RESEARCH PAPER

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Reactive oxygen species scavenger N-acetyl cysteine reduces methamphetamine-induced hyperthermia without affecting motor activity in mice

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Hyperthermia is a potentially lethal side effect of Methamphetamine (Meth) abuse, which involves the participation of peripheral thermogenic sites such as the Brown Adipose Tissue (BAT). In a previous study we found that the antioxidant N-acetyl cysteine (NAC) can prevent the high increase in temperature in a mouse model of Meth-hyperthermia. Here, we have further explored the ability of NAC to modulate Meth-induced hyperthermia in correlation with changes in BAT. We found that NAC treatment in controls causes hypothermia, and, when administered prior or upon the onset of Meth-induced hyperthermia, can ameliorate the temperature increase and preserve mitochondrial numbers and integrity, without affecting locomotor activity. This was different from Dantrolene, which decreased motor activity without affecting temperature. The effects of NAC were seen in spite of its inability to recover the decrease of mitochondrial superoxide induced in BAT by Meth. In addition, NAC did not prevent the Meth-induced decrease of BAT glutathione. Treatment with S-adenosyl-L-methionine, which improves glutathione activity, had an effect in ameliorating Meth-induced hyperthermia, but also modulated motor activity. This suggests a role for the remaining glutathione for controlling temperature. However, the mechanism by which NAC operates is independent of glutathione levels in BAT and specific to temperature. Our results show that, in spite of the absence of a clear mechanism of action, NAC is a pharmacological tool to examine the dissociation between Meth-induced hyperthermia and motor activity, and a drug of potential utility in treating the hyperthermia associated with Meth-abuse.

Introduction

Heat illness, or hyperthermia, is a life threatening condition,[1,2] which can be caused by environmental exposure, infection, head trauma, central thermic dysregulation, or by drugs, including drugs of abuse such as amphetamine, amphetamine derivatives including methamphetamine (Meth) and 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), and cocaine.[3,4] In fact, hyperthermia may be the primary causa-mortis in cases of drug poisoning. Animal studies confirm that drug-related hyperthermia is lethal in some species, although other toxic effects may predominate at different drug doses or rates of administration. Hyperthermia can also lead to rhabdomyolysis, followed by kidney failure and further mortality.[5] Although hyperthermia is among the major components leading to death in association with drug overdose, documentation is highly under-represented, essentially because heat-related deaths are not easily noticed. In addition, the criteria used to determine heat-related causes of death vary, leading to underreporting or to reporting heat as a factor contributing to death rather than it being underlying cause.[5]

From 1999 to 2010, the Centers for Disease Control and Prevention reported 7,415 heat-related deaths in the United States.[5] According to a previous statistics, 65% of the heat-related deaths were due to excessive environmental heat exposure, but lacked actual documentation of hyperthermia. Of the remaining cases, about 29% were associated with external causes, which include unintentional poisonings and drug abuse overdose.[5-9] However,

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the underreported character of hyperthermia and the increase of drug abuse-related deaths may signify that the incidence is actually higher.

One study examining 250 cases of drug-related heatstroke reported that nearly 50% of the patients had a maximal recorded body temperature of 40–41°C and a 69% survival rate,[10] about 25% had a temperature of 41.1–42.1°C with a 53% survival rate, and the remaining had temperatures >42.1°C with a survival rate of only 30%.[10] The highest reported core body temperature in a patient who survived was 46.5°C (115.7°F),[11] associated with a history and clinical course consistent with acute Meth overdose.[11] Current therapeutic approaches include cardiovascular stabilization, tranquilization and external cooling of psychostimulant-poisoned patients.[3,12] Specifically, drug-related heatstroke patients require strong initial hydration to correct the drastic decrease of volemia, to maintain renal blood flow, and to prevent kidney failure.[11] Importantly, due to a poor understanding of the factors that trigger hyperthermia in drug abuse, pharmacological interventions to ameliorate acute drug-related hyperthermia are not available.

Several mechanisms may contribute to drug-induced hyperthermia. It is known that activation of particular dopamine receptors in the central nervous system (CNS) mediates, at least partially, drug-induced hyperthermia,[3,13] suggesting that dopamine-blocking neuroleptic drugs may be helpful.[3,10,13] However, the role of peripheral thermogenic sites has thus far being underappreciated. The correlation between increased core body temperature and high motor activity has led to the hypothesis that muscle activity is a source of heat in drug abuse. The use of muscle relaxants such as dantrolene sodium (DNT) to treat drug-hyperthermia has been suggested.[14] In addition, we have recently demonstrated that, in mouse, mitochondria-rich brown adipose tissue (BAT) is responsible for 40% of the Meth-induced increase in temperature.[15] Acute changes in BAT mitochondria after Meth injection, were prevented by denervation of the tissue, which was able to decrease the increment in temperature.[15] In addition, we have shown that the administration of the anti-oxidant N-acetyl cysteine (NAC), which blocks reactive oxygen species (ROS), prior to the injection of Meth, significantly delayed the onset of high temperature.[15] These findings suggested a role for Meth-induced ROS, and BAT mitochondria, in the pathogenesis of Meth-hyperthermia.

Here, we follow up on these observations, using a mouse model of acute Meth-induced hyperthermia to test the extent to which NAC may be useful to treat drug-induced hyperthermia, in perspective with other 2 potential temperature modulating agents, DNT and S-adenosylmethionine (SAMe). NAC administration before or after Meth injection reduced the degree of hyperthermia and had a positive impact in BAT by preventing Meth-induced changes in mitochondria. Surprisingly, NAC had these beneficial effects without restoring ROS or glutathione levels in BAT, and also without interfering with locomotor activity. Our results suggest that NAC is a pharmacological modulator of core body temperature and may have experimental as well as clinical potential to understand and prevent heat illness and death in acute intoxication.

Material and Methods

Mice and telemetry - All procedures were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute and were carried out on male C57BL/6J mice (20–25 g/3 months old) and maintained on regular chow diet (Harlan Teklad LM-485 Diet 7012 (58% carbohydrate [kcal], 17% fat, metabolized energy 3.1 kcal/g)). Access to food and water were *ad libitum*. For telemetry studies, mice were anesthetized with isoflurane (induction 3–5%, maintenance 1–1.5%) and surgically implanted with radio transmitter telemetry devices (TA-F10, Data Sciences Inc., Saint Paul, MN) into the peritoneal cavity for core body temperature (CBT) and motor activity (MA) measurements. Mice were allowed to recover for 2 weeks and then submitted for freely moving telemetry recording (each group n = 4–6) simultaneously with the CLAM system (see below). Mice were individually housed in clear respiratory chambers in a room maintained at 25 ± 0.5°C on a 12:12 h light–dark cycle (lights on at 6:00 a.m.). The respiratory chambers were positioned onto receiver plates (RPC-1; Data Sciences) and radio signals from the implanted transmitter were continuously monitored and recorded. The animal's CBT and MA (number of horizontal movements) were monitored with a fully automated data acquisition system (Dataquest A.R.T., Data Sciences, Inc.) for at least 72 hrs before Meth and other treatments, to ascertain that baseline levels of temperature were stable and that no ongoing febrile response confounded results. *Respiratory exchange ratio (RER)* - Indirect calorimetry was performed simultaneously with telemetry in 3–4 days-acclimated, singly housed, standard diet-fed mice using a computer-controlled, open-circuit system (Oxymax System) that is part of an integrated Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Animals were tested in clear respiratory chambers (20 × 10 × 12.5 cm) with a stainless steel elevated wire floor. Each of these chambers was equipped with a food tray connected to a balance. Room air was passed through chambers at a flow rate of ~0.5 L/min. Exhaust air from each chamber is sampled at 30-min intervals for 1 min. Sample air was sequentially passed through O_2 and CO_2 sensors (Columbus Instruments) for determination of O_2 and CO_2 content, from which measures of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were estimated. Outdoor air reference values were sampled after every 4 measurements. Gas sensors were calibrated prior to the onset of experiments with primary gas standards containing known concentrations of O_2, CO_2, and N_2 (Airgas Puritan Medical, Ontario, CA). Respiratory exchange ratio (RER) was calculated as the ratio of VCO_2 / VO_2. The caloric value (CV) was calculated using the following constant: (3.815 + 1.232xRER). This was used to calculate Heat (kcal/hr), as CV x VO_2 in liters/unit time. VCO_2 and VO_2 were normalized with respect to individual animals' body weight and corrected to an effective mass value. Mice undergoing indirect calorimetry were also acclimated to the respiratory chambers for 3–4 d before the onset of the study. Data was recorded under ambient room temperature clamped at 25°C, beginning from the onset of the 24 hr light cycle, for 3 d.

In vivo administration of Meth - Mice were exposed to a single intraperitoneal (ip) injection of endotoxin-free high purity D-Methamphetamine HCl (Alltech Associates Inc., Deerfield, IL) at a dose similar to that of human abuse (5 mg/kg)[16-18] 4 hours into the light cycle. The animals were sacrificed 1 hr, 4 hrs or 24 hrs after treatment.

In vivo treatment with Dantrolene (DNT) - Dantrolene sodium (Sigma Aldrich, St. Louis, MO) was injected ip at a concentration of 5 mg/kg alone or simultaneously with Meth, as described in other models.[19]

In vivo treatment with N-Acetyl Cysteine (NAC) - N-Acetyl- D-Cysteine (Sigma Aldrich) was administered ip1hr before, or 1 hr, 2 hrs or 4 hrs after the injection of Meth or Vehicle, at a dose of 1000 mg/kg as a 20% solution described previously.[20]

In vivo treatment with S-adenosyl methionine (SAMe) - S-adenosyl methionine (Sigma Aldrich) was administered ip 1hr before, or 1 hr after the injection of Meth or Vehicle, at a dose of 200 mg/kg in 200 ml, as described previously.[21]

In vivo detection of ROS - To detect ROS in the BAT, mice were ip injected with dihydroethidium (DHE, Cayman chemicals, Ann Harbor, MN) at 150 μg/mouse in 200 μl volume, simultaneously to Meth and BATs were harvested (see below) one hour later for imaging. Tissues were placed in 4% paraformaldehyde overnight, and then mounted on Tissue-Tek OCT freezing medium (Sakura Finetek, Torrance, CA). Thirty-micron sections were obtained in a cryostat CM1950 (Leika Biosystems, Buffalo Grove, IL). Sections were air-dried overnight in the dark, and then subjected to immunohistochemistry.

Brown Adipose Tissue Harvest - Animals were deeply anesthetized with isoflorane (induction 5%) and perfused with ice-cold PBS containing 5uM EDTA. An incision between the scapulae was performed, and the interscapular BAT was exposed and excised.

Immunohistochemistry and confocal microscopy - The harvested tissue was mounted on Tissue-Tek OCT compound (Sakura Finetech, VWR, Radnor, PA), slowly frozen on dry ice, and maintained at −80°C until use. Primary labeling was performed on sections with pre-titrated dilutions of FITC-labeled Phalloidin (Sigma Aldrich), Alexa-Fluor647-labeled anti-TOM20 (clone FL-145, Santa Cruz Biotechnologies, Dallas, TX), and 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Grand Island, NY). Thirty-micron sections were air dried in the dark, and then incubated in 10% normal goat serum (Vector) for 60 min at room temperature. Tissue was permeabilized with 0.2% Triton X-100 and 0.1 M-glycine in PBS for 7 min at room temperature. Between staining steps, segments were washed in PBS containing 0.1% Tween-20. Each section was placed on a glass slide, mounted, and pressed overnight. Sample visualization was performed with a confocal microscope (2100 Radiance; Bio-Rad Laboratories). The 40× and 63× objectives were used to collect high-resolution images with an x–y resolution of 0.15 μm and a z-distance resolution of 0.80 μm. To characterize cells, z-series optical sectioning was performed with the 40× or 63× objectives, creating image stacks that spin the thickness of the total section. z-Series image stacks were collected at 0.5 μm z-distance increments. In the z-series images, green autofluorescence and background were sorted from bright staining. Three-dimensional analysis of regions of interest was performed on ImageJ (National Institutes of Health). Quantification of structures of interest was performed using Zen Microscope software (Zeiss, Peabody, MA).

Tissue Glutathione Assay - The total GSH and GSSH content of the BAT was determined by means of Cayman's GSH assay kit (Cayman Chemical Co, Ann Harbor, MI), according to the instructions of the manufacturer. Deproteinization was performed on pre-weighted BAT tissue using 1.5 M Perchloric acid treatment, which was followed by a pH neutralization using 1M KOH.

Primary cultures of brown adipocytes - Brown adipocytes and their precursors were isolated from P0-P1 mouse pups by collagenase A digestion as described,[22] and grown to confluence for 48 h. Medium was replaced with differentiation medium containing 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin, which was changed every other day, until full differentiation was achieved (5–6 days). Differentiated brown adipocytes were plated at 10^6 cell/ml and stimulated with Vehicle, Meth (120 mM), Norepinephrine (NE −100 nM) as a control of sympathetic input, or NAC (50 mM), or a combination of Meth and NAC for up to 24 hrs.

Mitochondrial potential by JC1 labeling - Differentiated brown adipocytes were labeled by JC-1 (Invitrogen) at 600 nM for 5 min at 37°C, followed by 3 washes and a 20–30 min incubation in Krebs'–Ringer's saline containing the stimulants. Cells were then excited at 480 nm wavelength and imaged at both green (JC-1 monomers, low potential) and red (JC-1 aggregates, high potential) channels.[23] Using a dual-view module Tecan Infinite F500 apparatus (Tecan Systems, Inc., San Jose, CA) both green and red fluorescence were simultaneously acquired.

qRT-PCR - Total RNA was purified from samples using RNeasy Plus mini kit (Qiagen, Valencia, CA). cDNA was obtained using RT2 First strand kits (SABiosciences, Qiagen, Frederick, MD) following manufacturer's instructions. Primers were designed for mouse by us, or were based on studied by others.[24] Primer design was performed using the PrimerExpress software (Applied Biosystems, Foster City, CA), or through the Genescript online tool (https://www.genscript.com/ssl-bin/app/primer). Mitochondrial complexes components were evaluated using the PCR array designed by SABiosciences/Qiagen (Valencia, CA) with a focus on Mitochondrial Energy Metabolism (Catalog # PAMM-008Z). All molecules investigated were calculated into relative amounts of mRNA in the samples, by subtracting the average cycle threshold (Ct) of the primary signal for GAPDH from that for each molecule of interest to give changes in Ct (dCt). The degree of changes in expression (the differences in dCt, or ddCt) was determined by using log2 relative units. Calculations and statistics were performed using PCR Array Data Analysis Software (SABiosciences).

Statistical Analysis - Longitudinal data on temperature, motor activity, RER and calorie intake were all acquired in parallel and compared using Repeated Measures ANOVA, followed by Bonferroni's test for multiple comparisons. Multiple regression analysis was performed for all the longitudinal data. Other results were compared using one-way ANOVA, also followed by Bonferroni's test. AUC was calculated for each longitudinal variable

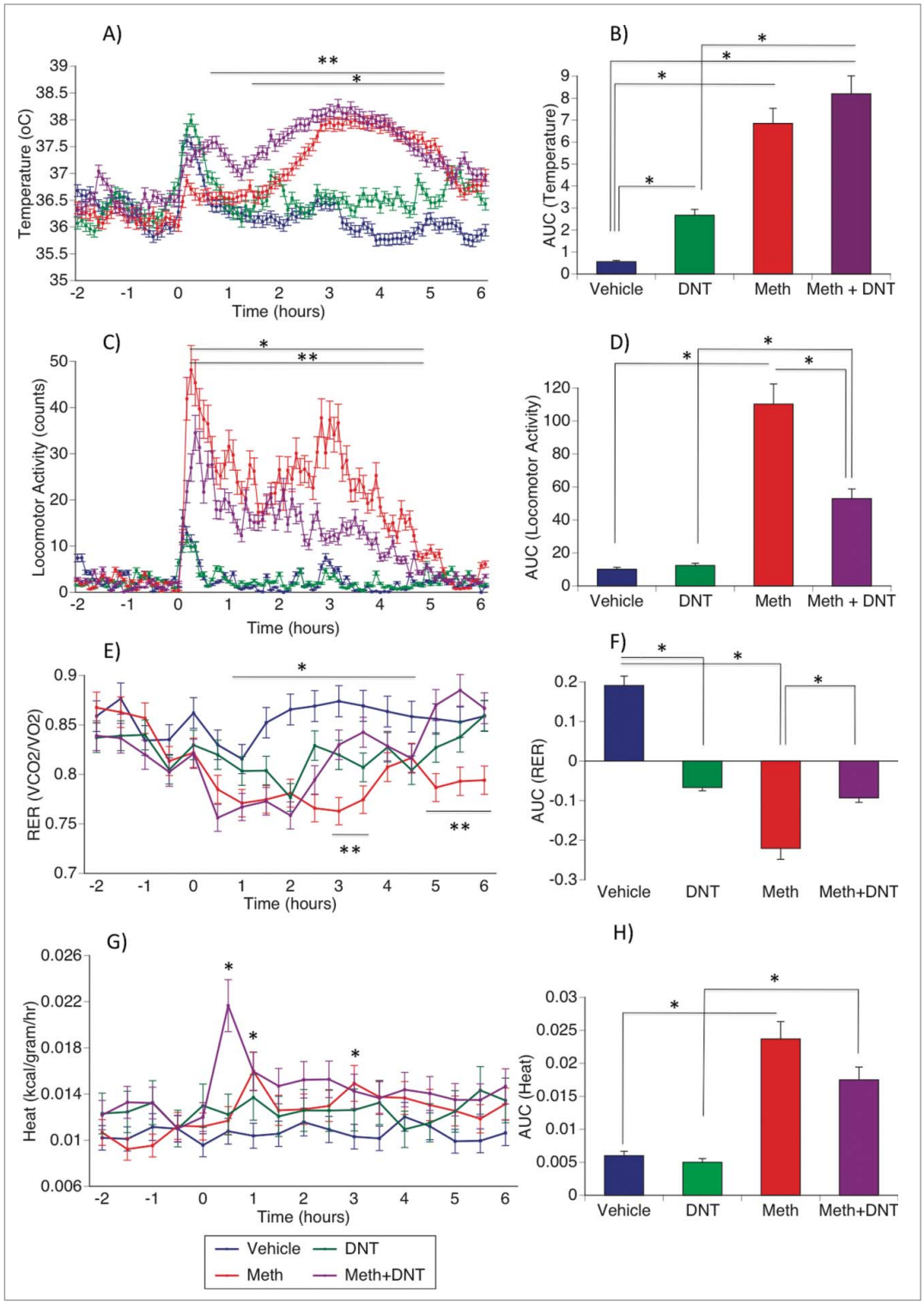

Figure 1. Effect of Dantrolene (DNT) on temperature, motor activity and RER in Meth –injected mice. Measurements were performed with the aid of telemetry, and taken at baseline (-2 to 0 hr) and over the course of 6 hrs following Meth injection. Meth was injected in C57Bl/6 mice at 5 mg/kg concentration ip (red lines and bars), in comparison to vehicle (blue lines and bars). The animals also received 5 mg/kg of Dantrolene (DNT), alone (green lines and bars) or simultaneously with Meth (purple lines and bars). (**A**) Core body temperature, (**B**) Area under the curve (AUC) for temperature, (**C**) Locomotor activity, (**D**) Calculation of the AUC of locomotor activity, (**E**) Respiratory Exchange Ratio (RER), (**F**) AUC of RER, (**G**) Heat, extrapolated from CV and body mass and (**H**) AUC for Heat. Baseline values for each variable were calculated by the average between -2 and 0, for determination of the AUC for a total of 6 hours, from time 0, using both positive and negative peaks. Values represent the Average ± SEM of one representative experiment with 4 mice/group, out of a total of 3 experiments performed.

for a total of 6 hours, from time 0, using baseline as values averaged between −2 and 0 hour time points, and taking into account both positive and negative peaks. These statistical tests were performed using Prism 5.0 Software (Graphpad software, San Diego, CA). Comparisons in qRT-PCR measurements were further performed using PCR Array Data Analysis Software (SABiosciences).

Results

A. Hyperthermia is dissociated from motor activity in mice injected with Meth

We have used an established mouse model to evaluate pharmacological approaches to reduce hyperthermia induced by the acute administration of Meth. In this mouse model, the acute administration of Meth, at sublethal concentrations that are equivalent to human abuse (5 mg/kg),[16,17] induce a transient increase in temperature to 38–39°C, starting between 1 and 2 hours, peaking at 4 hours and lasting for up to 8 hours after Meth injection.[15] This model has proven to be useful in examining the

mechanisms of thermogenesis and changes in mitochondria that are common in drug users, and in examining therapeutic strategies *in vivo*. We examined 2 drugs that target peripheral sites of thermogenesis, DNT, which decreases activity of skeletal muscles by inhibiting Calcium release channels and used as a therapy in human malignant hyperthermia,[25,26] and NAC, which has been extensively used to demonstrate the role of ROS in various processes,[27-29] and which we have previously shown to cause a decrease temperature in correlation with changes in BAT.[15]

First we examined the ability of DNT to modulate Meth-induced hyperthermia in the mouse model. As seen in **Figure 1A**, DNT did not decrease Meth-induced hyperthermia. The application of repeated measures ANOVA showed a difference between the Meth group and Meth^{+}DNT ($P = 0.001$), which was detectable between 30 minutes and 2 hours after Meth injection, when DNT had a potentiating effect on the increase of temperature caused by Meth (**Fig. 1A**). This was confirmed by the regression analysis, where the slopes between these 2 groups were significantly different ($P = 0.0079$). However, the analysis of the AUC using one-way ANOVA, followed by Bonferroni's posthoc tests did not detect an impact of DNT on the elevation of temperature caused by Meth (**Fig. 1B**). As expected, DNT significantly decreased locomotor activity as revealed by repeated measures ANOVA ($P = 0.0003$) (**Fig. 1C**), which was confirmed by the analysis of the AUC (**Fig. 1D**). Meth decreased RER in comparison to control animals, from 1 hrs to 5 hrs after injection ($p < 0$ .05). The application of repeated measures ANOVA or regression analysis to RER in DNT-treated animals, showed that DNT did not revert the decrease in RER induced by Meth, apart from apartial effect at 3–3.5 hrs after Meth, and from 5 to 6 hrs after Meth (**Fig. 1E**). The analysis of the AUC revealed that this partial effect of DNT on Meth-induced RER decrease was significant ($p = 0.027$, **Fig. 1F**). Regarding heat output, multiple comparisons applied to repeated measures ANOVA revealed that DNT increased heat production, at 30 minutes after injection, both alone or in Meth-treated animals ($p = 0.005$, $p = 0.002$, respectively), but this was followed by a return to baseline (**Fig. 1G**). The analysis of the AUC for Heat did not identify a significant impact of DNT on the heat production induced by Meth (**Fig. 1H**).

In a previous study we have shown that the administration of NAC before Meth injection was able to prevent hyperthermia, suggesting a role for ROS in the control of temperature in drug abuse. Our prior observations also linked Meth-induced hyperthermia and BAT, ROS levels in BAT, and the ability of NAC to modulate temperature. Here we tested the effect of NAC at various time points, both before and after Meth injection. Interestingly, NAC alone caused temperature to decrease significantly (**Fig. 2A and 2B**, green line and green bar). In addition, confirming what we have previously reported,[15] the injection of NAC 30 minutes prior to the injection of Meth delayed the onset and prevented the increase in temperature caused by Meth (**Fig. 2A and 2B**). Repeated measures ANOVA revealed significant differences between all the NAC-treated, Meth-injected animals when compared to Meth alone ($P < 0$ .05). A single dose of NAC 1 or 2 hrs after Meth (when temperature starts rising), or at 4hrs after Meth (when the temperature is at its peak), was able to effectively cause a transient drop of temperature, which was followed by a partial recovery ($P = 0.0001$, $P = 0.0005$, $P = 0.00098$, respectively, **Fig. 2A**). Regression analysis showed a negative slope for NAC alone (-0.4629), but interaction between NAC and Meth was not identified. In addition, the regression analysis did not identify a difference between NAC-treated Meth groups and Meth alone. However, the analysis of the Area under the curve (AUC) for temperature revealed that the NAC treatment at any time-point was able to efficiently decrease the temperature in comparison to the Meth-injected group (**Fig. 2B**). Regarding motor activity, repeated measures ANOVA showed a significant impact for Meth ($P <$ 0 .05). Importantly, NAC did not decrease motor activity in Meth-injected mice (**Fig. 2C**), by any of the applied analytical methods, suggesting that the control of temperature and motor activity are dissociated in the mouse model of Meth administration. This was confirmed by the analysis of the AUC for motor activity (**Fig. 2D**). Meth decreased RER as detected by repeated measures ANOVA ($p < 0.0001$), which was significant from 1 hour to 3 hours after Meth-injection (**Fig. 2E**). The NAC treatment, at any of the administered regimens, did not impact the decrease in RER caused by Meth, according to posthoc tests or to regression analysis (**Fig. 2E**). On the other hand, the analysis of the AUC for RER showed that the NAC treatment aggravated the decrease in RER that was induced by Meth (**Fig. 2F**). These data suggest that the alterations in metabolic substrate utilization caused by Meth were not shifted by NAC. The Meth administration caused a significant heat output in comparison to controls, according to repeated measures ANOVA ($p < 0.0001$), and detectable at 1 hour and at 3 hrs after injection (**Fig. 2G**). which was not significantly changed by NAC at any time point (**Fig. 1G and 1H**).

Overall, these results suggest that the increase in temperature caused by Meth can be modulated by NAC, but not DNT, independently from motor activity. Given that NAC was able to control body temperature, we further examined the effects of NAC in changes caused by Meth in the highly redox, mitochondria-rich BAT, a thermogenic site that was shown to contribute to Meth hyperthermia.[15]

B. NAC prevents Meth-induced mitochondrial loss in BAT but does not affect ROS efflux

We evaluated ROS levels and mitochondrial integrity in the BAT, 1 and 2 hrs after the administration of Meth in NAC-treated mice (-30 minutes). Detection of intracellular ROS is difficult due to the reactive nature and instability of these molecules. Here we utilized a system to detect ROS *in vivo* by injecting the superoxide-sensitive dye Dihydroethidium (DHE), which can permeate cell membranes and react with superoxide, forming a red fluorescent product, 2-hydroxyethidium, which intercalates with DNA.[30,31] Using this system, we detected superoxide in BAT and examined how Meth and NAC modify its distribution (**Fig. 3**). We identified ROS in the BAT of vehicle-treated animals, which was localized within TOM20+ mitochondria

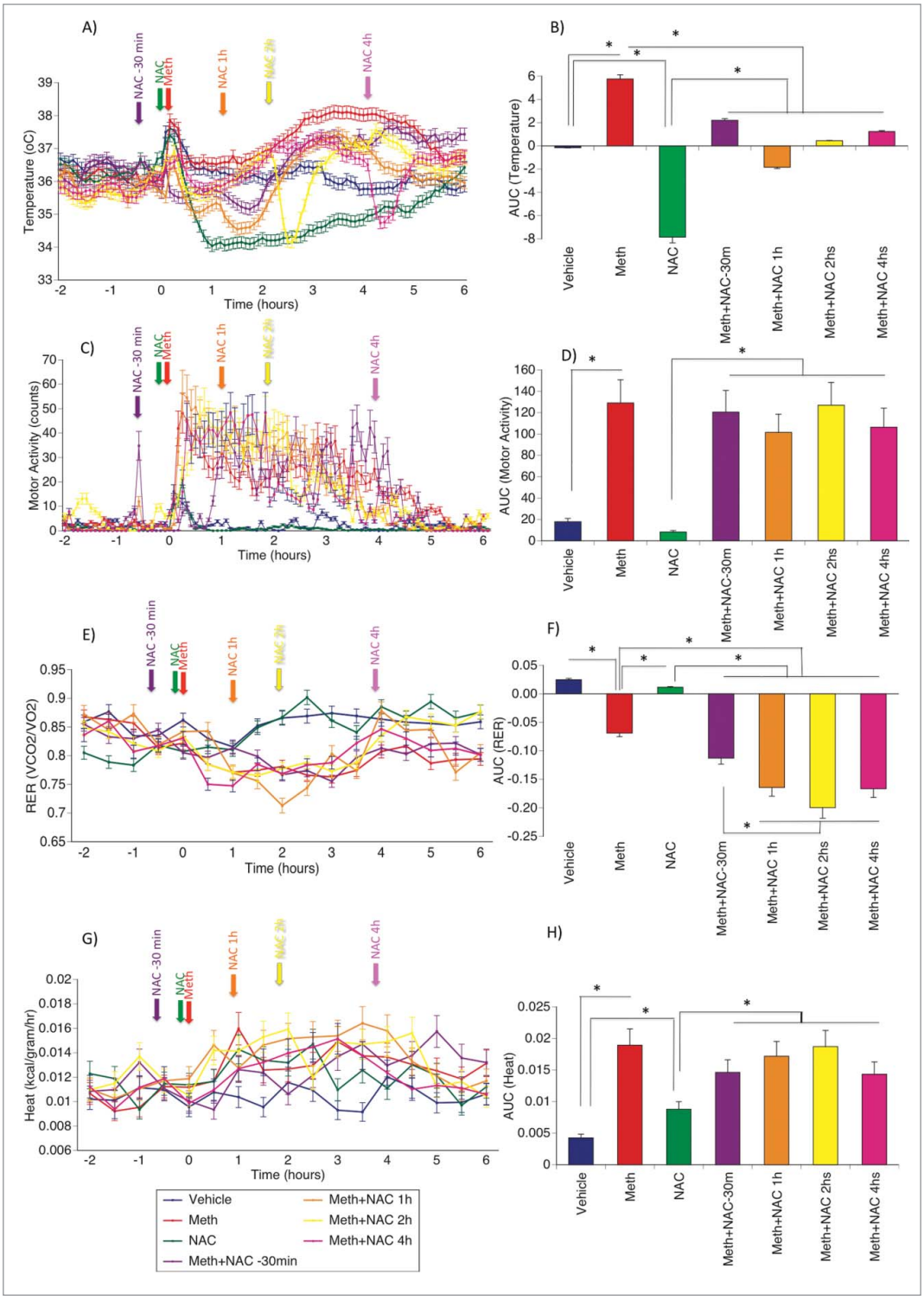

Figure 2. Effect of NAC on Meth hyperthermia, Motor activity and on RER. Mice were treated with NAC (1000 mg/kg) 30 min before, or 1, 2 or 4 hrs after Meth (5 mg/kg), and were monitored during 24 hours for Temperature and Respiratory Exchange Ratio. The figure shows the 6 hours in which the actions of Meth were observed. (**A**) Core body temperature, (**B**) Calculation of AUC for temperature, (**C**) Motor Activity, (**D**) AUC for Motor Activity. (**E**) RER, (**F**) AUC for RER, (**G**) Heat, extrapolated from CV and body mass and (**H**) AUC for Heat. Arrows indicate color-coded critical injection time-points. Red arrows indicate Meth injection (5 mg/kg), and colored arrows indicate NAC (1000 mg/kg) injection at indicated time-points. Baseline values for each variable were calculated by the average between -2 and 0, for determination of the AUC for a total of 6 hours, from time 0, using both positive and negative peaks. Values represent the Average ± SEM of one representative experiment with 5 mice/group, out of a total of 3 experiments performed. * $p < 0.05$.

(**Fig. 3C, 3D, and 3E**). Following that, we have enumerated ROS+ and TOM20+ mitochondria in tissue sections, in order to quantify the impact of NAC on ROS and on the number of these organelles (**Fig. 4**). We observed that in control animals NAC preserved ROS-loaded TOM20+ mitochondria (**Fig. 3I, 3J, 3K** and **Fig. 4A**). Meth caused a depletion of ROS-loaded organelles (P = 0.0003, **Figs. 3O and 4A**), and also a decrease in TOM20, detectable one hour after Meth (P = 0.009, **Figs. 3P and 4B**). NAC treatment of Meth-injected animals (1-hour post Meth) was not able to prevent the decrease of ROS from mitochondria that was induced by Meth (**Figs. 3U and 4A**), but it did avert the loss and even boosted the numbers of TOM20-expressing mitochondria ($p < 0$ .0001, **Fig. 3V** and 4B). Similar results were found 2 hrs after Meth (data not shown). This result indicates that NAC preserves mitochondria without affecting ROS.

C. NAC prevents Meth-induced mitochondrial stress in cultures

Because mitochondrial activity is associated with thermogenesis in BAT,[32,33] we examined in more detail the impact of Meth

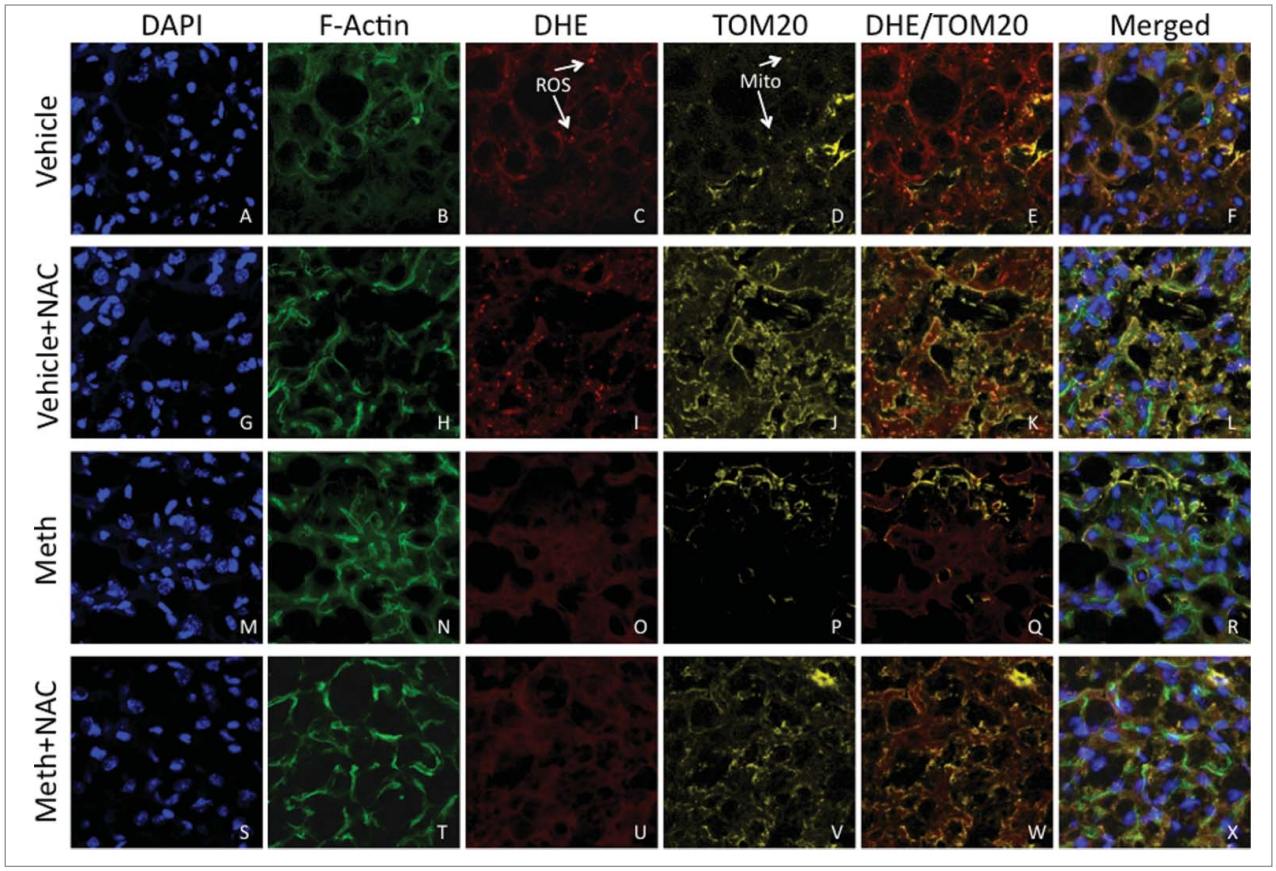

Figure 3. Effect of NAC and Meth on mitochondrial ROS and TOM20 expression. DHE was administered intraperitoneally 30 minutes before Meth (5 mg/kg), or NAC (1000 mg/kg). BAT was harvested from perfused animals 1 hour after Meth, or Vehicle, and the tissue was processed for confocal microscopy. DAPI (**A, G, M, S**), FITC-labeled Phalloidin (**B, H, N, T**), DHE (**C, I, O, U**), and AlexaFluor 647-labeled TOM20 (**D, J, P, V**) images were acquired, and then DHE was merged with TOM20 for co-localization (**E, K, Q, W**), or also with Phalloidin and DAPI (**F, L, R, X**). The pictures are flattened images from 7micron-z stacks from one representative animal in each group. Groups were injected with Vehicle (**A, B, C, D, E, F**), Vehicle+NAC (**G, H, I, J, K, L**), Meth (**M, N, O, P, Q, R**) or Meth^{+}NAC (**S, T, U, V, W, X**). White arrows in (**C**) indicate ROS-loaded organelles, and in (**D**) indicate TOM20+ mitochondria.

and NAC on mitochondria in differentiated primary brown adipocytes. Primary cultures were generated to evaluate compound-induced changes in controlled conditions, in the absence of sympathetic factors and in the absence of other cell types found in BAT, such as macrophages and endothelial cells, whose response to the drug could potentially have an impact in the thermogenic activity of brown adipocytes.

We performed JC-1 assays on differentiated brown adipocytes 24hrs after exposure to Meth and/or NE to determine whether NAC impacted the long-term induced changes in membrane potential. Given the potential role of NAC as a ROS scavenger, we examined the potential effects of Meth through ROS by adding H_2O_2 to the cultures. In addition, Meth can potentially induce mitochondrial changes indirectly through NE, as described previously.[15] Thus, we examined cultures stimulated with NE, in parallel. JC-1 permeates the membrane and exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Therefore,

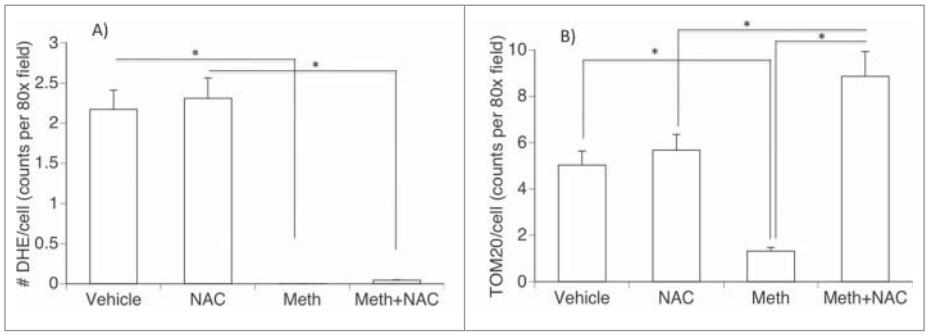

Figure 4. Impact of Meth and NAC on the number of ROS-loaded mitochondria in BAT. The numbers of (**A**) DHE+ and (**B**) TOM20+ structure organelles were obtained from flattened and normalized 80x magnification z-stack pictures using Zen image analyzer, in a fixed size range. Values represent the average ± SD of the number of structures counted by the software, and compared by One-way ANOVA, followed by Bonferroni's test. * $p < 0.05$.

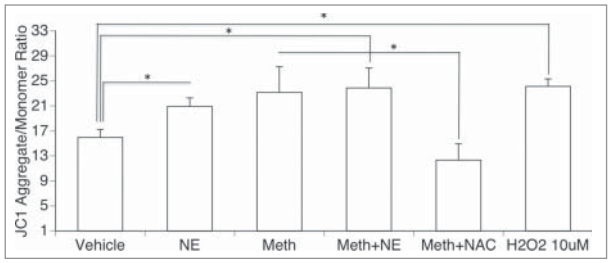

Figure 5. Effect of Meth and NAC on mitochondrial membrane potential. Meth, NE, H2O2, and NAC effects in primary brown adipocyte cultures, 24 hrs after treatment. Membrane potential was examined using JC1 as a marker of membrane polarization. Differentiated brown adipocyte primary cultures treated with Meth and/or NAC, or H2O2 for 24 hrs were treated with JC1 and fluorescence intensity was examined. Ratio between JC1 aggregates and monomers was calculated, as described in Material and Methods. ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$, bars indicate significant comparisons.

mitochondrial polarization is indicated by an increase in the red/green fluorescence ratio. We found that mitochondrial polarization was significantly increased directly by NE (One-way ANOVA, P = 0.026) and substantial but not significantly by Meth (P = 0.057)(**Fig. 5**). NAC was able to prevent mitochondrial polarization in Meth-stimulated cells (P = 0.016 compared to the Meth alone stimulation).

Next, we examined the extent to which NAC treatment affects the gene expression levels of components of inner mitochondrial membrane compartments, corresponding to Complexes I (**Fig. 6**) to V (**Fig. 7**) of the electron transport chain.[34] These components form proton carriers from the matrix to intra-membrane spaces, inducing the formation of a proton gradient, which dissipates when protons re-enter the mitochondrial matrix through complex V, also known as ATP synthase, which phosphorylates ADP into ATP. The expression levels of these components were examined in primary adipocytes stimulated with Meth, and/or NAC, using NE as a control for the effect of sympathetic input in BAT. The different gene products are indicated in X-axis of **Figs. 6 and 7**, separated in categories according to the class of components of the electron transport chain. The individual genes were compared between groups using One-way ANOVA, followed by Bonferroni's multiple comparison tests, and the significance ($p < 0$ .05) is indicated in the figures. We found that, similar to NE, Meth was able to upregulate the vast majority of components from complex I to IV in brown adipocyte primary cultures (**Figs. 6 and 7**), suggesting an action of Meth directly on brown adipocytes, in addition to the action of

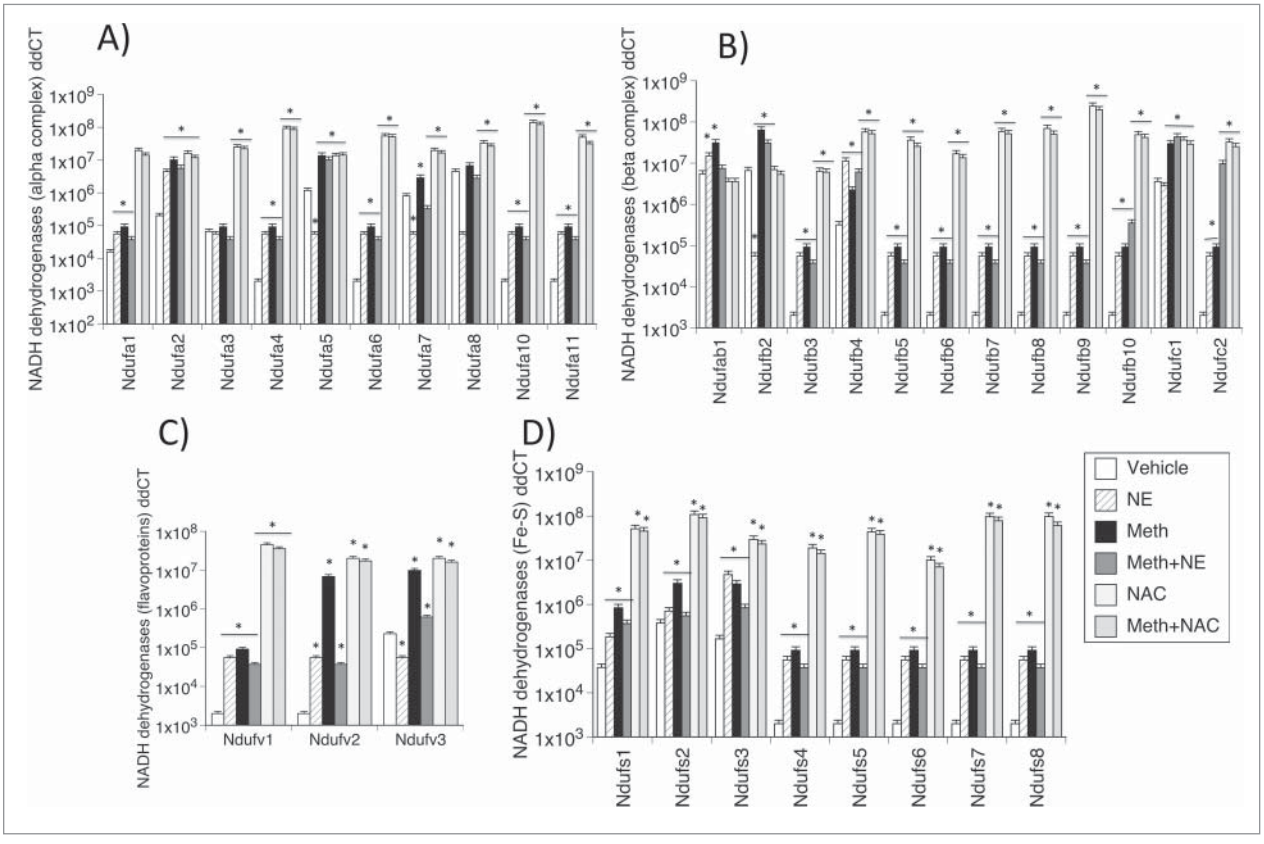

Figure 6. Effect of Meth on genes from mitochondrial Complex I. Transcriptional levels of gene components of the electron transport chain Complex I were measured by SyBrGreen qRT-PCR in primary cultures of brown adipocytes, 24 hrs after stimulation. Relative levels were normalized against GAPDH. Levels of (**A**) α complex NADH dehydrogenases, (**B**) β complex NADH dehydrogenases, (**C**) Flavoprotein-NADH dehydrogenases, and (**D**) Fe-S NADH dehydrogenases. Values represent the Average ± SEM of 3 replicates/group in 2 independent experiments. *$p < 0.05$ Compared to respective control.

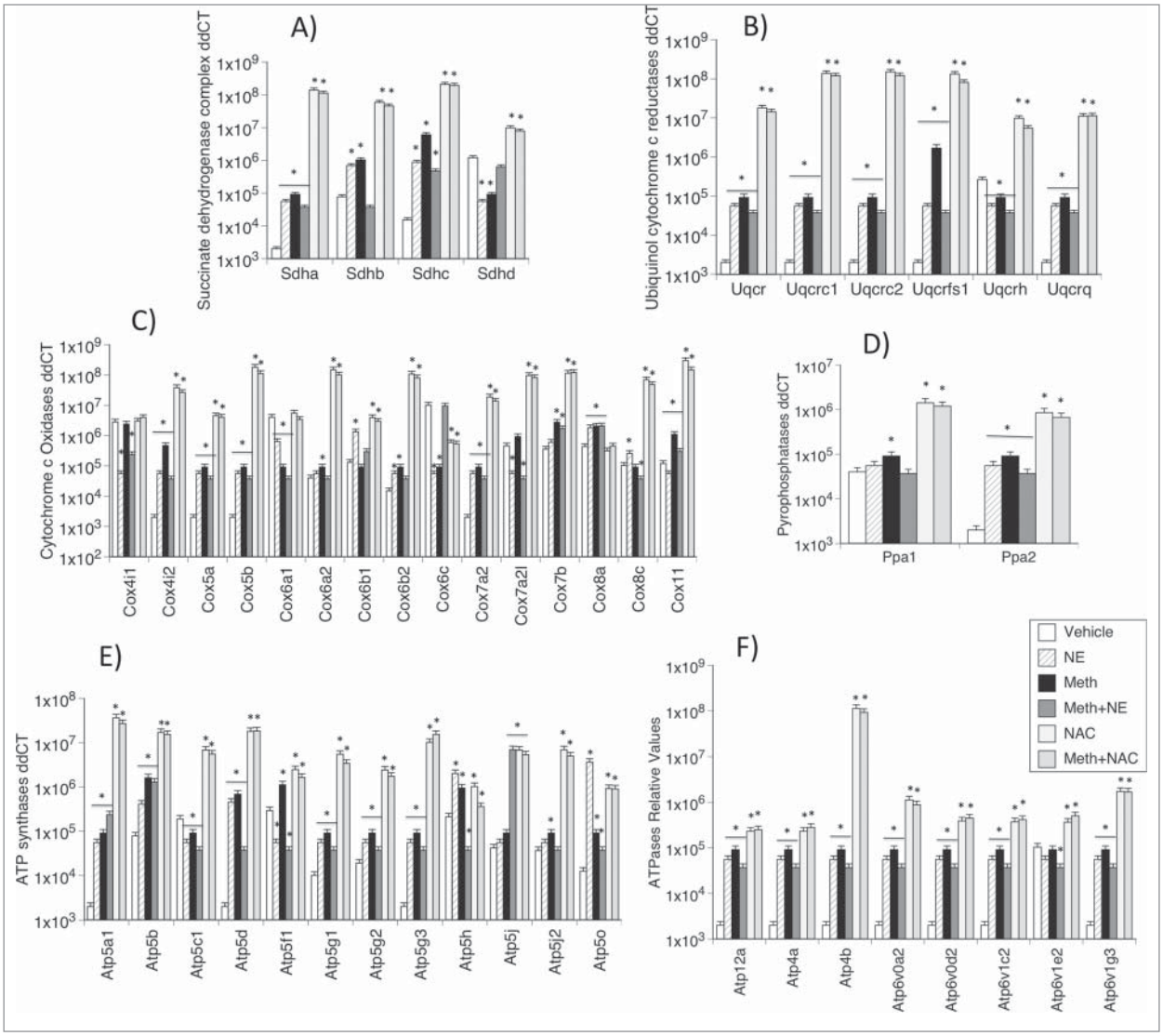

Figure 7. Effect of Meth on genes from mitochondrial Complex II to V. Transcriptional levels of mitochondrial electron-transport gene components of complexes II to V were measured in brown adipocyte primary cultures stimulated for 24 hours with NE and Meth using SyBrGreen qRT-PCR. Relative values were normalized based on GAPDH. Levels of (**A**) Complex II succinate dehydrogenases, (**B**) Complex III ubiquinol-cytochrome c reductases, (**C**) Complex IV cytochrome c oxidases, (**D**) pyrophosphatases, (**E**) ATP synthases, and (**F**) ATPases. Values represent the Average ± SEM of 3 replicates/group in 2 independent experiments. *$p < 0.05$ Compared to respective controls.

NE. Interestingly, NAC itself, alone or in combination with Meth, had a strong effect on expression of mitochondrial complexes components, dramatically enhancing their levels (**Figs. 6 and** 7). The effect of NAC was not changed by the presence of Meth in the cultures.

D. The protective effects of NAC in Meth-induced hyperthermia are not associated with alterations in glutathione levels

To explore the mechanisms by which NAC can reduce Meth-hyperthermia and preserve mitochondrial integrity in BAT, we measured the levels of glutathione in both, its reduced (GSH) and oxidized (GSSG) states. NAC is regarded as a source of cysteines for the synthesis of glutathione, which has an important anti-oxidant role, and a role as an electron donor to reduce disulfide bonds in various proteins. We examined whether NAC had an effect on glutathione levels within BAT at 2 hours after the injection of Meth, which corresponds to the time point when core body temperature started to rise (**Fig. 8**). The levels of both reduced and oxidized forms of glutathione were significantly decreased by the administration of Meth ($P = 0.02$ and 0.025, respectively, **Fig. 7A and 7B**). However, somewhat surprisingly, NAC treatment prior to or 1 hr after Meth did not significantly impact glutathione levels in BAT.

We next tested if the administration of S-adenosyl methionine (SAMe), which acts by improving the efficiency of the

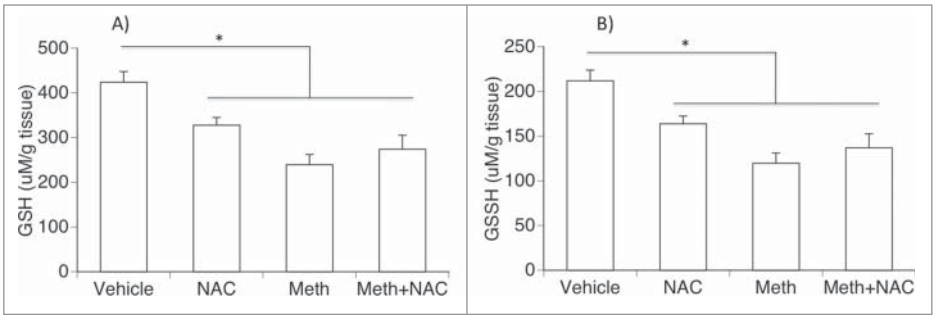

Figure 8. Effects of NAC and Meth on intra-BAT glutathione levels - BAT tissue was harvested 2 hours after the injection of Meth or Vehicle, deproteinized and processed for measurements of (**A**) reduced glutathione (GSH) and (**B**) oxidized glutathione (GSSH), using a colorimetric assay. Results represent the average ± SD, analyzed by One-Way ANOVA, followed by Bonferroni's test. *$p < 0.05$, compared to indicated groups.

glutathione pathway, rather than increasing glutathione levels, could modulate temperature and Meth-induced hyperthermia. SAMe has the ability to boost the efficacy of glutathione –mediated ROS detoxification, mediated by the activity of glutathione S-transferase (GST). For that, we treated animals with 200 mg/kg of SAMe, 1 hr before, and 1 hr after Meth. Interestingly, like NAC, the injection of SAMe caused a transient hypothermia in controls, further suggesting a role for ROS in the control of body temperature. Analysis using repeated measures ANOVA showed that the administration of SAMe had a significant effect in decreasing the temperature, both in controls and in animals treated with Meth ($p = 0.0003$, $p < 0.05$ between groups) **Fig. 9A**). Posthoc tests indicated that the effect of SAMe treatment on Meth-induced hyperthermia was detectable between 3,5 and 5.25 hrs after Meth injection. However, regression analysis of the temperature suggested that that the effect of SAMe on Meth, when compared to Meth, was not enough to bring temperature to control levels ($p = 0.0844$). The calculation of the AUC confirmed the difference between groups (ANOVA $p<0.0001$), and the posthoc test identified significance between Meth^{+}SAMe and SAMe ($p = 0.028$), but not between Meth^{+}SAMe and Meth ($p = 0.0733$) (**Fig. 9B**).

In contrast to NAC treatment, SAMe did significantly decrease motor activity after Meth injection (Repeated measures ANOVA P = 0.0005; **Fig. 9C**), but not in controls. Posthoc tests revealed that effect of SAMe on Meth-induced motor activity was detectable between 2.5 and 5 hours after Meth. However, regression analysis did not identify the effect of SAMe in locomotor activity in controls or in Meth treatment (**Fig. 9C**). The analysis of the AUC showed that SAMe did impact motor activity in Meth treatment ($p = 0.04$), by 43% (**Fig. 9D**). The effect of SAMe on Meth-induced decrease of RER was marginal (**Fig. 9E and 9F**). SAMe treatment decreased the heat output induced by Meth, which was mainly detectable between 1 and 2 hours following Meth (Repeated measures ANOVA P = 0.0006, regression analysis 0.0018 between Meth and Meth^{+}SAMe)(**Fig. 9G**), which was confirmed by the analysis of the AUC (p = 0.00097 between Meth and Meth^{+}SAMe) (**Fig. 9H**).

Discussion

We have previously demonstrated that the hyperthermia induced by Meth is partially mediated by changes in BAT, which include signs of mitochondrial stress that are dependent on sympathetic enervation.[15] However *in vitro*, the actions of Meth on brown adipocytes can occur directly and to some extent require ROS, which is released by several cell types in response to Meth.[35-37] We have employed pharmacological approaches to control Meth hyperthermia in the mouse model, using DNT, a muscle relaxant, and NAC, a commonly used antioxidant and free radical scavenger. These 2 drugs showed a very distinct action that demonstrates that temperature and motor activity (and also metabolic substrate utilization), may be independently regulated. For instance, while DNT decreased movement but did not affect core body temperature following Meth, NAC was remarkable for its ability to transiently down modulate the high temperatures induced by Meth injection, without affecting motor activity, revealing the potential of the later to interfere with very specific mechanisms regulating hyperthermia in drug abuse. The injection of NAC alone caused hypothermia, which could account for the drop in temperature induced by this drug in Meth-injected mice. However, it was clear that the effects of NAC were accompanied by important changes in mitochondria within the BAT, which is a relevant thermogenic site in Meth-abuse.[15]

NAC is classically an antioxidant and free radical scavenger. As a mechanism, NAC can act as a precursor for GSH biosynthesis in glutathione regeneration.[38] The pre-treatment with NAC acted to block the onset of hyperthermia following Meth. Thus, we tested the ability of NAC to rescue the hyperthermic process after its initiation. We also examined whether this drug had an impact in BAT, and mitochondrial redox state. For that, NAC was administered prior to, or at different time points after, the injection of Meth. As previously described, NAC injection 30 minutes before Meth prevented the increase of temperature, while the administration of NAC after Meth caused a transient but significant down regulation of core body temperature. This suggests a potential use of NAC to clinically recover hyperthermic states due to drug abuse.

We examined whether NAC had an impact in the BAT, which we showed to be relevant in Meth hyperthermia.[15] Indeed, NAC had an action on brown adipocytes eliciting a protective effect against Meth - induced loss and morphological disruption of mitochondria, measured by the expression of TOM20. However, NAC did not prevent the efflux of superoxide from BAT mitochondria, as detected by DHE in vivo. The decay of TOM20 protein expression in BAT immediately following Meth suggests that the drug is a potent stress factor over mitochondria in that site. TOM20 degradation is mediated by the ubiquitin

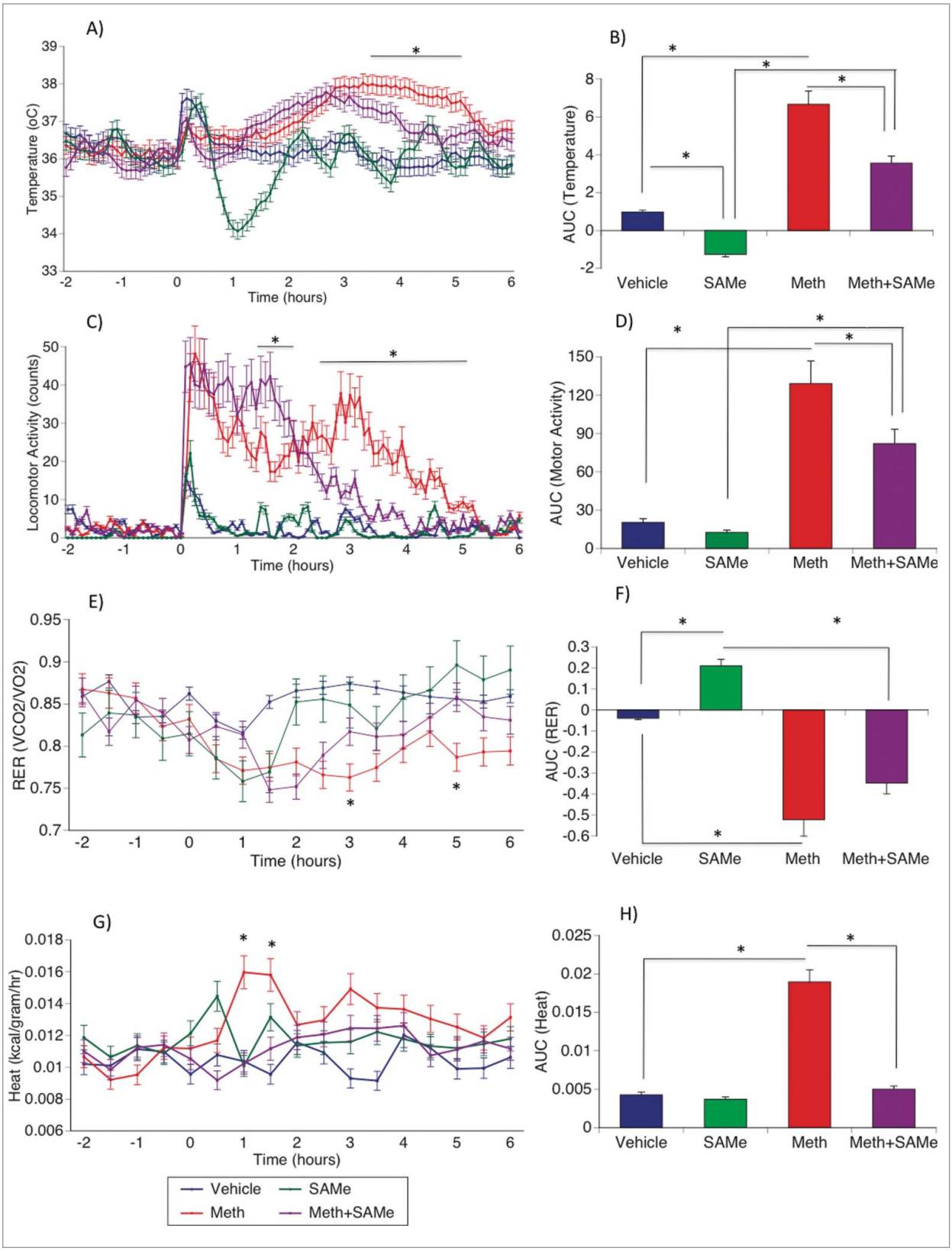

Figure 9. For figure legend, see page 238.

proteasome, triggered by changes in mitochondrial polarization.[39] We do observe long-term mitochondrial polarization in brown adipocytes in vitro, which can be a result of oxidative stress, mildly driven by NE, and to some extent driven by Meth directly. However, given that the loss of TOM20 is observed just 1 hrs after Meth, the immediate effects of Meth on mitochondria polarization are likely stronger, and need to be examined. NAC treatment in vivo preserved the mitochondrial integrity based on the expression of TOM20. In vitro, it was also able to prevent mitochondrial polarization, which was on itself, induced by ROS. One of the potential mechanisms that by which NAC can provide a regulatory outcome in the presence of ROS, is based on the restoration of glutathione levels.

The oxidation-reduction (redox) state of the tissue environment is a result of the balance between the levels of ROS and endogenous thiols such as glutathione, which protect cells from oxidative damage.[40] The dramatic release of ROS induced by Meth may exceed compensatory changes in the normally low levels of glutathione in BAT, causing it to decrease. It has been described that Meth also depletes glutathione from regions of the brain,[41] providing a mechanism of neurotoxicity by Meth-induced oxidative stress, which can be corrected by NAC.[42] However, in the BAT, NAC did not prevent the decrease in glutathione levels, or replenished glutathione storages. On the other hand, NAC was able to prevent the loss of TOM20 in mitochondria, and highly upregulated the transcription of complex I to V electron transport chain components. Interestingly, NAC preserved mitochondria, but did not prevent the depletion of ROS from those organelles.

BAT mitochondria have been found to have an intrinsically highly oxidation status and a basal ROS emission that is about 250% higher than other mitochondria-rich sites, such as skeletal muscle,[43] which causes the UCP1-mediated uncoupling in BAT to be highly sensitive to changes in the local environment.[43] Perhaps not surprisingly, BAT was found to have low glutathione levels compared to muscle.[43] We did confirm slight but significantly lower levels of glutathione in BAT compared to sites such as muscle and brain (data not shown), which suggests the special importance of the redox balance in the BAT microenvironment for controlling thermogenic functions in brown adipocytes.

Both NAC and SAMe can act by neutralizing ROS. Thus, the effect of both drugs on temperature, causing hypothermia in controls, and counteracting Meth-induced hyperthermia suggest that ROS can participate in the control of temperature, and in the induction of Meth-hyperthermia. The interesting aspect of NAC consists in the ability to modulate temperature without affecting muscle activity. The effects of NAC are detectable in BAT, where H_2O_2 alone and Meth trigger mitochondrial polarization and activity. Mechanistically, ROS can signal the regulation of chromatin modification mechanisms[44,45] that could locally affect the transcription of relevant genes. Importantly, the major signaling pathway of ROS involves NFkB,[46] which is abolished *in vivo* by the dose of NAC used here.[20] Whether NFkB is involved in the development of hyperthermia by Meth, in BAT or elsewhere, is uncertain.

The control of Meth-hyperthermia by NAC does not seem to involve increase of the glutathione levels to scavenge ROS in BAT. NAC also did not affect glutathione in muscle (data not shown). On the other hand, NAC increased glutathione in the brain (data not shown), which suggest that central mechanisms controlling Meth-hyperthermia are relevant. SAMe is a physiologic precursor of thiols and sulfurated compounds, which can also increase glutathione, but mostly acts to improve its efficiency by increasing the levels of glutathione S-transferase.[47] The effects of SAMe suggested that the remaining glutathione following Meth, can be important for correcting the course of hyperthermia after Meth. Like NAC, SAMe induced hypothermia in controls, which could play a counteracting effect. Yet, the drastic effect of NAC in controlling temperature, compared to SAMe, when combined to Meth, and the strong dissociation of these effects from locomotor activity suggest that alternative and specific pathways are being affected. Importantly, the dissociation between the control of temperature and motor activity in NAC treatment does not exclude the participation of muscles in the upregulation of temperature by drugs of abuse, for instance through uncoupling,[48] rather than movement. Vasoconstriction and heat dissipation could also be involved in the development of hyperthermia.[49] In addition, CNS-controlled mechanisms that are specific to the control of temperature are critical.[50] However, Dopamine receptor antagonists [3,13], which are effective in controlling hyperthermia in some models, have an action on motor activity.[51] The mechanisms by which NAC affect peripheral and central pathways, remains to be addressed. The potential of other anti-oxidants to perform similarly regarding controlling Meth-hyperthermia also remains to be examined.

The oxidative stress induced by Meth may disturb biological processes, although cellular redox reactions are often tightly regulated to play physiological roles. Cysteines are important in such regulation, as they exhibit chemical properties that make protein thiols sensitive to redox state, but also predispose them to oxidative damage by ROS. These reactions impact proteins by altering conformation, and critical chemical properties, thus altering function and outcome, by for instance promoting disulfide shuffling and alternative folding.[52,53] The degree to which this process can affect protein activity, varies according to the role and importance of the cysteine residue for carrying out protein

Figure 9 (**See previous page**). Effects of SAMe in Meth-induced changes in temperature, locomotor activity and RER. Mice were treated with SAMe (200mg/kg) 1h before Meth (5mg/kg), and were monitored for a total of 6 hours for Temperature and Respiratory Exchange Ratio. (**A**) Core body temperature, (**B**) AUC for temperature, (**C**) Locomotor Activity, (**D**) AUC of Locomotor activity, for a total of 6 hrs, (**E**) Respiratory Exchange Ratio (RER), (**G**) Heat, extrapolated from CV and body mass and (**H**) AUC for Heat. Baseline values for each variable were calculated by the average between -2 and 0, for determination of the AUC for a total of 6 hours, from time 0, using both positive and negative peaks. Arrows indicate critical injection time-points. Values represent the Average ± SEM of one representative experiment with 4 mice/group. *$p < 0.05$.

function, regardless of glutathione, but still under reversible redox control or, alternatively, as a result of oxidative damage.[54,55] Thus, Meth as a ROS-inducer and trigger, and NAC as a controller, can potentially be acting on the same residues, but in different ways. BAT is a tissue that may be particularly sensitive to such mechanism, for being a high redox environment with a high mitochondrial content.[56] However, other sites and pathways can be affected by such mechanism. For instance, many metabolic enzymes, kinases, phosphatases, and transcription factors contain critical thiol-bound cysteine residues that alter protein activity. In transcription factors such as NF-κB and c-jun, cysteine residues are needed for binding to DNA.[57-59] Thus, the modification of thiol/cysteines in proteins may be a mechanism to turn on or off signaling pathways. Such control could be achieved by the administration of a source of cysteines such as NAC prior to the initiation of the thermogenic program, explaining the outcome of the administration of NAC in Meth hyperthermia. It is also important to acknowledge that NAC can be a potential glutathione analog, directly working as a substrate to glutathione transferase, without affecting glutathione synthesis.[60] Thus, the role of NAC in providing cysteines could bypass the replenishment of glutathione levels, quickly quenching ROS as well as affecting other proteins. However, the exact targets of protein modifications caused by NAC and that can impact temperature in BAT and elsewhere, remain to be uncovered. Further studies are critical to understand the basis for temperature control by cysteines and identify the key molecules affected by this mechanism, for reducing Meth-induced hyperthermia.

We have examined correlations between the control of Meth-hyperthermia by NAC and changes in BAT that occurred independently of changes in motor activity. The dissociative character of the control of temperature in relation to motor activity in NAC treatment could be centrally performed. Even though core body temperature and motor activity are generally synchronized, studies in rats suggest a disconnection between these 2 processes,[61] but rather a centrally organized connection between temperature and metabolic rate through hormone levels.[62] Whether NAC acts on a temperature-specific compartment of the response to Meth, and whether its mechanism of action is through ROS or through thiol modifications on specific sets of proteins, in the BAT or in the CNS, are unanswered questions.

NAC has been used in humans, including at high orally administered doses for the treatment of acute acetaminophen toxicity,[63,64] and to prevent DNA damage and mutagenesis associated to tumor development,[65] as well as to the effects of radiotherapy.[66] Differently from glutathione, NAC can cross the blood brain barrier, and in the CNS can have beneficial effects. For instance, it has been shown that NAC reduces neuronal loss in ischemic and reperfusion brain injury.[67,68] It has been utilized in several neurological disorders that involve oxidative stress as a pathological cause (for a Review, see [69]). Specifically, in a rat model of Meth abuse, NAC pre-treatment decreased neurotoxicity associated to the drug, and, contrasting to our results in mice, it reduced motor activity.[70] Regarding the effect of NAC in temperature, one report shows that NAC ameliorates febrile states associated to inflammation.[71] *In vitro*, the benefits of NAC were also demonstrated for preventing the MDMA-induced neuronal death.[72] The desired effects on temperature of NAC in our model were achieved using ip injections. Other routs of administration may be investigated, the potential of NAC to rescue hyperthermia due to other etiologies, and the examination of whether other anti-oxidants can play a role in controlling temperature in Meth abuse, also remain to be addressed.

NAC has potential applications to heat illness secondary to the use of Meth, but most importantly, it may be an important pharmacological model to explore mechanisms controlling temperature. The effects of NAC are observed in BAT, a peripheral thermogenic site that plays a role in Meth-hyperthermia, where it acts to prevent signs of mitochondrial damage. BAT has been recently shown to be relevant in humans, in correlation with metabolic responses to cold and energy homeostasis.[73-77] A thermogenic role for BAT in human adults further stimulated in Meth abuse must be examined. However, given the properties of NAC, the effects of this drug may be extensive to other thermogenic sites. Our experiments suggest a role for ROS, and BAT mitochondria in hyperthermia induced by Meth in mice. In addition, the results also put forward that NAC is an important pharmacological tool in the mouse model of Meth abuse, where it is able to affect temperature without affecting motor activity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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