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Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylenedioxymethamphetamine administration

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Abstract

Metabolic mapping of discrete brain regions using cytochrome oxidase histochemistry was used as a marker for alterations in mitochondrial function and cytochrome oxidase enzymatic activity in response to high doses of amphetamine derivatives. The activity of cytochrome oxidase, complex IV of the electron transport chain, was determined at three different time-points following administration of high doses of methamphetamine or 3,4-methylenedioxymethamphetamine (MDMA) (four injections of 10–15 mg/kg administered over 8 h). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), nucleus accumbens (29–30%) and substantia nigra (31–43%), 2 h following administration of either methamphetamine and MDMA. This decrease in cytochrome oxidase activity was transient and returned to control levels within 24 h. Since the methamphetamine and MDMA-induced decrease in cytochrome oxidase activity was localized to dopamine-rich regions, increased extracellular concentrations of dopamine may contribute to the inhibition of metabolic function via its metabolism to form quinones or other reactive oxygen species. These results support previous studies demonstrating that psychostimulants induce a rapid and transient decrease in striatal ATP stores and provide further evidence that these drugs of abuse can disrupt mitochondrial function.

Keywords: Neurotoxicity; Cytochrome oxidase; Amphetamine; Histochemistry; Energy metabolism

1. Introduction

Increased metabolic stress compromises bioenergetic processes and has been hypothesized to contribute to lasting changes in the dopamine and serotonin (5-hydroxytryptamine, 5-HT) systems following high-dose methamphetamine administration. Evidence of metabolic stress following methamphetamine includes increased extracellular concentrations of lactate (Stephans et al., 1998) and a decrease in striatal ATP concentrations (Chan et al., 1994). Poblete and Azmitia (1995) have reported that 3,4-methylenedioxymethamphetamine (MDMA) increases the breakdown of glycogen in vitro, indicating that similar changes in metabolic function occur following administration of other substituted amphetamines. Acute administration of methamphetamine or its parent compound amphetamine, has been shown to rapidly (within 1 h) increase local cerebral glucose utilization in multiple brain regions (Pontieri et al., 1990; Porrino et al., 1984). In addition, high-dose treatment with methamphetamine or MDMA results in decreased cerebral glucose metabolism weeks to months following drug administration, suggesting lasting impairments in metabolic systems (Huang et al., 1999; McBean et al., 1990; Sharkey et al., 1991). Although these studies indicate that energy metabolism is altered following methamphetamine or MDMA administration, no studies have demonstrated directly that mitochondrial function itself is acutely or chronically compromised following high doses of psychostimulants.

Metabolic mapping using cytochrome oxidase histochemistry can be used to compare relative levels of enzy-

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matic activity in discrete brain regions (Hevner et al., 1995). The density of cytochrome oxidase staining is highly correlated with cytochrome oxidase activity as measured in tissue homogenates, but the histochemical technique has the advantage of higher anatomic resolution compared with biochemical measurements in tissue homogenates (Hevner et al., 1995). Unlike mapping using 2-deoxyglucose, which measures rapid changes in glucose utilization, cytochrome oxidase histochemistry can be used to measure changes in energy usage over a period of hours to weeks (Wong-Riley, 1989). The degree of cytochrome oxidase staining is believed to reflect the overall functional activity of neurons in that intense staining is associated with areas that have both a high level of excitatory input and high tonic firing rates (Kageyama and Wong-Riley, 1982; Mjaatvedt and Wong-Riley, 1991).

The primary goal of this study was to identify and characterize mitochondrial dysfunction following methamphetamine or MDMA administration. Specifically, the activity of complex IV (cytochrome oxidase) was determined following administration of high doses of methamphetamine or MDMA to determine if these psychostimulants had rapid or lasting effects on mitochondrial enzyme function. Based on the suggestion that psychostimulants increase the formation of nitric oxide (Abekawa et al., 1996; Zheng and Laverty, 1998), a known complex IV inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), and the finding of depleted energy stores following methamphetamine administration (Chan et al., 1994), it was hypothesized that high-dose methamphetamine or MDMA would decrease cytochrome oxidase histochemical staining in a brain region specific manner correlating with the ability of these drugs to increase extracellular concentrations of dopamine and 5-HT. Cytochrome oxidase activity was examined 2 h, 24 h, and 7 days following administration of the last dose of methamphetamine or MDMA. These time-points were chosen, in part, based on a previous study demonstrating a loss of striatal ATP at 1.5 h, but not 24 h following the same course of methamphetamine treatment (Chan et al., 1994). In addition, several studies have demonstrated that substituted amphetamines result in a rapid depletion of neurotransmitter content, followed by a transitory recovery (24 h post drug), and a lasting monoamine loss (generally measured 3 or 7 days post drug). Therefore, these time-points were also chosen to determine if changes in cytochrome oxidase activity correlate temporally with the known time-course of methamphetamine- and MDMA-induced monoamine loss.

2. Materials and methods

2.1. Subjects and drug administration

Male Sprague–Dawley rats ($N = 61$, 200–275 g) were housed, two to three animals per cage, in clear plastic shoe boxes. Animals were maintained on a 12-h light/dark cycle (lights on at 6:00 AM) with food and water available ad libitum throughout the experiments. All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 80-23). For all experiments, methamphetamine (10 mg/kg), MDMA (15 mg/kg), or an equivalent volume of vehicle (saline) was administered i.p. every 2 h for a total of four injections. Four animals died following administration of MDMA ($n = 2$) or methamphetamine ($n = 2$).

The protocol used in the study required the transcardial perfusion of rats with fixative, a technique that is not compatible with the measurement of dopamine and 5-HT tissue content. However, hyperthermia following psychostimulant administration has been shown to be a reliable predictor of toxicity (Bowyer et al., 1994; Che et al., 1995; Craig and Kupferberg, 1972). Therefore, rectal temperatures (taken every 30–60 min for 8 h following the first injection) were determined by the use of an RET-2 copper–constantan thermocouple rectal probe (Physitemp Instruments; Clifton, NJ) and a TH-8 thermalert thermometer (Sensortek; Clifton, NJ).

2.2. Cytochrome c oxidase histochemistry

Rats were killed 2 h ($n = 5$ per group), 24 h ($n = 7–10$ per group), or 7 days ($n = 5–6$ per group), following the last injection of drug or vehicle. Subjects were anesthetized with chloral hydrate and perfused transcardially with fixative (0.3% glutaraldehyde, 4% paraformaldehyde, and 2% sucrose in 0.1 M phosphate buffer, pH 7.4). Whole brains were post-fixed for 1 h, rinsed in 0.1 M phosphate buffer with 4% sucrose and cryoprotected by submersion in buffer with increasing concentrations of sucrose. Brains were sliced (40 μm) on a cryostat (−20°C) and rinsed on a shaker at 4°C overnight in phosphate buffer with 4% sucrose. To ensure that differences in cytochrome oxidase staining were not due to variations across staining runs, slices from comparable brain regions for the three drug treatment groups (saline, methamphetamine, MDMA) were placed in individual Netwell™ tissue processing wells (Electron Microscopy Sciences, Fort Washington, PA) and processed together for cytochrome oxidase activity according to a modification of the method by Wong-Riley (1979). Slices were then incubated (38°C) in phosphate-buffered saline (4% sucrose) with 3,3′-diaminobenzidine and cytochrome *c* for 3 h. Following repeated washing in buffer, slices were mounted on gel-coated slides and coverslipped for later analysis.

2.3. Data analysis

Images were captured from slides using a CCD camera (Sierra Scientific, Sunnyvale CA) and a Northern Light

box (Model B90, Imaging Research, St. Catherines, Ontario, Canada). The relative optical density (ROD score) of staining, a semi-quantitative measure of cytochrome oxidase activity, in various brain regions was determined using an MCID image analysis system (Imaging Research). Each area of interest was outlined in its entirety and the average pixel density determined for that nucleus. Approximately 4–12 densitometry measurements were made for each brain region within an animal. These comprised at least the left and right sides of two consecutive slices assayed in duplicate trays. For larger nuclei, additional consecutive slices were analyzed. The number of slices analyzed (one to three pairs) depended upon the size of the region examined (i.e., more slices were taken for regions with greater anterior/posterior length such as the caudate). This was to insure that staining for cytochrome oxidase activity did not show regional variability within nuclei examined (i.e., the anterior vs. posterior striatum). Variability between these measurements was very low. Although occasional hemispheric differences were noted, no systematic differences between hemispheres or across nuclei were found. Therefore, the densitometry measurements were averaged to give a single ROD score for each brain region within a given rat.

The density of background staining in white matter (corpus callosum) was subtracted from the total ROD score obtained in gray matter regions to give a measure of specific staining of cytochrome oxidase activity. Corrected ROD scores for tissue taken from animals killed at a given time-point were compared by two-way mixed factor analysis of variance (ANOVA) with drug treatment as the between-subjects factor and brain region as the within-subjects factor. Significant interactions were further analyzed by Newman–Keuls' test. Peak core temperature during drug administration was analyzed by one-way ANOVA followed by Newman–Keuls' test.

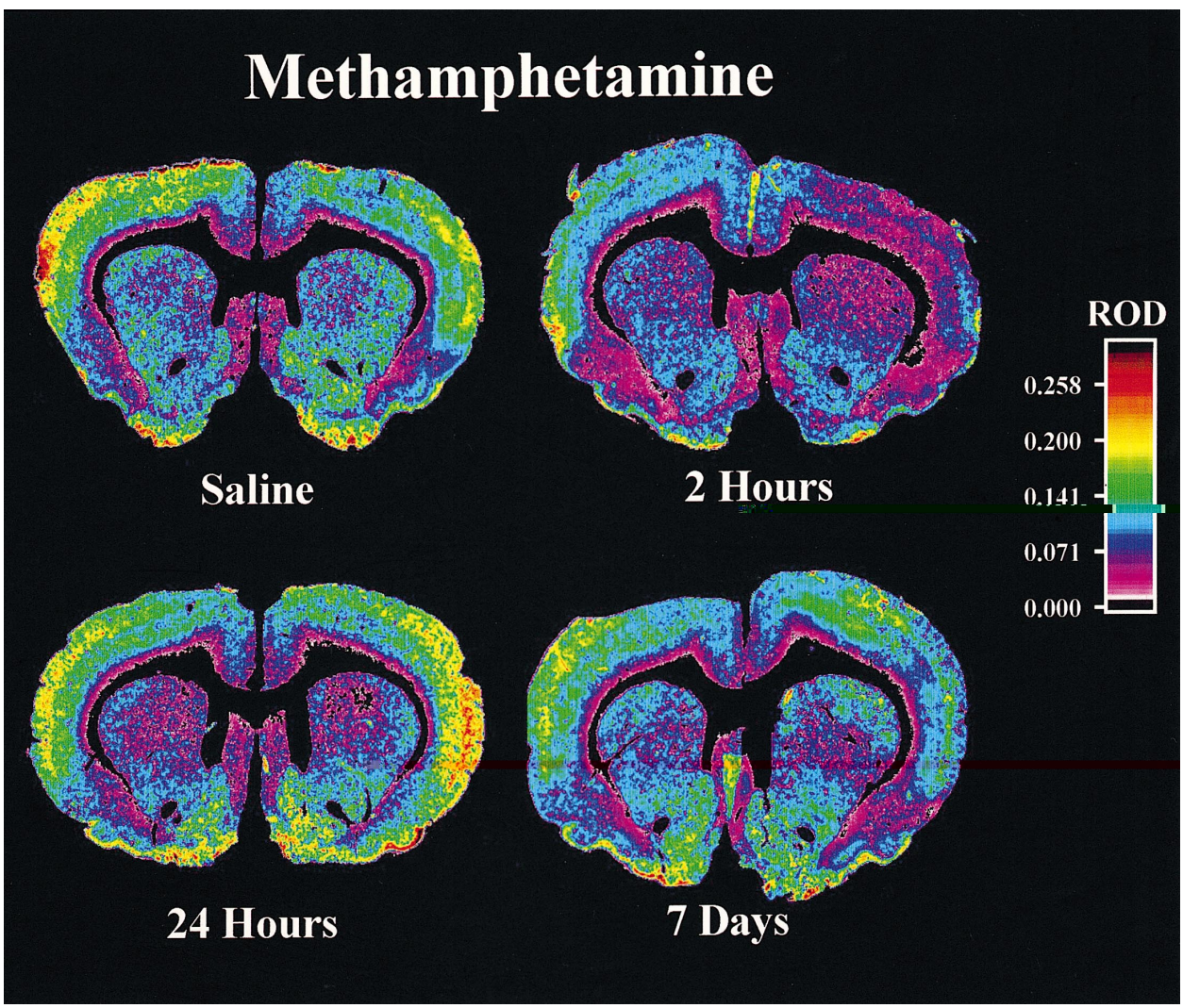

Fig. 1. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or methamphetamine (10 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following methamphetamine administration (panel B) compared to a saline control rat (panel A).

2.4. Materials

The following drugs and chemicals were purchased from Sigma (St. Louis, MO): D-methamphetamine, MDMA, 3,3′-diaminobenzadine, paraformaldehyde, and cytochrome *c* (derived from horse heart). Glutaraldehyde (50% solution, biological grade) was obtained from Electron Microscopy Sciences. Doses of methamphetamine and MDMA are expressed as the salt. Sodium phosphate (monobasic and dibasic) was obtained from Fisher Scientific (Fair Lawn, NJ).

3. Results

The density of cytochrome oxidase staining varied significantly across brain region with the greatest staining in the nucleus accumbens and the lowest staining in the entopeduncular nucleus (main effect of region $F(9,108) = 71.5$, $P < 0.01$). There was a rapid decrease in cytochrome oxidase staining in the striatum (23–29%), substantia nigra (31–43%), and in both the core and shell subregions of the nucleus accumbens (29–30%) 2 h following administration of the last dose of either methamphetamine (Fig. 1) or MDMA (Fig. 2) (main effect of drug: $F(2,12) = 6.5$, $P < 0.01$; interaction: $F(18,108) = 2.4$, $P < 0.01$) (Fig. 3a). This decrease in cytochrome oxidase activity was transient and reversed to control levels within 24 h (Fig. 3b) and remained at basal levels 7 days post drug (Fig. 3c). Although there was a general trend towards a decrease in activity in most regions, cytochrome oxidase histochemistry was not significantly altered at any time-point in the other six regions examined (hippocampus, entopeduncular nucleus, motor thalamus, subthalamic nucleus, motor cortex, prefrontal cortex). In preliminary studies, no clear differences between the substantia nigra pars compacta and

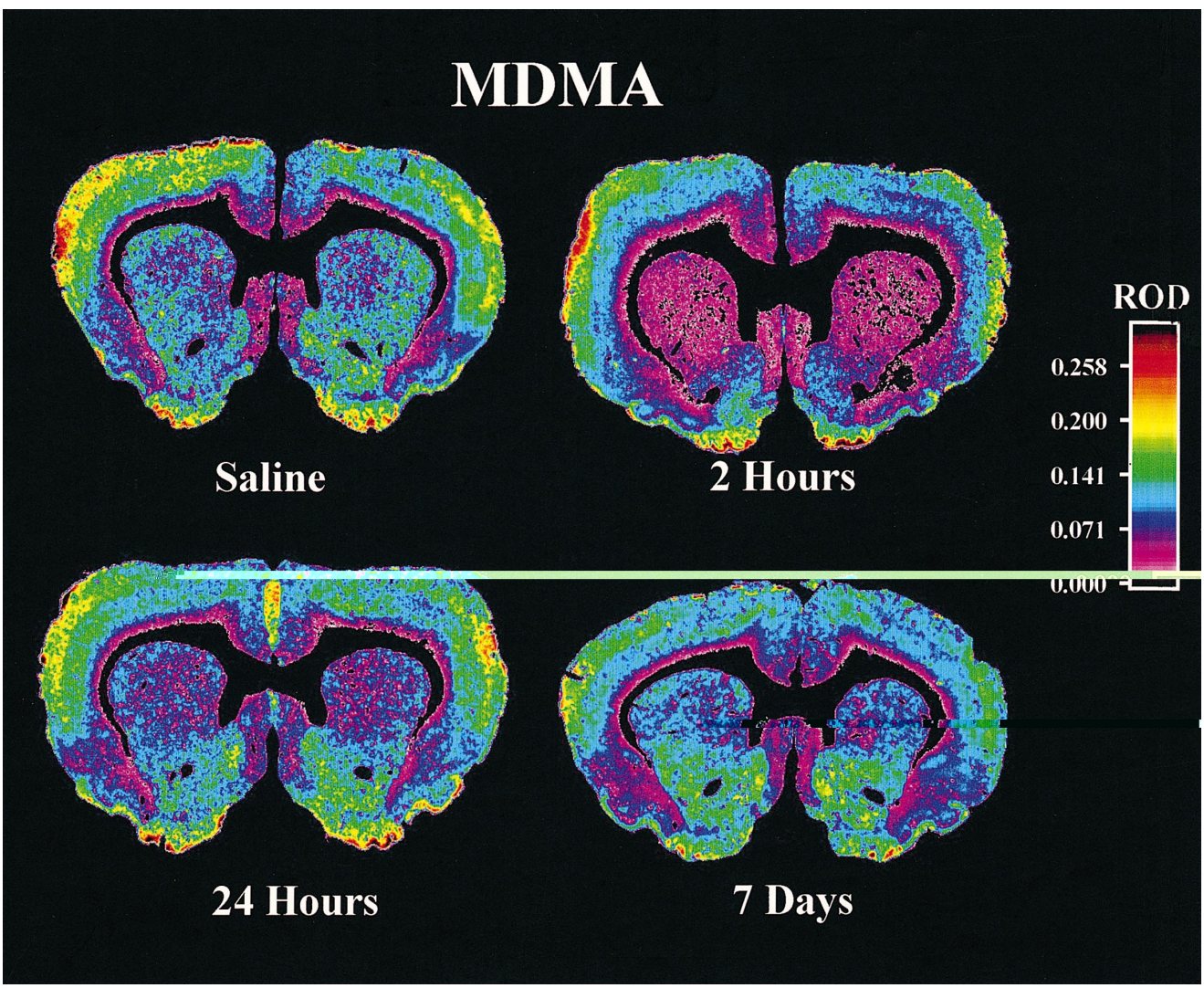

Fig. 2. Cytochrome oxidase histochemistry in representative coronal slices from animals treated with saline (top left) or MDMA (15 mg/kg) and killed 2 h (top right), 24 h (bottom left), or 7 days (bottom right) following the last dose of drug. Note the decrease in density of staining within the caudate and nucleus accumbens 2 h following MDMA administration (panel B) compared to a saline control rat (panel A).

pars reticulata were seen. Because boundaries between the nigral subregions (pars compacta and pars reticulata) were not always distinct in stained slices, cytochrome oxidase activity was determined for the entire substantia nigra.

Rectal temperatures rapidly increased following each injection of methamphetamine (10 mg/kg) (Fig. 4). In contrast, hyperthermia experienced by MDMA (15 mg/kg)-treated rats was stable across drug administration. Although administration of either drug produced hyperthermia when compared to saline-treated rats (peak temperature 37.6 ± 0.09°C), peak temperatures attained following methamphetamine administration (41.2 ± 0.17°C) were higher than those found following MDMA (39.6 ± 0.26°C) ($F(2,48) = 102$, $P < 0.01$).

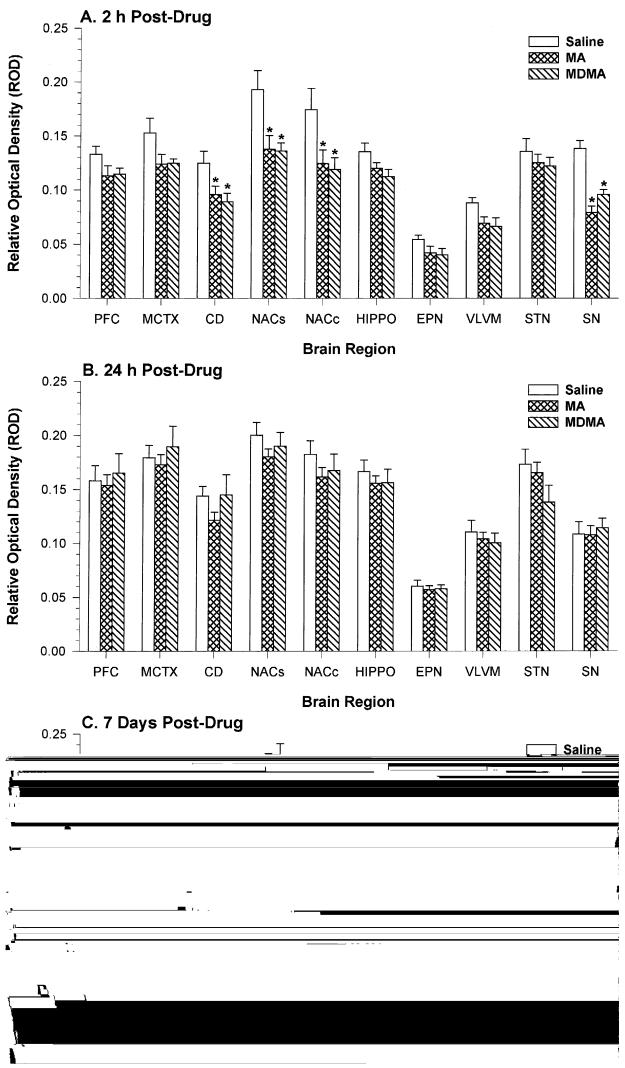

Fig. 3. Effect of repeated methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on the density of cytochrome oxidase histochemical staining (ROD score) (A) 2 h ($n = 5$), (B) 24 h ($n = 7$–10), or (C) 7 days ($n = 5$–6) following the last dose of drug. Cytochrome oxidase activity varied across brain region in saline treated control animals. Cytochrome oxidase activity was significantly decreased 2 h following methamphetamine or MDMA administration in dopamine terminal regions (CD, NACs, NACc) and in the SN ($^{*}P < 0.05$). No differences in cytochrome oxidase staining were found 24 h or 7 days post drug. Anatomical abbreviations are as follows: PFC, prefrontal cortex; MCTX, motor cortex; CD, caudate; NACs, nucleus accumbens shell; NACc, nucleus accumbens core; HIPPO, hippocampus; EPN, entopeduncular nucleus; VLVM, motor thalamus; STN, subthalamic nucleus; SN, substantia nigra.

Fig. 4. Effect of methamphetamine (10 mg/kg) or MDMA (15 mg/kg) on core body temperature (°C). Systemic administration (arrows) produced a hyperthermic response compared to rats injected with saline vehicle ($n = 17$) ($P < 0.01$). Core body temperature peaked 30 min following each injection of methamphetamine ($n = 19$). In contrast, rats treated with MDMA ($n = 15$) had a hyperthermic response that remained fairly constant throughout the 8 h of drug administration.

4. Discussion

The acute administration of methamphetamine and MDMA resulted in a rapid and transient decrease in complex IV activity. Both methamphetamine and MDMA decreased cytochrome oxidase staining to a similar extent in the caudate, nucleus accumbens, and substantia nigra. These changes occurred within 2 h following the final drug injection and returned to basal levels within 24 h. No significant alterations in cytochrome oxidase activity were found in six other brain regions examined. Chan et al. (1994) reported that methamphetamine administration to mice (using the same dosing regimen), depleted ATP levels by 20% in the striatum, but not the hippocampus. In addition, ATP levels were depleted 1.5 h following methamphetamine administration, and returned to control values within 24 h (Chan et al., 1994). Thus, the inhibition of complex IV activity found in the current study correlates both temporally and anatomically with the ATP depletion that has been found previously to occur after high-dose methamphetamine administration. Together, these studies provide evidence that acute high-dose psychostimulant administration produces a rapid and transient disruption of metabolic processes that is regionally selective.

Several mechanisms could underlie the compromise in metabolic function that follows methamphetamine or MDMA administration. Evidence indicates that the release of nitric oxide and subsequent activation of the nitric oxide synthase pathway follow both methamphetamine and MDMA administration (Abekawa et al., 1996; Zheng and Laverty, 1998). Since nitric oxide is a known cytochrome oxidase inhibitor (Cleeter et al., 1994; Lizasoain et al., 1996), stimulant-induced activation of this pathway may directly inhibit complex IV activity. Additionally, psychostimulants may increase neuronal energy utilization through the sustained activation of monoamine transporters, hyperlocomotion, and the production of hyperthermia, all of which have been described as responses to psychostimulant administration (Huether et al., 1997). Since the majority of ATP in the neuropil is devoted to the maintenance of ion gradients and the restoration of the membrane potential following depolarization (Erecinska and Silver, 1989; Hevner et al., 1992; Wong-Riley, 1989), sustained activation following prolonged neurotransmitter release may lead indirectly to the depletion of substrates for the electron transport chain. Such a decrease in available precursors may slow or halt the production of ATP through a decline in complex IV activity.

Stimulant-induced increases in the extracellular concentrations of monoamines may also contribute to mitochondrial inhibition. Elevated extracellular dopamine may compromise mitochondrial function via autoxidation to form quinones and/or the enzymatic degradation of dopamine to form H_2O_2 and the generation of hydroxyl radicals (Graham et al., 1978; McLaughlin et al., 1998). This hypothesis is especially interesting given the finding that significant decreases in cytochrome oxidase activity were restricted to dopamine-rich brain regions (striatum, nucleus accumbens, and substantia nigra). Reactive oxygen species and dopamine-derived quinones are known to directly inhibit mitochondrial enzymes associated with energy production (Ben-Schachar et al., 1995; Yagi and Hatefi, 1987; Zhang et al., 1990). Although dopamine and 5-HT-mediated inhibition of energy production has not been demonstrated to occur in vivo, incubation of rat brain mitochondria in vitro with dopamine decreases State 3 (ATP-synthesis coupled) respiration, and incubation with dopamine–quinones increases State 4 respiration (Berman and Hastings, 1999). These studies indicate that reactive dopamine by-products may increase proton leakage across the mitochondrial membrane and inhibit the production of energy stores. Although Berman and Hastings (1999) reported that the L-DOPA-mediated decrease in State 3 respiration was not due to altered complex IV activity, L-DOPA does inhibit complex IV, but not complex I, activity in vitro (Pardo et al., 1995). In addition, Jiang et al. (1999) have recently reported that tryptamine-4,5-dione, a metabolite of 5-HT oxidation, inhibits cytochrome oxidase and NADH-coenzyme Q1 reductase in vitro by covalently modifying sulfhydryl groups on these enzyme complexes. These data indicate that the sustained release of 5-HT following psychostimulant administration also may contribute to the inhibition of mitochondrial function.

In the current study, the effects of high-dose psychostimulants on complex IV activity were rapid and transient. In contrast, studies using similar doses of methamphetamine and MDMA have found persistent depletions of dopamine and/or 5-HT (Shankaran et al., 1999; Stephans and Yamamoto, 1994; Yamamoto and Zhu, 1998). Although the time courses of metabolic inhibition and persistent nerve terminal loss are discordant, a rapid and transient disruption of mitochondrial function may produce neurotoxicity. For example, Chan et al. (1994) reported that inhibition of metabolism, by pretreatment with 2-deoxyglucose, exacerbated both the methamphetamine-induced ATP loss and long-term reduction of striatal dopamine content. Conversely, pretreatment with nicotinamide attenuated both the rapid decrease in striatal ATP and the lasting dopamine depletions following amphetamine administration (Wan et al., 1999). In addition, the intrastriatal perfusion of substrates for the electron transport chain (ubiquinone or nicotinamide) for several hours following methamphetamine administration attenuated the long-term loss of dopamine content, again linking a metabolic deficit with loss of monoamine nerve terminals (Stephans et al., 1998). Furthermore, the local inhibition of complex II via intrastriatal perfusion with malonate synergized with the local administration of methamphetamine to enhance dopamine toxicity compared to the perfusion of either drug alone (Burrows et al., 2000). These data indicate that a depletion of energy stores is critically linked with the neurotoxic effects of stimulant drugs. Although no direct measures of toxicity were made in the present study, the hyperthermic response to psychostimulant administration was comparable to that previously shown to be associated with lasting monoamine depletions (Bowyer et al., 1994; Che et al., 1995). Additional studies are needed to examine the possible relationship between the inhibition of complex IV activity and lasting monoamine depletions that can occur following methamphetamine or MDMA administration.

In conclusion, the rapid inhibition of metabolic function in dopamine-rich regions, as determined by a decrease in complex IV activity following methamphetamine and MDMA administration correlates with previous studies suggesting that psychostimulant administration compromises energy balance in the brain. This change in cytochrome oxidase activity could reflect protein turnover, a loss of enzyme function, or uncoupling of oxidative phosphorylation. Future studies are needed to determine the underlying mechanism of the transient loss of cytochrome oxidase activity. These studies add to the growing importance for understanding the consequences of psychostimulant administration in light of the recent evidence of dopamine terminal dysfunction in human methamphetamine abusers (McCann et al., 1998a,b; Wilson et al.,

1996) and the physiological abnormalities in humans who have used MDMA (Allen et al., 1993; Bolla et al., 1998; Morgan, 1999).

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