2S) with an affinity (K_d) higher than that demonstrated in the controls.

3.3. GR binding assay

Binding assay determination of GR levels on cytosolic preparations from hippocampus showed a significant decrease in density (B_{max}) without substantial variations in affinity (K_d) after exposure to the FST (Table 2). Pre-treatment with α-MSH at both doses of 1 μg/rat and 4 μg/rat did not modify GR levels in hippocampus of non-stressed animals, but it was able to inhibit the effect of FST on GR binding at the higher dose (4 μg/rat).

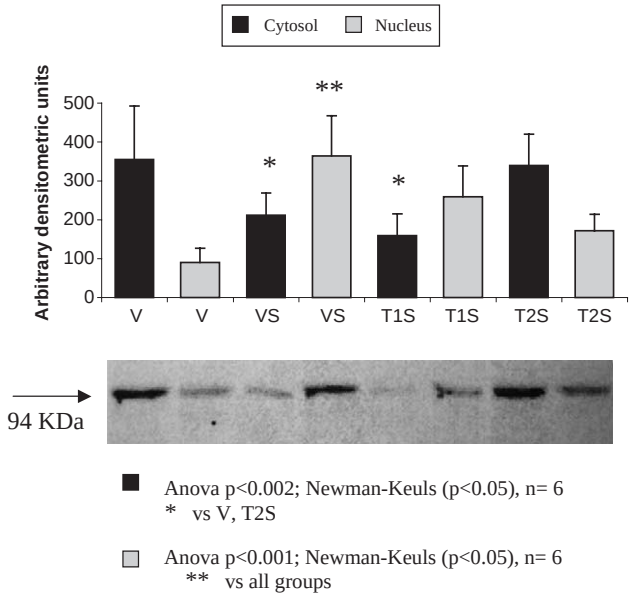

Fig. 2. Western blot analysis of GR levels in both the cytosolic and nuclear tissue fraction. V=saline vehicle, VS=vehicle+stress, T1S=1 μg/rat α-MSH+stress, T2S=4 μg/rat α-MSH+stress.

3.4. GR protein cellular distribution

The Western blot analysis of GR levels in both the cytosolic and nuclear tissue fractions from rat hippocampus showed that in basal condition, GR protein is prevalently located in cytosolic tissue fraction (Fig. 2). Exposure to FST produced a large redistribution of GR from the cytosolic to the nuclear tissue fraction. Administration of α-MSH at the dose of 1 μg/rat before exposure to FST did not affect the redistribution of GR induced by stress, while the 4 μg/rat dose induced a receptor distribution comparable to that of the control group.

4. Discussion

The rat plasma CORT determination was a highly sensitive stress parameter, since even the s.c. injection of saline in handled rats induced a significant increase in the CORT levels. Furthermore, according to Kelliher et al. (2003), the FST proved to be a stronger stress in this regard. Both doses of α-MSH (1 and 4 μg/rat) partially inhibited increased CORT levels induced by saline, but only the higher dose blocked the effect of FST, a finding that suggests that only the higher dose activated negative feedback on stress-induced CORT release.

Assessment of SERT activity in cortical synaptosomes also revealed that SERT is a very sensitive target of stress. Even the saline injection stimulated the [^{3}H]-5-HT re-uptake, while the FST strongly activated this response. Among the few articles related to the effect of stress on SERT activity, variable responses are reported, which depend on the type of stress (Mennini et al., 1993; Watanabe et al., 1993), the brain area investigated (Martin et al., 2000; Mennini et al., 1993), or the rat strain (Martin et al., 2000). In particular, FST is a stressor reported to exert different actions: (i) an inhibitory effect on 5-HT re-uptake in some brain areas, such as the dorsal raphe nucleus, lateral septum and hippocampus (Roche et al., 2003), (ii) a stimulatory effect on 5-HT release and turnover in frontal cortex (Connor et al., 2000; Rueter and Jacobs, 1996). Our findings may seem to be in conflict with several other investigations in which the intra-cerebro-ventricular (i.c.v.) administration of ACTH or α-MSH in rat discrete hypothalamic areas induced anxiety, aggression, grooming, stretching, yawning and stimulated sexual behavior (Argiolas et al., 2000; Gonzalez et al., 1996; Chaki et al., 2003), while the i.c.v. administration of a selective MC4-R non-peptide antagonist (MCL0129) reversed the anxiogenic and depressive behavior induced by FST (Chaki et al., 2003). This discrepancy may be explained by the different α-MSH administration procedure, i.e., i.c.v. or s.c.. I.c.v. administration implies a direct and immediate effect on selected brain areas. S.c. administration 15 min before FST, instead, is able to activate a generalized feedback mediated and delayed effect, since α-MSH crosses the blood–brain

barrier (Banks and Kastin, 1995) and, as an hormone, acts on pituitary and hypothalamus.

Moreover the high CORT plasma levels induced by FST may influence the neuronal 5-HT system at various levels. Some papers have shown a down-regulation in hippocampal SERT binding of young rodents, but not in middle-aged and older groups. These data suggest that an increased serotonergic activity may be present in young rats exposed to high CORT levels, while in older animals no such alteration in SERT binding sites would occur, with a consequent decrease of serotonergic function (Maines et al., 1999; Slotkin et al., 1996). A slow release CORT treatment did not modify cortical SERT binding, but enhanced 5-HT turnover (Bush et al., 2003). On the other hand, an increase in SERT expression in immortalized human B-lymphoblastoid cells has been described (Glatz et al., 2003).

In our experiment the cortical SERT activation induced by saline injection or, at a higher degree, by FST, is partially counteracted by α-MSH administration at both doses. The inhibitory effect of α-MSH on stress-induced SERT activation seems to be different from the CORT plasma level rise inhibition, since the higher dose (4 μg/rat) only inhibits stress-induced increase of CORT plasma concentration, while both doses (1 μg and 4 μg/rat) significantly inhibit stress-induced cortical SERT activation.

Together with the stress-induced increase of plasma CORT, a reduction in hippocampal cytosolic GR binding sites was evidenced.

The hippocampus is a target of stress hormones and it is known to contain high levels of GRs (Fuxe et al., 1985). It is likely that maintenance of these receptors is critical for a normal neuronal function in such a significant region of the brain (Jacobson and Sapolsky, 1991). Although it is expected that the elevated basal level of CORT would occupy more receptors in the brain, the cytosolic GR binding assay does not enable us to establish if the decrease of GR density in hippocampus reflects a major change in receptor occupancy or a rapid down-regulation of GRs. Western blot analysis indicated that the decrease in cytosolic GR observed after FST can be accounted for by the activation of GR. In fact, while the exposure to stress decreased cytosolic GR, there was a concurrent increase in GR levels in the nuclear fraction. These results are consistent with our Western blot analysis of GR in the two specific cellular compartments after acute CORT administration in rats (data not shown) and with Western blot studies of GR in various cell lines after acute glucocorticoid treatment which also find a redistribution of GR from the cytosolic to nuclear fraction (Meyer and Schimdt, 1997; Sanchez, 1992).

Finally, our data show that α-MSH pre-treatment just prior to FST blocked every effect of stress on GR if the dose of 4 μg/rat was injected. Several studies have documented the effects of α-MSH on HPAA and neuroendocrine function in rodents and monkeys. I.c.v. injection of α-MSH reportedly attenuated the CORT response to neurogenic stress in rats (Milligan et al., 1998). Recently it was shown that an α-MSH antagonist agouti-related protein (AGRP), when administered centrally, stimulates ACTH, cortisol and prolactin in monkeys; these stimulatory effects are blocked by infusion of α-MSH (Xiao et al., 2003). In addition, to demonstrate neuroendocrine effects of exogenous α-MSH infusion, several studies provided evidence that endogenous α-MSH participates in neuroendocrine regulation. I.c.v. administration of an α-MSH antiserum was shown to enhance IL-1β-induced stimulation of ACTH and CORT release and basal and stress-induced prolactin secretion, a feature that supports a physiological role for endogenous α-MSH in these processes (Khorram et al., 1984; Papadopoulos and Wardlaw, 1999). The observation that the peptide is able to block the effect of IL-1β on HPAA activity, but does not interfere with the stimulation of ACTH release by CRH infusion, is consistent with a hypothalamic effect of α-MSH on the HPAA. The distribution of MC-Rs in the hypothalamus supports a potential role for endogenous α-MSH in this brain region. In fact, two of the five known melanocortin receptors (MC3-R and MC4-R) are found in the hypothalamus, including the arcuate and paraventricular nucleus, areas involved with POMC and CRH synthesis and HPAA response to stress (Lyson and McCann, 1993; Mountjoy et al., 1994; Roselli-Rehfuss et al., 1993).

On the basis of our results we suggest this sequence of stress-related events: FST strongly activated 5-HT cortical terminals with increased 5-HT release. The higher 5-HT concentration into the synaptic cleft stimulated SERT activity, which increases 5-HT re-uptake. In the meantime the stress-induced HPAA activation increased CORT plasma levels. The high plasma CORT levels reached the central nervous system and took part in serotonergic pathway activation, thus increasing 5-HT release, turnover and re-uptake. Furthermore high CORT levels bound hippocampal GR, which induced cytosolic versus nuclear cellular fraction migration. A prolonged and/or repeated stress exposure might have exhausted 5-HT pathways and produced a depressive pathology (FST or 'despair' test) in which the prolonged CORT feedback activation could have led to HPAA disregulation. The α-MSH administration before FST exposure, through a feedback mechanism on pituitary and hypothalamic nuclei, inhibited the stress-induced SERT stimulation and restored the SERT basal activity. α-MSH counteracted stress-induced HPAA activation, with a normalization in CORT plasma levels and in GR regulation, and then prevented the CORT effects on serotonergic terminals. Combined, these multi-target sites of α-MSH activity support the crucial role of α-MSH to regulate the long-lasting behavioral adaptive responses to stress.

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