TEMPERATURE
2016, VOL. 3, NO. 4, 557–566
http://dx.doi.org/10.1080/23328940.2016.1166310

RESEARCH PAPER

OPEN ACCESS

Potentiation of Ecstasy-induced hyperthermia and FAT/CD36 expression in chronically exercised animals

Sandra L. Hrometz[a], Jeremy A. Ebert[b], Karen E. Grice[b], Sara M. Nowinski[c], Edward M. Mills[c], Brian J. Myers[d], and Jon E. Sprague[e]

[a]Department of Pharmaceutical Sciences, College of Pharmacy, Natural and Health Sciences, Manchester University, Fort Wayne, IN, USA; [b]Department of Pharmaceutical and Biomedical Sciences, The Raabe College of Pharmacy, Ohio Northern University, Ada, OH, USA; [c]Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, TX, USA; [d]Department of Chemistry and Biochemistry, The Getty College of Arts & Sciences, Ohio Northern University; Ada, OH, USA; [e]The Ohio Attorney General's Center for the Future of Forensic Science, Bowling Green State University, Bowling Green, OH, USA

ABSTRACT

Fatal hyperthermia as a result of 3,4-methylenedioxymethamphetamine (MDMA) use involves non-esterified free fatty acids (NEFA) and the activation of mitochondrial uncoupling proteins (UCP). NEFA gain access into skeletal muscle via specific transport proteins, including fatty acid translocase (FAT/CD36). FAT/CD36 expression is known to increase following chronic exercise. Previous studies have demonstrated the essential role of NEFA and UCP3 in MDMA-induced hyperthermia. The aims of the present study were to use a chronic exercise model (swimming for two consecutive hours per day, five days per wk for six wk) to increase FAT/CD36 expression in order to: 1) determine the contribution of FAT/CD36 in MDMA (20 mg/kg, s.c.)-mediated hyperthermia; and 2) examine the effects of the FAT/CD36 inhibitor, SSO (sulfo-N-succinimidyl oleate), on MDMA-induced hyperthermia in chronic exercise and sedentary control rats. MDMA administration resulted in hyperthermia in both sedentary and chronic exercise animals. However, MDMA-induced hyperthermia was significantly potentiated in the chronic exercise animals compared to sedentary animals. Additionally, chronic exercise significantly reduced body weight, increased FAT/CD36 protein expression levels and reduced plasma NEFA levels. The FAT/CD36 inhibitor, SSO (40 mg/kg, ip), significantly attenuated the hyperthermia mediated by MDMA in chronic exercised but not sedentary animals. Plasma NEFA levels were elevated in sedentary and exercised animals treated with SSO prior to MDMA suggesting attenuation of NEFA uptake into skeletal muscle. Chronic exercise did not alter skeletal muscle UCP3 protein expression levels. In conclusion, chronic exercise potentiates MDMA-mediated hyperthermia in a FAT/CD36 dependent fashion.

ARTICLE HISTORY
Submitted 26 February 2016
Revised 10 March 2016
Accepted 10 March 2016

KEYWORDS
chronic exercise; FAT/CD36; hyperthermia; MDMA; sulfo-N-succinimidyl oleate

Introduction

3,4-methylenedioxymethamphetamine (MDMA; Ecstasy, Molly) use is associated with multiple adverse effects, including tachycardia, arrhythmia, rhabdomyolysis and hyperthermia.[1] In the rodent model, peak hyperthermia following subcutaneous MDMA administration occurs between 60 and 90 min after dosing.[2] However, dramatic increases in plasma norepinephrine (NE), non-esterified free fatty acids (NEFA) and insulin occur within 30 min of MDMA administration.[2,3] The hyperthermia resulting from MDMA administration is due to a combination of peripheral vasoconstriction (which prevents heat dissipation)[4] and activation of mitochondrial uncoupling proteins (UCPs).[5,6] UCPs are localized to the inner mitochondrial membrane that separates the mitochondrial matrix from the intermembrane space. When activated, UCPs dissipate the proton gradient across the mitochondrial inner membrane, resulting in increased proton conductance and the release of energy as heat.[7] NEFA in SKM mitochondria serve as ligand activators for UCP facilitated proton leak.[8,9] Mills et al.[5] demonstrated that mice lacking UCP3 had an attenuated hyperthermic response to MDMA.

The 35-fold increase in plasma NE levels[2] following MDMA administration leads to activation of peripheral

CONTACT Jon E. Sprague jesprag@bgsu.edu 39 College Park, Bowling Green State University, Bowling Green, OH 43403, USA
© 2016 Sandra L. Hrometz, Jeremy A. Ebert, Karen E. Grice, Sara M. Nowinski, Edward M. Mills, Brian J. Myers, and Jon E. Sprague. Published with license by Taylor & Francis.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

α_1 and β_3-adrenergic receptors (AR).[10-12] Activation of α_1AR mediates peripheral vasoconstriction.[13] Activation of β_2 and β_3 AR stimulates hormone sensitive lipase (HSL)-dependent lipolysis of adipose tissue triglycerides (TG) and liberates NEFA into the bloodstream,[14,15] which in turn activate UCPs.[8,9] Insulin stimulates translocation of FAT/CD36 to the plasma membrane surface in SKM to mediate fatty acid (FA) uptake.[16] By increasing plasma insulin and NE (to stimulate β_2 and β_3-ARs), MDMA increases available NEFA in SKM mitochondria to enhance UCP3 activity and therefore heat production. The strength of the relationship between increased plasma NEFA levels and an increased thermogenic response to MDMA has been shown previously in rats fed a high-fat diet.[17] NEFA uptake into SKM requires specific transport proteins including fatty acid translocase (FAT/CD36), plasma membrane associated fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATP 1–6).[18,19] The specific fatty acid (FA) transporter, FAT/CD36 is the major participant in FA uptake in SKM, adipose and cardiac myocytes.[20,21] mRNA and protein expression of FAT/CD36 and UCP3 has been shown to increase secondary to exercise.[22,23] In the present study, we hypothesized that FAT/CD36 and UCP3 expression would increase subsequent to chronic exercise and result in a potentiation of MDMA-mediated hyperthermia. The FAT/CD36 inhibitor, SSO (sulfo-N-succinimidyl oleate[24] was utilized to further characterize the contribution of FAT/CD36 in the hyperthermia mediated by MDMA.

Material and methods

Animals

Male Sprague-Dawley rats (Charles Rivers, Wilmington, MA, USA) weighing 175–200 g were housed in groups of two or three (cage size: 21.0 × 41.9 × 20.3cm) and given ad libitum access to standard lab chow (Harlan Teklad 22/5 Rodent Diet, Harlan, Indianapolis, IN, USA, 15% kcal fat) and water. Experiments began after a one week acclimation period. An ambient temperature of 24°C was maintained for all experiments a 12:12 hour light-dark cycle was utilized. Experimental procedures were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996) and approved by the Ohio Northern University Animal Care and Use Committee.

Drugs and chemicals

Racemic MDMA was generously donated by Dr. David E. Nichols of Purdue University (West Lafayette, IN, USA) as the HCl salt. Rabbit polyclonal α-GAPDH was generously donated by Dr. Kimberly Kline of University of Texas at Austin (Austin, TX, USA).N-hydroxysulfosuccinimide or Sulfo-NHS ester was purchased from Pierce Chemical (Rockford, IL, USA). Unless otherwise indicated, all other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO, USA). MDMA was diluted in normal saline (NS) and SSO in dimethylsulfoxide (DMSO).

Synthesis and characterization of sulfo-N-succinimidyl oleate (SSO)

Preparation of SSO was carried out using a procedure adopted from Harmon and colleagues (Harmon et al., 1991). Briefly, *N*-hydroxysulfosuccinimide was added to a flame-dried 50 mL round-bottomed (RB) flask containing a magnetic stir bar. The flask was placed under an atmosphere of argon. The compound was dissolved in dry *N,N*-dimethylformamide. Dicyclohexylcarbodiimide was added followed by oleic acid. The reaction stirred 16 h then was filtered through a medium glass fritted funnel (attached to house vacuum) into a flame-dried 250 mL RB. An inverted funnel attached to an argon line was placed above the funnel to blanket the apparatus from atmospheric moisture. The filtrate was sealed with a rubber septum and placed in an ice bath for 4 h while under argon. Distilled ethyl acetate was added via syringe over 20 min. The suspension stirred 30 min and was filtered under argon to collect the white solid on a medium fritted funnel. The funnel was transferred to an argon filled glove bag so that the solid could be transferred into a 100 mL RB flask. The white solid was evacuated ̃0.1 torr for 6.5 h to obtain 2.33 g (72% yield) of product. For the animal studies, SSO (3.29 g) was placed in an oven dried 250 mL serum bottle. 82 mLs of dimethylsulfoxide (DMSO) (anhydrous 99.9+ %) were added and were sealed under argon. For control animals, 100 mLs of DMSO (from same bottle used to prepare the SSO solution) was added to a serum bottle, which was also sealed under argon. Nuclear magnetic resonance (NMR) spectra were recorded on a 200 MHz Varian Mercury Plus instrument using residual solvent peaks as an internal reference. After storing the prepared SSO under argon in a

desiccator containing Drierite for four wk, elemental analysis was performed by Atlantic Microlab, Inc. (Narcross, GA, USA) and high-resolution mass spectral analysis was performed by Indiana University (Bloomington, IN, USA). After six wk of storage, little decomposition of material was observed by high performance liquid chromatography.

Exercise studies

Protocol for chronic exercise and sedentary experimental groups

Rats were acclimated and randomly divided into two test groups (n = 24–28): chronic exercise and non-exercise sedentary, respectively. The swim protocol for our chronic exercise training was adapted from previous literature.[25] Rats were placed in a large 132.5 L tubs (82.6 × 50.2 × 47.3cm). Once the tubs were filled with lukewarm water (36°C), the rats were not able to grasp the sides or climb out. The tubs were filled to a depth of 33 cm which allowed the rats to swim or tread water without their tails touching the bottom of the tank. Swim time for chronic exercised animals was increased gradually over a period of two wk, up to a maximum swim time of two h per day, five days per wk for six wk. Animals were towel dried and returned to their cages after swimming. Sedentary rats remained in their cages for the duration of the study. Body weights were measured weekly, prior to water exposure.

Study design

The chronic exercise (n = 6 per treatment group) and sedentary (n = 7 per treatment group) animals were randomly divided to one of four test groups: DMSO/Saline (control), SSO/Saline (SSO only), DMSO/MDMA (MDMA only) and SSO/MDMA. Animals were first pretreated with SSO (40mg/kg/day, i.p.) or its vehicle, (DMSO) daily for four days. On day four, animals were injected with either SSO or DMSO 30 min prior to MDMA (20 mg/kg, s.c.) or saline injection. Core temperatures were measured at baseline (before MDMA treatment) and every 15 min after MDMA injection for 60 min. Animals were not exercised for at least 24 hours before MDMA administration. At the conclusion of the study (60 min post-MDMA injection), animals were sacrificed. The 60 minute time frame used was a result of our previous studies demonstrating that peak hyperthermia occurs within 60 minutes of MDMA administration and that the mediators (namely NEFA and NE) are elevated within 30 minutes of treatment.[2] The gastrocnemius muscle was removed immediately and flash frozen in liquid nitrogen and cardiac puncture was performed to obtain blood. The dose and pattern of SSO administration was based upon literature of in vivo use of a closely related molecule, sulfo-N-succinimidyl palmitate.[26] Our lab performed a dose-response curve to determine an SSO dose which demonstrated changes in plasma NEFA levels 30 min after MDMA administration, without overt toxicity.

Temperature measurements

Core (rectal) temperature was measured in all animals before injections and after MDMA administration using a Thermalert TH-8 (Physitemp Instruments, Clifton, NJ, USA) monitor with a (RET-2) rectal probe attached to the thermocouple. White petrolatum (Gallipot, St. Paul, MN, USA) was applied to the probe before insertion. The probe was inserted 3 cm into the rectum while the rat was gently restrained. A steady readout was obtained within 30 s of probe insertion.

Statistical analysis

Core temperatures were compared within each treatment group with an analysis of variance (ANOVA) with a Dunnett's post hoc test, while data between the groups were evaluated by an ANOVA with a Student Newman-Keuls post hoc test at each time point. When only two groups were compared, a *t* test was used. Statistical significance was set *a priori* at $p < 0.05$.

Statistical analysis and temperature area under the curve (TAUC)

The temperature measurements were also converted to temperature area under the curve (TAUC) in a fashion similar to those described elsewhere.[27] TAUC is a composite measurement of temperature and was determined for each animal by taking the temperatures measured at baseline (time of MDMA administration) and 15, 30, 45 and 60 min post-MDMA administration. This composite measure represents the area under the curve of a plot of temperature (°C) versus time (min), and is expressed as °C x min.

Tissue preparation protocols

Preparation of mitochondrial protein lysates

Gastrocnemius samples (̃100 mg) were minced in cold CP-1 buffer (100 mM KCl, 50 mM Tris-HCl, 2 mM EGTA, pH 7.4, 4°C), homogenized and centrifuged (500 × *g* for 10 min at 4°C). Supernatant was collected, filtered (40 μm cell strainers) and centrifuged (10,500 × *g* for 10 min at 4°C). Mitochondrial pellets were collected and lysed in RIPA buffer with protease inhibitors (200 nM PMSF, 1μg/mL Leupeptin, 1μg/mL Pepstatin A, 0.1 kinetic units Aprotinin) on ice for 30 min. Samples were centrifuged (16,000 × *g* for 10 min at 4°C) to clear membranes and debris, and supernatant was transferred to a new tube.

Preparation of whole tissue protein lysates

Gastrocnemius samples (̃100 mg) were placed in an Eppendorf tube in 300μL RIPA buffer with protease inhibitors and a metal bead. Tubes were then loaded on the bead mill and homogenized at 30 shakes/s for 10 min at 4°C. Beads were removed, and samples were centrifuged at 16,000 × *g* for 10 min at 4°C, then supernatant was transferred to a new tube.

Western blot analysis

Protein lysates were normalized for protein concentration by BCA Assay (Pierce). Samples were normalized to 10 μg protein per sample for mitochondrial lysates and 30 μg protein per samples for whole tissue lysates with RIPA buffer (50 mM Tris-HCl (pH 7.5), 1% NP-40, 0.5 % Sodium Deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8) and protein sample buffer (7.6 mg/mL Tris, 20 mg/mL SDS, 0.5 mg/mL Bromophenol Blue, 2.5% Glycerol, 1.8% 2-mercaptoethanol) was added. Mitochondrial protein lysates were run on 12.5% SDS-polyacrylamide gel, while 30 μg whole tissue protein lysates were run on a 10% SDS-polyacrylamide gel and both gels were then transferred to nitrocellulose. Membranes were blocked for 24 h in 5% milk in Tris buffered saline with Tween (TBST; (10 mM Tris-Base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) at 4°C, then incubated in primary antibody overnight at 4°C. Primary antibodies were as follows: rabbit polyclonal anti-UCP3 (abcam #3477) 1:3000, rabbit polyclonal anti-Mitofusin 2 (abcam #50838) 1:1000, rabbit polyclonal anti-CD36 (abcam #36977) 1:1000, rabbit polyclonal anti-GAPDH 1:5000. Membranes were then washed, and incubated in secondary donkey anti-rabbit-HRP (GE Healthcare) 1:3000. Membranes were washed in TBST and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce). FAT/CD36 and UCP3 expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Mitofusin2 expression, respectively and compared via densitometric analysis using ImageJ software.

Non-esterified free fatty acid (NEFA) determination

Plasma non-esterified fatty acid levels were determined by the acyl-CoA synthetase – acyl-CoA oxidase (ACS-ACOD) method at the Diagnostic Laboratory of the College of Veterinary Medicine at Cornell University (Ithaca, NY). Briefly, acyl-coenzymeA (acyl-CoA) was combined with the plasma to create CoA thiol esters. Acyl-CoA oxidase was then added, generating hydrogen peroxide which, along with included peroxidase, oxidatively condensed 3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline and 4-aminoantipyrine. This created a purple adduct which allowed for measurement of non-esterified fatty acids due to maximal absorption at 550 nm.

Results

Effect of chronic exercise on body weight

After completing the six weeks of exercise, the weight of chronically exercised animals (482.22 g +/− 7.97) was significantly ($p < 0.05$) lower than sedentary animals (512.56 g +/− 9.67). Both the exercised and sedentary animals gained weight throughout the study, the exercised animals gained weight at a slower rate.

Effect of exercise and SSO on MDMA-induced hyperthermia

In both the chronic exercise and sedentary groups, MDMA administration resulted in a significantly higher core body temperature than all other treatments (control, SSO only and SSO/MDMA) at the 45 and 60 min time points. Additionally, pretreatment with the FAT/CD36 inhibitor, SSO, significantly attenuated MDMA-induced hyperthermia at 45 and 60 min for both the chronic exercise and sedentary groups (Fig. 1).

The TAUC analysis reveals MDMA-induced hyperthermia is significantly greater in the chronic exercise group compared to animals in the sedentary group ($p < 0.02$; Fig. 2). However, treatment with SSO significantly attenuated MDMA-induced hyperthermia ($p < 0.05$) in the chronic exercise group only

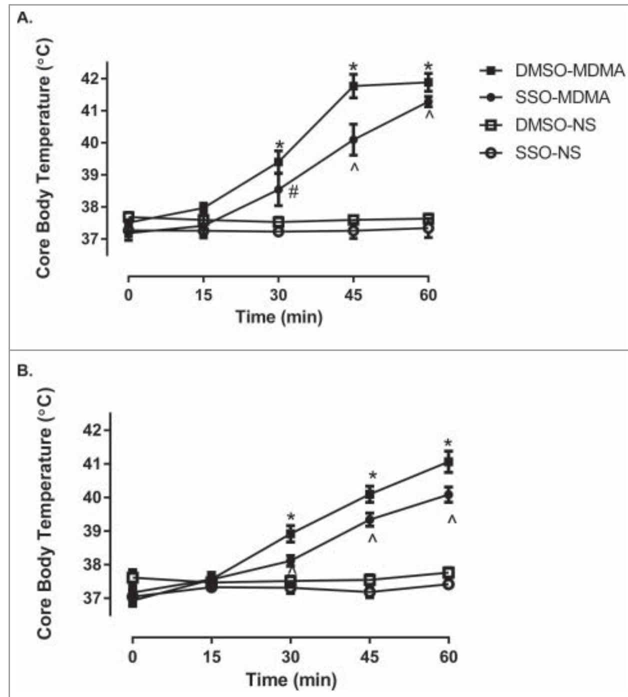

Figure 1. Core body temperature vs. time in 1A) chronic exercise (CE) and 1B) sedentary (SED) animals. Each value is expressed as the mean +/− SEM (n=6–7). *Denotes significantly greater than all other treatment groups ($p < 0.05 – 0.001$). **Denotes significantly greater than DMSO-NS (control) and SSO-NS (SSO only; $p < 0.01 – 0.001$). ^Denotes significantly greater than DMSO-NS and SSO-NS ($p < 0.001$). #Denotes significantly greater than SSO-NS ($p < 0.05$). MDMA was administered at time zero.

(Fig. 2). Core body temperatures for the control and SSO treatments were not significantly different within the chronic exercise ($p > 0.05$) and sedentary groups ($p > 0.05$). Likewise, the core body temperatures for the control ($p > 0.05$) and SSO only ($p > 0.05$) treatments were not significantly different between the sedentary and chronic exercise animals (data not shown).

Effect of exercise and SSO on MDMA-induced changes in plasma non-esterified fatty acids (NEFA)

NEFA levels were measured 60 min after MDMA administration. Overall, chronic exercise animals had significantly lower plasma NEFA levels than sedentary animals receiving the MDMA only or SSO/MDMA treatments. Specifically, compared to sedentary animals, the chronic exercise animals had significantly lower plasma NEFA levels after MDMA administration ($p < 0.002$). In the chronic exercise animals, pretreatment with SSO before MDMA (SSO/MDMA) resulted in significantly lower NEFA levels compared to the sedentary animals ($p < 0.02$). In the chronic exercise animals,

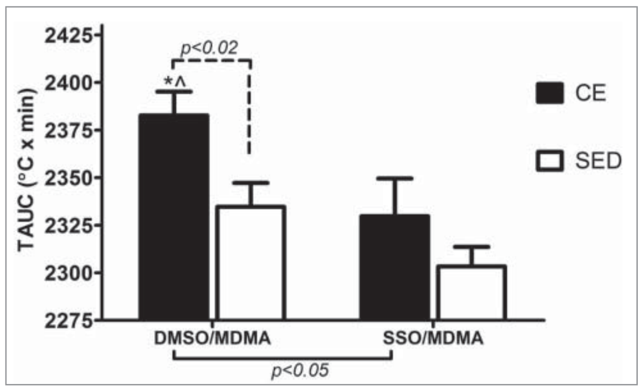

Figure 2. Total area under the curve (TAUC) through 60 min after MDMA administration for chronic exercise (CE) and sedentary (SED) training with MDMA alone and SSO/MDMA treatment. Each value is expressed as the mean +/− SEM (n = 6–7). *Denotes significantly greater than SSO/MDMA treatment in animals within the CE group ($p < 0.05$). ^ Denotes significantly greater than MDMA only in the SED group ($p < 0.02$).

NEFA levels in SSO/MDMA treatment group were significantly higher than the MDMA only treatment ($p < 0.005$) (Fig. 3). NEFA levels between chronic exercised (0.8 ± 0.9 mEq/L) and sedentary controls (0.6 ± 0.03 mEq/L) were not significantly different.

Effect of exercise on FAT/CD36 and UPC3 expression in skeletal muscle

FAT/CD36 and UCP3 protein expression in gastrocnemius muscle biopsies from 3 control animals (DMSO/NS) in the exercise and sedentary groups

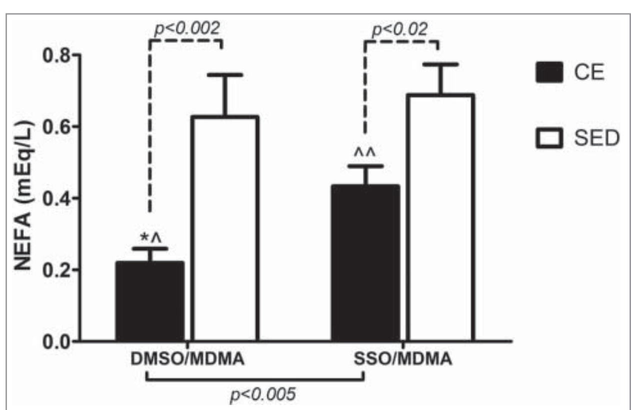

Figure 3. NEFA levels collected 60 min after MDMA administration. Values are the mean +/− SEM (n = 6 – 7). *Denotes significantly less than SSO/MDMA treatment in animals within the CE group ($p < 0.005$). ^Denotes significantly less than SED animals treated with MDMA only ($p < 0.002$). ^Denotes significantly less than SED animals treated with SSO/MDMA ($p < 0.02$). NEFA levels between the CE (0.8 ± 0.9 mEq/L) and SED (0.6 ± 0.03 mEq/L) controls (DMSO/NS) were not significantly different.

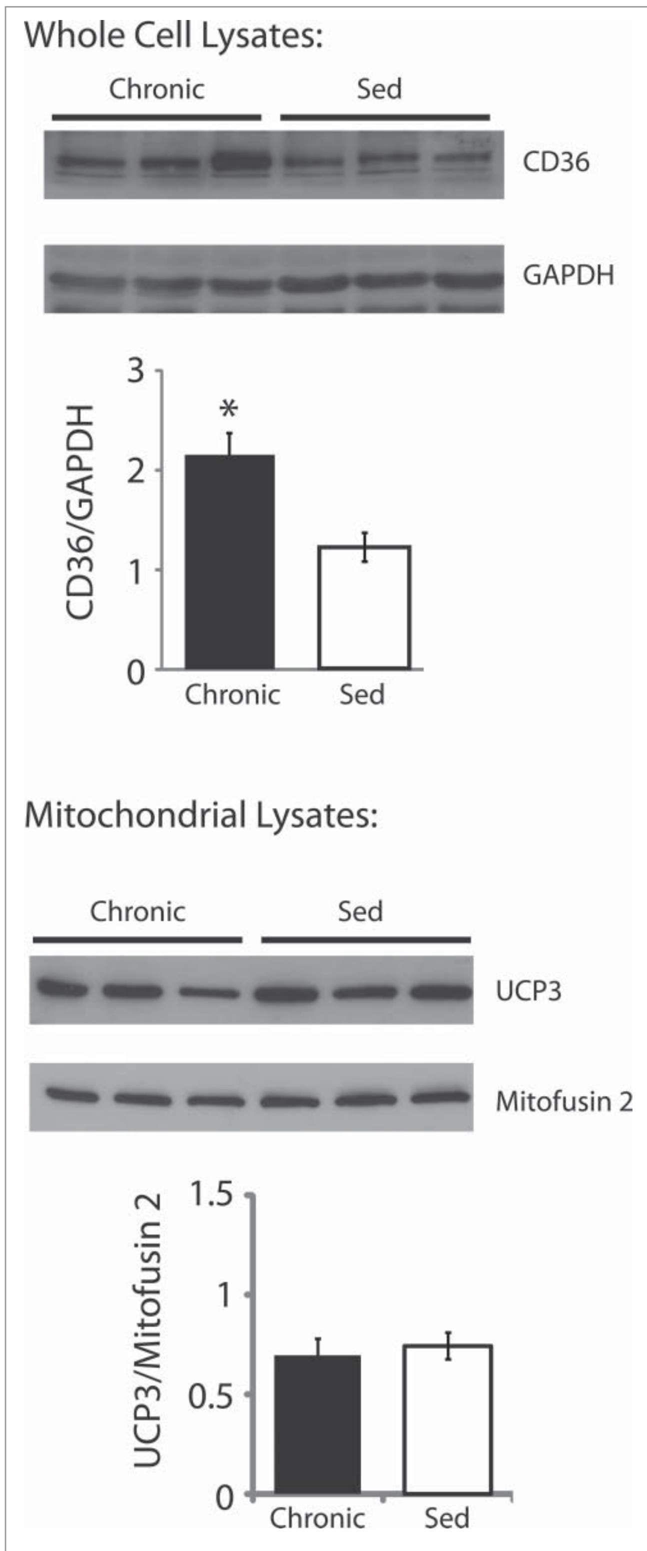

Figure 4. Western blot analysis (upper panels) of gastrocnemius skeletal muscle samples in chronic exercise (CE) and sedentary (SED) animals. Lower panels are SDS-PAGE gels that were stained for protein loading controls. N = 3 per group. 4A) CD36/FAT protein levels. 4B) UCP3 protein levels. * Denotes significantly greater than SED ($p < 0.05$).

were analyzed via western blotting. Chronic exercise resulted in a significant increase in FAT/CD36 protein expression compared to sedentary animals ($p < 0.05$) (Fig. 4A). Conversely, UCP3 protein expression was not significantly changed in either the exercise group or the sedentary control group (Fig. 4B).

Discussion

Based upon experiments using isolated mitochondrial preparations, UCP proteins have been demonstrated to require FA for their protonophoric, and therefore thermogenic functions.[8] We have previously shown that plasma FA levels increase (at 30 min) prior to peak (at 60 min) sympathomimetic hyperthermia and that a high fat diet increases both systemic FA levels and MDMA-mediated hyperthermia.[17]

White adipose tissue (WAT) is the body's largest fuel reservoir and stores lipid FA species of varied hydrocarbon chain lengths and degrees of saturation as TGs in organellar lipid droplets. FA are widely considered to be essential ligand activators of UCPs.[8] Obese Zucker rats have been shown to display a potentiated thermogenic response to MDMA.[3] In the present study, we demonstrate that chronic exercise reduced the animal's total body weight; yet, augmented the hyperthermia induced by MDMA. The finding that body weight is reduced and MDMA-induced hyperthermia is potentiated in the chronic exercise animals suggests that mitochondrial FA uptake and utilization is facilitated in the chronic exercise animals.

FAs liberated from TG in WAT are released into the circulation before being transported into SKM cells by FAT/CD36. Once in SKM, the FAs activate UCP3.[18,19] FAT/CD36 translocation from intracellular pools to the cell surface of SKM and mitochondria has been shown to be insulin sensitive.[18] Insulin signaling is vital for FA uptake into SKM, and insulin has been demonstrated to play a role in mediating the thermogenic effects of MDMA.[3]

The expression and activity of FAT/CD36 has been shown to increase with SKM contraction secondary to electrical stimulation in rats,[19,21,22,28] swim exercise in mice[31] and aerobic exercise in humans.[29,30] The types of aerobic exercise employed to increase FAT/CD36 expression included a one-time bout of stationary bike cycling for two h in male and female subjects[29] and six wk of high intensity interval training in untrained females.[30] A single bout of swim exercise until

exhaustion also increased SKM FAT/CD36 protein expression in mice.[31] Here, we demonstrate that chronic swim exercise also increases the expression of FAT/CD36 in SKM.

Previous research has shown that exercise potentiates the toxicity and hyperthermia of MDMA.[32,33,34] Reports on the effect of exercise on skeletal muscle UCP expression are inconsistent. In male Sprague-Dawley rats, a bout of 200 min of treadmill running resulted in increased expression of UCP3 in SKM.[23] This same group also subjected rats to a one time bout of swim exercise (30 min periods for up to 200 min interrupted by ten min rest periods) and reported an increase in UCP3 mRNA, but did not report a change in UCP3 protein expression.[23] In male Sprague-Dawley rats, a two wk acclimation period (15 min of treadmill running daily) followed by a single bout of treadmill running (75 to 180 min) was shown to result in UCP3 upregulation.[35] Human subjects undergoing six wk of either endurance or sprint-running training showed decreases in UCP3 expression in specific SKM fiber types. Compared to sprint training, endurance training reduced UCP expression significantly more in type I fibers and significantly less in type IIx fibers.[36] Endurance-trained runners (human) that switched to speed endurance training for four wk showed an insignificant increase ($p = 0.06$) in protein expression of SKM UCP3.[37]

In the present study, we demonstrate that swim exercise for six wk results in significantly augmented expression of FAT/CD36, but did not have an effect on the expression of UCP3 (Fig. 4). As expected, both sedentary and chronic exercise animals had significant hyperthermia following MDMA administration. However, the chronic exercise animals had potentiated hyperthermia compared to sedentary animals. We speculate that chronic exercise animals with increased expression of FAT/CD36, experienced greater uptake of NEFAs into mitochondria, resulting in a potentiated hyperthermia. This speculation is further supported by results obtained using SSO. When animals were pretreated with SSO to attenuate FA transport into SKM via FAT/CD36, MDMA-induced hyperthermia was attenuated in all animals (chronic exercise and sedentary; Fig. 1, and 2).[38,39] Additionally, NEFA data reveal information about the movement of NEFA following MDMA administration. Compared to sedentary animals, the chronic exercise animals had significantly lower plasma NEFA levels 60 min after MDMA and when MDMA was administered after SSO (SSO/MDMA; Fig. 3). These finding suggests that the increased expression of FAT/CD36 resulted in enhanced uptake of NEFA from the bloodstream and into plasma NEFA levels were significantly higher than chronic exercise animals receiving MDMA only.

A potential limitation of the present study is that our sedentary control group stayed in their home cages maintained at an ambient temperature of 24°C during the two hour swim period for the chronic exercise group. However, the acclimation of animals to an ambient temperature of 31°C for 30 days has previously been shown to result in a hypothermic response to doses of amphetamine that produced hyperthermia in animals maintained at 21°C for 30 days.[40] In order to maintain our desired thermogenic response, we maintained the sedentary control animals at an ambient temperature of 24°C.

Although FAT/CD36 expression increased with chronic exercise, cortisol could have also contributed to the thermogenic effect seen with MDMA. Chronic exercise in the form of forced swim is believed to be a source of stress and would result in the release of cortisol from the adrenal glands. Chronic stress in male Wistar rats (in the form of body restraint (6 h/day) for up to 5 wk, has been shown to increase the levels of corticotropin-releasing hormone in the anterior pituitary, with no change seen in plasma cortisol levels (Hashimoto et al., 1988). Thirty days of variable chronic unpredictable stress in male Sprague-Dawley rats has been shown to increase plasma levels of both cortisol and adrenocorticotropin hormone levels.[41] In the 30 day study, rats were randomly subjected to two different stressors per day and they included immobilization for 1 hr (both at room temperature and in a 4°C cold room), cage vibration for 1 hr, isolation in the form of individual caging for 24 hr, crowding (6 rats per cage) for 24 h, 30 min of forced swim in 26 – 30°C water or 5 min forced swim in 10 – 15°C water.[42] More recently, Johnson and Yamamoto have shown that 10 days of chronic unpredictable stress (which included twice daily stressors such as 15 or 50 min exposure to a cold room (4°C), physical restraint for 30, 50 or 60 min, cage agitation for 20, 40 or 60 min, lights on overnight, 2 or 3 hr of no lighting during the day, 3 or 4 min swim in 23°C water, isolation housing overnight and overnight food and water deprivation) increases basal plasma levels of cortisol.[43] Additionally, they show that prior exposure to 10 days of these chronic unpredictable stresses potentiates MDMA-induced hyperthermia.

The dose of SSO used only resulted in a significant decrease in MDMA-induced hyperthermia in animals with increased expression of FAT/CD36 (chronic exercise vs. the sedentary animals). Increased expression of CD36/FAT presumably allowed for increased uptake of NEFA into SKM, resulting in significantly enhanced MDMA-induced hyperthermia compared to animals with lower expression of CD36/FAT (sedentary animals). Blocking FAT/CD36 in the animals with FAT/CD36 overexpression resulted in significantly attenuated hyperthermia with MDMA, while the sedentary animals (with lower FAT/CD36 expression) did not have an attenuation in hyperthermia. This provides evidence of a more substantial effect of FAT/CD36 and NEFA uptake as a mediator of MDMA-induced hyperthermia over the effect of cortisol release in response to chronic stress.

Abbreviations

5-HT	serotonin
ANOVA	analysis of variance
AR	adrenergic receptors
ATGL	adipose triglyceride lipase
BCA	bicinchoninic acid assay
CE	chronic exercise animals
DMSO	dimethylsulfoxide
FA	fatty acid
FABPpm	plasma membrane fatty acid binding protein
FAT/CD36	fatty acid translocase
FATP	fatty acid transport proteins
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HSL	hormone sensitive lipase
MDMA	3,4-methylenedioxymethamphetamine
NE	norepinephrine
NEFA	non-esterified free fatty acid
PKA	protein kinase A
PMSF	phenylmethylsulfonyl fluoride
RIPA	radioimmunoprecipitation assay buffer
SDS	sodium dodecyl sulfate
SED	non-exercise sedentary animals
SKM	skeletal muscle
SSO	sulfo-N-succinimidyl oleate
TBST	tris buffered saline with Tween
TG	triglycerides
UCP	uncoupling protein
WAT	white adipose tissue

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

We are grateful for the generous gift of MDMA from Dr. David E. Nichols and rabbit polyclonal α-GAPDH from Dr. Kimberly KlineThis work was funded in part by a Bower, J. Bennett, H. Bennett Chair of Pharmacy Research Grant (SLH) and institutional funds by the College of Pharmacy, University of Texas at Austin (EMM).

References

[1] Rusyniak DE, Sprague JE. Toxin-induced hyperthermic syndromes. Med Clin N Am 2005; 89:1277-96; PMID:16227063; http://dx.doi.org/10.1016/j.mcna.2005.06.002

[2] Sprague JE, Yang X, Sommers J, Gilman TL, Mills EM. Roles of norepinephrine, free fatty acids, thyroid status, and skeletal muscle uncoupling protein 3 expression in sympathomimetic-induced thermogenesis. J Pharmacol Exp Ther 2007; 320:274-80; PMID:17012607; http://dx.doi.org/10.1124/jpet.106.107755

[3] Banks ML, Buzard SK, Gehret CM, Monroy AN, Kenaston MA, Mills EM, Sprague JE. Pharmacodynamic characterization of insulin on MDMA-induced thermogenesis. Eur J Pharmacol 2009; 615:257-61; PMID:19482019; http://dx.doi.org/10.1016/j.ejphar.2009.05.021

[4] Pedersen NP, Blessing WW. Cutaneous vasoconstriction contributes to hyperthermia induced by 3,4-methylenedioxymethamphetamine (Ecstasy) in conscious rabbits. J Neurosci 2001; 21:8648-54; PMID:11606652

[5] Mills EM, Banks ML, Sprague JE, Finkel T. Pharmacology: uncoupling the agony from ecstasy. Nature 2003; 426:403-4; PMID:14647371; http://dx.doi.org/10.1038/426403a

[6] Dao CL, Nowinski SM, Mills EM. The heat is on: molecular mechanisms of drug-induced hyperthermia. Temperature 2014; 1:183-91; http://dx.doi.org/10.4161/23328940.2014.985953

[7] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004; 84:277-359; PMID:14715917; http://dx.doi.org/10.1152/physrev.00015.2003

[8] Echtay KS, Winkler E, Frischmuth K, Klingenberg M. Uncoupling proteins 2 and 3 are highly active H^+ transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquione). Proc Natl Acad Sci USA 2001; 98:1416-21; PMID:11171965; http://dx.doi.org/10.1073/pnas.98.4.1416

[9] Brand MD, Esteves TC. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell Metab. 2005; 2:85-93; PMID:16098826; http://dx.doi.org/10.1016/j.cmet.2005.06.002

[10] Himms-Hagen J, Cerf J, Desautels M, Zaror-Behrens G. Thermogenic mechanisms and their control. Experientia Suppl 1978; 32:119-34; PMID:348485; http://dx.doi.org/10.1007/978-3-0348-5559-4_14

[11] Kuusela P, Rehnmark S, Jacobsson A, Cannon B, Nedergaard J. Adrenergic stimulation of lipoprotein lipase gene expression in rat brown adipocytes differentiated in culture: mediation via β 3- and alpha1-adrenergic receptors. Biochem J 1997; 321:759-67; PMID:9032464; http://dx.doi.org/10.1042/bj3210759

[12] Zhao J, Cannon B, Nedergaard J. alpha1-Adrenergic stimulation potentiates the thermogenic action of beta3-adrenoreceptor-generated cAMP in brown fat cells J Biol Chem 1997; 272:32847-56; PMID:9407062; http://dx.doi.org/10.1074/jbc.272.52.32847

[13] McDaid J, Docherty JR. Vascular actions of MDMA involve alpha1 and alpha2-adrenoceptors in the anaesthetized rat. Br J Pharmacol 2001; 133:429-37; PMID:11375260; http://dx.doi.org/10.1038/sj.bjp.0704094

[14] Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, Sattler W, Magin TM, Wagner EF, Zechner R. Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. J Biol. Chem 2002; 277:4806-15; PMID:11717312; http://dx.doi.org/10.1074/jbc.M110355200

[15] Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 2004; 306:1383-6; PMID:15550674; http://dx.doi.org/10.1126/science.1100747

[16] Luiken JJ, Dyck DJ, Han XX, Tandon NN, Arumugam Y, Glatz JF, Bonen A. Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. Am J Physiol Endocrinol Metab 2002; 282:E491-5; PMID:11788383; http://dx.doi.org/10.1152/ajpendo.00419.2001

[17] Mills EM, Weaver KL, Abramson E, Pfeiffer M, Sprague JE. Influence of dietary fats on Ecstasy-induced hyperthermia. Br J Pharmacol 2007; 151:1103-8; PMID:17533413; http://dx.doi.org/10.1038/sj.bjp.0707312

[18] Bonen A, Chabowski A, Luiken JJ, Glatz JF. Mechanisms and regulation of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. Physiol 2007; 22:15-28

[19] Campbell SE, Tandon NN, Wol; degiorgis G, Luiken JJ, Glatz JF, Bonen A. A novel function for fatty acid translocase (FAT)/CD36: involvement in long chain fatty acid transfer into the mitochondria. J Biol Chem 2004; 279:36235-41; PMID:15161924; http://dx.doi.org/10.1074/jbc.M400566200

[20] Luiken JJ, Schaap FG, van Nieuwenhoven FA, van der Vusse GJ, Bonen A, Glatz JF. Cellular fatty acid transport in heart and skeletal muscle as facilitated by proteins. Lipids 1999; 34:S169-75; PMID:10419138; http://dx.doi.org/10.1007/BF02562278

[21] Bonen A, Dyck D, Ibrahimi A, Abumrad N. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. Am J Physiol 1999; 276:E642-9; PMID:10198299

[22] Bonen A, Luiken JJ, Arumugam Y, Glatz JF, Tandon NN. Acute regulation of fatty acid uptake involves the cellular redistribution of fatty acid translocase. J Biol Chem 2000; 275:14501-8; PMID:10799533; http://dx.doi.org/10.1074/jbc.275.19.14501

[23] Zhou M, Lin BZ, Coughlin S, Vallaga G, Pilch PF. UCP-3 expression in skeletal muscle: effects of exercise, hypoxia and AMP-activated protein kinase. Am J Physiol Endocrinol Metab 2000; 279:E622-9; PMID:10950831

[24] Coort SL, Willems J, Coumans WA, van der Vusse GJ, Bonen A, Glatz JF, Luiken JJ. Sulfo-N-succinimidyl esters of long chain fatty acids specifically inhibit fatty acid translocase (FAT/CD36)-mediated cellular fatty acid uptake. Mol Cell Biochem 2002; 239:213-9; PMID:12479588; http://dx.doi.org/10.1023/A:1020539932353

[25] Hart KJ, Shaw JM, Vajda E, Hegsted M, Miller SC. Swim-trained rats have greater bone mass, density, strength, and dynamics. J Appl Physiol 2001; 91:1663-8; PMID:11568148

[26] Kusaka Y, Tanaka T, Okamoto F, Terasaki F, Matsunaga Y, Miyazaki H, Kawamura K. Effect of sulfo-N-succinimidyl palmitate on the rat heart: myocardial long-chain fatty acid uptake and cardiac hypertrophy. J Mol Cell Cardiol 1995; 27:1605-12; PMID:8523423; http://dx.doi.org/10.1016/S0022-2828(95)90524-3

[27] Miller DB, O'Callaghan JP. Elevated environmental temperature and methamphetamine neurotoxicity. Environ Res 2003; 92:48-53; PMID:12706754; http://dx.doi.org/10.1016/S0013-9351(02)00051-8

[28] Koonen DP, Benton CR, Arumugam Y, Tandon NN, Calles-Escandon J, Glatz JF, Luiken JJ, Bonen A. Different mechanisms can alter fatty acid transport when muscle contractile activity is chronically altered. Am J Physiol Endocrinol Metab 2004; 286:E1042-9; PMID:15140757; http://dx.doi.org/10.1152/ajpendo.00531.2003

[29] Holloway GP, Bezaire V, Heigenhauser GJ, Tandon NN, Glatz JF, Luiken JJ, Bonen A, Spriet LL. Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. J Physiol 2006; 571:201-10; PMID:16357012; http://dx.doi.org/10.1113/jphysiol.2005.102178

[30] Talanian JL, Holloway GP, Snook LA, Heigenhauser GJF, Bonen A, Spriet LL. Exercise training increases sarcolemmal and mitochondrial fatty acid transport proteins in human skeletal muscle. Am J Physiol Endocrinol Metab 2010; 299:E180-8; PMID:20484014

[31] Lukaszuk B, Bialuk I, Górski J, Zajączkiewicz M, Winnicka MM, Chabowski AA. Single bout of exercise increases the expression of glucose but not fatty acid transporters in skeletal muscle of IL-6 KO mice. Lipids 2012; 47:763-72; PMID:22623023; http://dx.doi.org/10.1007/s11745-012-3678-x

[32] Duarte JA, Leao A, Magalhaes J, Ascensao A, Bastos ML, Amando FL, Vilarinho L, Quelhas D, Appell HJ, Carvalho F. Strenuous exercise aggravates MDMA-induced

skeletal muscle damage in mice. Toxicol 2005; 206: 349-58; http://dx.doi.org/10.1016/j.tox.2004.07.012

[33] Tao R, Shokry IM, Callanan JJ, Adams HD, Ma Z. Mechanisms and environmental factors that underlying the intensification of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy)-induced serotonin syndrome in rats. Psychopharmacol 2015; 232:1245-60; http://dx.doi.org/10.1007/s00213-014-3759-z

[34] Zaretsky DV, Zaretskaia MV, Durant PJ, Rusyniak DE. Treadmill running restores MDMA-mediated hyperthermia prevented by inhibition of the dorsomedial hypothalamus. Brain Res 2015; 1608:75-81; PMID:25725382; http://dx.doi.org/10.1016/j.brainres.2015.02.037

[35] Jiang N, Zhang G, Bo H, Qu J, Ma G, Cao D, Wen L, Liu S, Ji LL, Zhang Y. Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function. Free Radic Biol Med 2009; 46:138-45; PMID:18977294; http://dx.doi.org/10.1016/j.freeradbiomed.2008.09.026

[36] Russell AP, Wadley G, Hesselink MK, Schaart G, Lo S, Léger B, Garnham A, Kornips E, Cameron-Smith D, Giacobino JP, et al. UCP3 protein expression is lower in type I, IIa and IIx muscle fiber types of endurance-trained compared to untrained subjects. Pflugers Arch 2003; 445:563-9; PMID:12634927; http://dx.doi.org/10.1007/s00424-002-0943-5

[37] Iaia FM, Hellsten U, Nielsen JJ, Fernstrom M, Sahlin K, Bangsbo J. Four weeks of speed endurance training reduces energy expenditure during exercise and maintains muscle oxidative capacity despite a reduction in training volume. J Appl Physiol 2009; 106:73-80; PMID:18845781; http://dx.doi.org/10.1152/japplphysiol.90676.2008

[38] Harmon CM, Luc P, Beth AH, Abumrad NA. Labeling of adipocyte membranes by sulfo-N-succinimidyl derivatives of long-chain fatty acids: inhibition of fatty acid transport. J. Membr. Biol. 1991; 121: 261-8; PMID:1865490; http://dx.doi.org/10.1007/BF01951559

[39] Luiken JJ, Turcotte LP, Bonen A. Protein-mediated palmitate uptake and expression of fatty acid transport proteins in heart giant vesicles. J Lipid Res 1999; 40:1007-16; PMID:10357832

[40] Harri MNE. Amphetamine toxicity in temperature-acclimatised mice. Acta Pharmacol Et Toxicol 1976; 38:1-9; http://dx.doi.org/10.1111/j.1600-0773.1976.tb03092.x

[41] Hashimoto K, Suemaru S, Takao T, Sugawara M, Makino S, Ota Z. Corticotropin-releasing hormone and pituitary-adrenocortical responses in chronically stressed rats. Regulatory Peptides 1988; 23:117-26; PMID:2852828; http://dx.doi.org/10.1016/0167-0115(88)90019-5

[42] Herman JP, Adams D, Prewitt C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. Neuroendocrinol 1995; 61:180-90; http://dx.doi.org/10.1159/000126839

[43] Johnson BN, Yamamoto BK. Chronic unpredictable stress augments +3,4-methylenedioxymethamphetamine-induced monoamine depletions: the role of corticosterone. Neurosci 2009; 159:1233-43; http://dx.doi.org/10.1016/j.neuroscience.2009.01.067