RESEARCH ARTICLE

N-Acetyl Cysteine Mitigates the Acute Effects of Cocaine-Induced Toxicity in Astroglia-Like Cells

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Abstract

Cocaine has a short half-life of only about an hour but its effects, predominantly on the central nervous system (CNS), are fairly long-lasting. Of all cells within the CNS, astrocytes may be the first to display cocaine toxicity owing to their relative abundance in the brain. Cocaine entry could trigger several early response changes that adversely affect their survival, and inhibiting these changes could conversely increase their rate of survival. In order to identify these changes and the minimal concentrations of cocaine that can elicit them in vitro, rat C6 astroglia-like cells were treated with cocaine (2–4 mM for 1h) and assayed for alterations in gross cell morphology, cytoplasmic vacuolation, viability, reactive oxygen species (ROS) generation, glutathione (GSH) levels, cell membrane integrity, F-actin cytoskeleton, and histone methylation. We report here that all of the above identified features are significantly altered by cocaine, and may collectively represent the key pathology underlying acute toxicity-mediated death of astroglia-like cells. Pretreatment of the cells with the clinically available antioxidant N-acetyl cysteine (NAC, 5 mM for 30 min) inhibited these changes during subsequent application of cocaine and mitigated cocaine-induced toxicity. Despite repeated cocaine exposure, NAC pretreated cells remained highly viable and post NAC treatment also increased viability of cocaine treated cells to a smaller yet significant level. We show further that this alleviation by NAC is mediated through an increase in GSH levels in the cells. These findings, coupled with the fact that astrocytes maintain neuronal integrity, suggest that compounds which target and mitigate these early toxic changes in astrocytes could have a potentially broad therapeutic role in cocaine-induced CNS damage.
Introduction

Cocaine is an addictive and widely abused psychostimulant that can evade the protection of the blood brain barrier (BBB) to enter the brain and compromise its normal functioning. Cocaine's effects on biochemical processes in the CNS is an area of active research, and how these cocaine-induced changes impact neurons and astrocytes is not well understood. Although acute exposure to cocaine has been shown to alter gene expression [1], it is the changed cell biochemistry that appears to underlie many of the clinical symptoms. Identification of early biochemical symptoms such as vacuolation and changes in mitochondrial membrane potential may offer clues about underlying mechanisms and therapeutic avenues. While the long-term/chronic effects of cocaine, including post-translational modifications such as acetylation, methylation [2, 3], phosphorylation have been well established in the literature, early precipitating events that lead to these chronic changes following acute exposure are much less understood. Furthermore, cocaine's ability to interfere with normal signaling pathways in neurons [4] has narrowed the focus of research within CNS to neurons, despite evidence that astrocytes--cells that provide both physical and chemical support to neurons [5] and maintain the integrity of the BBB [6]--are also vulnerable. The present study is geared towards unraveling the acute morphological and epigenetic changes in astrocytes upon exposure to cocaine. Incorporating data from our previous studies that focused on the chronic effects of cocaine [7, 8] and considering that astrocytes outnumber neurons in most brain regions [9], we postulate that toxic effects of cocaine manifest in astrocytes prior to any neuronal damage. Cocaine's entry into the brain through the BBB, known for its astroglial interaction [10, 11], may also expose astrocytes to cocaine sooner and for longer periods than any other cell-type in the CNS thereby enhancing their vulnerability to cocaine-induced toxicity. Because neurons depend on astrocytes for survival [12, 13], loss of astrocytes due to cocaine toxicity could ultimately lead to loss of neurons/neuronal function [14]--a circumstance that could possibly be avoided in the initial stages of cocaine addiction by protecting astrocytes from the acute effects of cocaine-induced toxicity. This study tests the hypothesis that inhibition of the acute effects of cocaine in astrocytes increases their survival.

The objectives of the present study are to identify various early response changes associated with acute exposure of astroglia-like cells to physiologically-relevant doses of cocaine in vitro; to determine the minimal doses that compromise their viability; and to investigate the role of NAC in mitigating cocaine-induced toxicity in these cells and determining its mode of action. To this end, we used a CNS derived rat C6 astroglia-like cell line (CCL-107) which is astrocytic in origin and unlike other CNS cell lines, exhibits a high degree of similarity with human astrocytes in its gene expression [15] and enzymes [16]. Studies have also shown that this cell line contains undifferentiated glial cells [17] that release glial cell line-derived neurotrophic factors similar to astrocytes [18]. Taken together, these properties demonstrate that C6 cell cultures behave like an astroglia-like cell line. In the past, C6 cells have also been used extensively for in vitro drug abuse research [7, 8, 19–22] and in the study of astrocytic function [23–30].

Materials and Methods

Chemicals

All chemicals used were of analytical grade. RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin sulfate, amphotericin B, phosphate-buffered saline (PBS) and L-glutamine were obtained from Media Tech (Herndon, VA, USA). Cocaine (Ecgonine methyl ester benzoate) hydrochloride, crystal violet, dichlorodihydrofluorescin diacetate dye (H2DCFDA), L-glutaraldehyde, 0.5 M EDTA (ethylene diamine tetraacetic acid) solution, 5,5-dithiobis-2-nitrobenzoic
acid (DTNB), nicotinamide adenine dinucleotide phosphate (NADPH), 5-sulfosalicylic acid, NAC and trypan blue were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used according to various protocols.

**Cell Culture**

The CNS derived rat C6 astroglia-like cell line (CCL-107) was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained as a monolayer culture as described before [7].

**Immunocytochemistry of Glial Fibrillary Acidic Protein**

We assayed for the presence of glial fibrillary acidic protein (GFAP), an important marker protein expressed abundantly only in astrocytes, in C6 cells. Cells were cultured in 96-well plates (5x10^4/well) overnight following which they were fixed in 4% paraformaldehyde, permeabilized in 0.1% triton X 100 in PBS and incubated with rabbit-anti-rat GFAP 1° antibody (ab7260) (Abcam, Cambridge, MA) for 2 h at room temp. Samples were washed in PBS and subsequently incubated with goat anti-rabbit Alexa Fluor 488 conjugate for 2 h at room temp. These were counterstained for nuclei with propidium iodide (PI) and photomicrographed using an inverted microscope with a 40x objective, CCD camera. Data was acquired using ToupTek View (TouTek Photonics Co, Zhejiang, China).

**Treatment with Cocaine and NAC**

Cocaine hydrochloride stock (1M) and working stock solutions (80–160 mM) were prepared in PBS as described previously [7]. Cytotoxic studies were performed in polystyrene, flat bottom 96-well (0.32 cm²) microtiter plates (BD Labware, NJ, USA). Cells were seeded at a starting density of 2x10^4 cells per well in a total volume of 195 μl of complete RPMI 1640 growth medium with 10% FBS and allowed to adhere overnight in an incubator (37°C, 5% CO₂). Cocaine was added to the medium under sterile conditions using minimal volumes of the working stock solutions (5 μl/well) to achieve final concentrations of 2, 3 and 4 mM without disrupting pH. Untreated cells received equal volumes of PBS (5 μl/well) and served as vehicle controls. Treated samples were interspersed with controls in different wells of the same 96-well microtiter culture plates. Treatment with cocaine was carried out in an incubator (37°C; 5% CO₂) and lasted for 1h to mimic its biological half-life in the body [31, 32] and the fact that a single-dose of cocaine in addicts wears off after an hour for typical amounts and routes of intake (National Institute of Drug Abuse, Research Report Series, March 2010, Bethesda, Maryland, USA). In a subset of experiments, cells were pretreated with 5 mM NAC [33] for 30 min prior to cocaine exposure, while in another set of experiments, 5 mM NAC (30 min) was added to cells post 1h cocaine exposure. Cell viability and GSH levels were assayed at the end of an hour-long incubation using methods outlined below.

**Assessment of Morphology and Vacuolation**

To evaluate gross morphological changes including vacuolation, cells were stained with crystal violet (0.1%) and observed under an inverted phase contrast IX-70 Olympus microscope (Ontario, NY, USA) with a 40x objective. Photomicrographs were taken using an ocular videocamera system (MD35 Electronic eyepiece, Zhejiang JinCheng Scientific & Technology Co., Ltd, HangZhou, China) running C-Imaging System Software (Compix Inc. Cranberry Township, PA, USA).
Assessment of Cell Viability

Cell viability was assessed by the crystal violet dye uptake assay, described previously [34]. Briefly, at the end of the 1h treatment, 100 μl of 0.25% glutaraldehyde was added to each well and the cultures incubated for 30 min to fix cells to the polystyrene surface of culture plates. The plates were then gently washed three times and air-dried. Following staining with 0.1% aqueous crystal violet dye (15 min), the plates were washed and air dried again. The dye in each well was extracted with 100 μl of 50 mM sodium phosphate monobasic solution containing 50% ethyl alcohol. Optical density (OD) measurements of incorporated dye in viable cells were obtained at 540 nm using a microplate reader (Bio-Tek Instruments Inc, Wincoski, VT, USA). Cell viability was re-confirmed using 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) Cell Titer 96 AQueous One Solution Reagent kit (Promega, Madison, WI, USA) and titer plates were read for dye color in a micro plate reader at 490 nm.

Measurements of Intracellular ROS

Cocaine-induced ROS release was measured with H₂DCFDA dye in 96-well plates. Prior to cocaine exposure, 10 μM of the dye was loaded into cells [35] for 30 min and washed. Cells were subsequently treated with phenol red free media before exposure to cocaine. Plates were read using a micro plate fluorometer model 7620, Version 5.02, Cambridge Technology, Inc., (Watertown, MA, USA) with the excitation and emission filters set at 485 and 530 nm, respectively.

Assessment of Total Cellular GSH Levels

Total cellular GSH was assayed according to [36]. Briefly, following cocaine treatment, cells were fixed with 0.25% glutaraldehyde for 30 min followed by gentle washing and air drying. Cells were then deproteinized with 2% 5-sulfosalicylic acid (10 μl/well) for 30 min at 37°C followed by incubation with 90 μl of a reaction mixture containing (in mM): 0.416 sodium EDTA, 0.416 NADPH, 0.835 DTNB and 0.083 sodium phosphate buffer (pH 7.5) for 30 min at 37°C. Absorbance of color was measured on a micro plate reader at 412 nm.

Plasma Membrane Integrity Assay

Cell membrane integrity following exposure to various concentrations of cocaine was determined by measuring lactose dehydrogenase (LDH) release with CytoTox 96 non-radioactive assay kit (Promega) as per kit instructions provided by the manufacturer. Color intensity was measured using a micro plate reader at 490 nm.

Fluorescence Microscopy for Nuclear and F-Actin Staining

Briefly, following cocaine treatment, cells were fixed in 4% paraformaldehyde for 15 min and subsequently permeabilized in 0.25% triton X 100 in PBS for 15 min. Stock solutions for fluorescent dyes (Life Technologies, Carlsbad, CA, USA) were made in ethanol (5 mg/ml) and diluted in HBSS before being added to cells. Final concentrations for PI (excitation: 535 nm, emission 617 nm) and Alexa Fluor 488 Phalloidin (excitation: 488 nm; emission: 520 nm) were 5 μg/ml and 6.6 μM respectively. Images show Alexa Fluor 488 Phalloidin and PI nuclear counterstain to corroborate changes in cytoskeleton F-actin tertiary structure. Samples were analyzed photographically using XDY-1 inverted fluorescent microscope with a 40x objective, CCD camera and data acquired using ToupTek View (TouTek Photonics Co, Zhejiang, China). Fluorescence intensity was acquired using Image J software, National Institutes of Health (NIH), Bethesda, Maryland, USA.
Histone H3-K27 Methylation Assay

Global histone H3-K27 methylation assay kit (Epigentek, Farmingdale, NY, USA) was used to measure H3-K27 methylation in the cells. Briefly, cells (starting density: 1.5x10⁶) were seeded in 100 mm sterile culture dishes per 10 ml of complete DMEM containing 5.5 mM glucose and incubated overnight. They were exposed to cocaine (1h) on the following day and harvested by trypsinization. Histone extraction and methylation detection were done as per the protocol supplied by the manufacturer (Epigentek). OD measurements were obtained from a micro plate reader at 450 nm. The amount of methylation (ng/mg protein) in H3 histone was determined by the following equation: (OD/slope) × 1000. Slope was determined from the standard curve of the positive control (H3-K27) supplied with the kit.

Statistical Analysis

Experimental results are presented as the mean ± standard error of the mean (S.E.M.). The data were analyzed for significance by one-way ANOVA and then compared using Dunnett’s multiple comparison tests in GraphPad Prism Software, version 5 (San Diego, CA, USA). The lethal concentration of cocaine (mM) needed to kill 50% of cells (LC₅₀), was determined as described earlier [37].

Results

Detection of GFAP in C6 Cells

The C6 cells used in this study were initially derived from rat brain tumors [38] and shown to express GFAP [29], a characteristic marker protein of astrocytes. Thus, these cells, used extensively in drug abuse studies, can be considered astrocytic in function. We reconfirmed the astrocytic nature of C6 cells by assaying their GFAP expression. We found that C6 cells indeed expressed high levels of GFAP (Fig. 1).

Figure 1. Detection of glial fibrillary acidic protein marker in C6 astroglia-like cells. Cells were incubated with rabbit-anti-rat GFAP 1° antibody and then with goat anti-rabbit Alexa Fluor 488 conjugate for 2 h. Samples were counterstained for nuclei with PI and photomicrographed using an inverted microscope with a 40x objective.

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The Early Response Changes

Acute exposure of astroglia-like cells to cocaine (2–4 mM for 1h) elicited changes in morphological features, induction of vacuolation, ROS generation, alteration in GSH levels, cell membrane disruption, damage to cytoskeleton, and lysine methylation. These changes will be collectively dubbed as the “early response changes”, described below.

Morphological Changes and Sensitivity to Cocaine Exposure

One of the first and foremost effects of toxic drug exposure is a change in cell morphology. Microscopic observation of crystal violet stained astroglia-like cells revealed that an hour-long exposure to cocaine was enough to evoke profound alterations in general architecture including cell-shape. The whole cell morphology became irregular, intercellular gaps expanded and there was a marked vacuolization as a function of cocaine concentration (Fig. 2B-D). Despite these changes, there was no apparent evidence of nuclear lysis at any of the cocaine doses used. As changes in cell morphology are indicative of cell toxicity, it is evident that astroglia-like cells are extremely sensitive to acute exposure of cocaine.

Cell Viability

Acute exposure to cocaine caused a significant dose-dependent decrease in viability of astroglia-like cells at concentration greater than or equal to 2 mM (n = 9 assays, F = 210.8, P<0.01 at all concentrations with respect to control; Fig. 3A). The average cell viability (± S.E.M.) at 2, 3, and 4 mM cocaine was 79 ± 3.4, 37 ± 3.8 and 9 ± 2.4% respectively, compared with control (100%). The LC50 of cocaine in these cells was 2.7 mM. Study of cell viability by the MTS assay also yielded similar results with an LC50 of 2.8 mM cocaine (data not shown).

ROS Production and Depletion of Total GSH Levels

Excess ROS production in cells could arise from cocaine–mitochondrial interaction resulting in its dysfunction [7, 8]. Indeed, cells treated with 2, 3 and 4 mM cocaine for 1h showed a dose-
dependent increase in ROS release compared with control cells and at the highest concentration of cocaine tested (4 mM), there was as much as ~42% increase in ROS release (n = 4 assays; F = 3.5, P < 0.05; Fig. 3B) suggesting that acute exposure to cocaine causes excessive release of ROS in astroglia-like cells. GSH is one of the most abundant antioxidants in cells. We found, concomitant with increases in ROS production, significant decreases in GSH levels at 3 mM cocaine or higher (n = 6 assays, F = 24.4, P < 0.01; Fig. 3C). GSH levels were 17 ± 2.0 and 26 ± 2.7% of their control values (100%) at 3 and 4 mM cocaine, respectively.

Figure 3. Measurement of the acute effects of cocaine exposure in astroglia-like cells on cell viability (A), ROS production (B), total GSH levels (C), and LDH release (D). Cells were treated with the indicated concentrations of cocaine for 1h. Cell viability was assessed using the crystal violet dye (0.1%) uptake protocol (n = 9). ROS production was assessed by loading cells with a H2DCFDA dye (10 μM, 30 min), followed by cocaine exposure in phenol red–free media. Dichlorodihydrofluorescein (DCF) was measured on a micro plate fluorometer with the excitation and emission filters set at 485 and 530 nm respectively (n = 4). Depletion in GSH levels were quantified on a plate reader following exposure to cocaine (n = 6). For measurements of LDH release, cells in phenol red–free media were treated with cocaine following which they were incubated in equal volumes of media and substrate from the assay kit (50 μl, 30 min) before being read on a micro plate reader (n = 12). Data for all measures are represented as mean ± S.E.M. (n = number of assays), *P < 0.05; **P < 0.001–0.01 w.r.t to control.

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Plasma Membrane Damage and Disruption of F-Actin Filaments

LDH is a ubiquitous constituent of cell cytoplasm. Its presence in the extracellular supernatant is indicative of a compromised plasma membrane following an insult. We observed a significant dose-dependent release of LDH from astroglia-like cells in response to acute exposure to cocaine ($n = 12$ assays, $F = 4.0, P < 0.05$ at all concentrations with respect to control; Fig. 3D). LDH release was increased on average by $81 \pm 39$ and $123 \pm 44\%$ of their control values at 2 and 3 mM respectively and at 4 mM cocaine, there was a greater than 3-fold increase ($344 \pm 83\%$) in the amount of LDH. These data suggest that acute exposure to cocaine damages astroglia-like cell membrane. Yet, despite its effect on plasma membrane, cocaine did not induce nuclear or chromatin fragmentation (karyolysis) as evidenced by PI (red) staining. However, a significant dose-dependent ($n = 3, F = 28.4, P < 0.01$) decrease in phalloidin staining (Fig. 4I) suggests that acute cocaine exposure may disrupt F-actin cytoskeleton (green) in astroglia-like cells (Fig. 4A-D). The average decreases in phalloidin staining compared to control were $54 \pm 5, 35 \pm 3, 33 \pm 1\%$ at 2, 3 and 4 mM cocaine, respectively (Fig. 4I). These data implicate the cytoskeleton as one of the primary cellular targets of injury arising from acute exposure to cocaine.

NAC Pretreatment Prevents Cocaine-Induced Morphological Changes

Microscopic observation of crystal violet stained cells revealed that compared with untreated cells (Fig. 5A), there were no significant morphological differences in either the NAC pretreated control cells (Fig. 4B) or NAC pretreated cells exposed to cocaine (4 mM) (Fig. 5D). A noteworthy difference was the conspicuous absence of vacuoles in the NAC pre-treated cells that were exposed to cocaine compared with those that were exposed to cocaine alone (Fig. 5C, D). We verified that NAC by itself or in combination with cocaine did not cause LDH release nor did it disrupt F-actin cytoskeleton in cells (Fig. 4E). We found almost equal levels of phalloidin in NAC control cells and cells co-treated with cocaine ($P > 0.05$; Fig. 4I).

Attenuation of Cocaine Toxicity with NAC Pretreatment

A dose-dependent viability assay indicated that NAC alone (1–5 mM, 1h) was not toxic to astroglia-like cells [21]. Even at 5 mM NAC, treated cells were as healthy and viable as untreated ones (data not shown) prompting the use of two concentrations of NAC (2.5 and 5 mM) in assaying its protective role against cocaine-induced toxicity. Cells were pretreated with 2.5 or 5 mM NAC for 30 min before exposure to cocaine (2–4 mM) for 1h. The viability data revealed that while NAC pretreatment at 2.5 mM rendered 60–70\% cell protection against cocaine-induced toxicity (data not shown), 5 mM NAC could provide full protection (100\%, $n = 12$ assays, $F = 824, P < 0.01$; Fig. 6A) and was therefore chosen as the preferred dose for the remainder of this study. In an attempt to see if NAC pretreatment could decrease cocaine toxicity when cells are repeatedly exposed to cocaine, the media of cells treated with NAC and cocaine were exchanged for fresh media, before cocaine retreatment (2–4 mM, 1h). Our data (Fig. 6A) suggests that cells were viable despite repeated cocaine treatments (viability 81–100\%; $n = 12$ assays, $F = 288, P < 0.01$) in contrast with cells that were treated with cocaine alone. Interestingly, post NAC exposure caused a marginal but significant ($n = 16$ assays, $F = 365, P < 0.01$) increase in cell viability compared to cells treated with cocaine alone (Fig. 6B). The average increase in the viability due to post NAC exposure was 13, 25 and 6\%, respectively to 2, 3 and 4 mM cocaine exposure. The LC50 was increased from 2.7 to 3 mM cocaine.
GSH is a tripeptide, synthesized from L-cysteine, L-glutamic acid and glycine. NAC enters the cells easily and releases cysteine upon hydrolysis. The released cysteine can serve as a precursor for GSH biosynthesis in astrocytes. In order to determine if NAC pretreatment alters GSH levels in the presence of cocaine, data were analyzed under absorbance at 412 nm (Fig. 6C). GSH
levels in NAC pretreated control cells were significantly higher compared with naïve control cells (no cocaine). This observation supports the hypothesis that cells did indeed utilize the cysteine residue from NAC to synthesize glutathione during the pretreatment period. Exposure to cocaine further increased GSH levels at all concentrations tested, and these levels were significantly greater than those in cocaine exposed cells that were not pretreated with NAC ($n=8–12$ assays, $F=16.3, P<0.01$).

**Attenuation of Histone Methylation by NAC**

Histone methylation, a form of epigenetic chromatin remodeling, may have a major role in drug addiction. Although this process can occur in any of the histones H2, H3 or H4, methylation of histone H3 at K (lysine) 27 position in particular has been associated with gene repression [39] influencing genome function at large and linked to depression-like behavior in animals [40]. To investigate if cocaine treatment could methylate histone H3 at K27 in astroglia-like cells, we used the standard curve for methylated H3-K27 (slope: 0.085; Fig. 7A) to quantify the extent of methylation in our cultures. We found that the amount of methylation at K27 increased significantly as a function of increasing concentrations of cocaine ($n=6$ assays, $F=4.6, P<0.05$ at 3 and 4 mM cocaine; Fig. 7B). For instance, compared with control cells (0%), the increase in methylation observed with 3 and 4 mM cocaine was 85 ± 15% and 69 ± 26%, respectively. Interestingly, while pretreatment of naïve control cells with NAC (5 mM, 30 min) did not alter base line methylation, exposure of these cells to cocaine now abrogated its effects on methylation. Thus, in the presence of NAC, histone methylation levels in cocaine-exposed cells were comparable to those of control cells (no cocaine, Fig. 7B). These data clearly indicate that NAC pretreatment inhibits cocaine-induce methylation of histones in astroglia-like cells.
Discussion

According to the National Institute of Drug Abuse [41], ~3.6 million Americans abuse cocaine on a regular basis, many at an early age, exasperating the problem of cocaine dependency—a major cause of drug-related deaths within the US [42]. Cocaine is highly lipophilic and reaches various domains of the brain easily. Astrocytes, part of the frontline defense against chemical damage in the brain [43], are potential targets of substances of drug abuse. Studies have demonstrated that drugs of abuse like cocaine cause toxic effects in astrocytes by altering their...
morphology and cell size in vivo [44–46]. Cocaine entry into astrocytes could trigger several early response changes which adversely affect their survival. Despite their importance, no studies to date have looked at these acute changes in astrocytes upon cocaine exposure or determined the minimal doses of cocaine needed to compromise their viability in vitro. Because C6 astroglia-like cells exhibit several similarities to astrocytes in terms of gene expression [15], presence of enzymes [16] and GFAP expression (Fig. 1; [29]), we used them to identify the early response changes following acute cocaine exposure.

Cocaine Concentrations

Cocaine concentrations needed to elicit pharmacological responses in brain cells from human addicts have ranged from nano- to micromolar [47] and under in vitro conditions have extended well into the millimolar range [7, 48–51]. Hence, the use of even 10 mM cocaine–2 to 5 times the concentrations used in this study–at even longer incubation intervals are not atypical [48, 50, 52] despite cocaine’s relatively short half-life of ~1 hour in vivo [31, 32]. Furthermore, cocaine doses 1 mM or less, fail to show significant death of astroglia-like cells under in vitro conditions (data not shown), an observation consistent with previous reports [49].

The apparent discrepancy between measurement of cocaine levels in vivo and those required to elicit a cellular response in vitro may be attributed to, among other factors, thermodynamics, wherein the latter offers better control of environmental conditions and therefore greater accuracy of measurements. Nonetheless, the difficulty of correlating in vivo effects based on in vitro data needs to be clearly acknowledged. Likewise, measurements of cocaine levels in vivo also do not necessarily reflect actual amounts of cocaine consumed by an addict [48] owing to, for instance, the development of drug tolerance [53] in which a well-adapted abuser can take in as much as 5g of cocaine per day. Hence, an accurate assessment cocaine levels in the brain requires factoring in of issues such as drug tolerance and/or frequency of use [54] and/or its hydrolysis by blood esterases [55]. The cocaine concentrations used in the present study–deemed of physiological relevance–are not only based on LC50 and EC50 values found in the literature.

Figure 7. Histone methylation with acute cocaine treatment. Standard curve of methylated H3-K27 (A). Cells were pretreated with NAC (5 mM, 30 min; n = 6) followed by exposure to the indicated concentrations of cocaine for 1h (B). Data represent mean ± S.E.M. (n = number of assays), *P<0.05, significance of comparison with corresponding control; #P<0.05, significance of comparison between cocaine alone and cocaine + NAC.  
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[50, 51, 56, 57], but our own assessments of the minimal concentrations needed to induce detectable changes following an acute exposure [7, 8]. The present study establishes that the minimal concentrations of cocaine required to compromise astroglia-like cell viability significantly is 2 mM under in vitro conditions of acute exposure lasting 1h. On average, this translates to ingestion of ~3.3 g of cocaine by an adult human (assuming a total blood volume of ~5 L) and/or ~5 mg of cocaine by a juvenile-adult rat (120 g, assuming a blood volume of 64 ml/Kg), values that are well within the realm of possibility/ experimental research. In addition, we have confirmed that the observed pathology in astroglia-like cells following acute exposure to cocaine is recapitulated in both neuronal and non-neuronal tissue [58–60] and under other conditions of oxidative stress including methamphetamine neurotoxicity [61].

**Early Response Changes**

Drugs that inhibit mitochondrial enzymes interfere with cellular respiration often resulting in the production of excess ROS [62]. GSH, a major antioxidant in cells, counteracts the increased ROS levels to provide protection. We observed that cocaine treatment not only increased ROS production in astroglia-like cells (Fig. 3B), but concomitantly decreased GSH levels—a double insult (Fig. 3C). While increased ROS levels confirm that cocaine interferes with mitochondrial energy metabolism, an observation consistent with our previous studies [7, 8], decreases in astrocytic GSH levels have also been reported in the literature [63]. Depletion in GSH levels make cells more vulnerable to oxidative injury which could lead to cell death via cytoskeleton and plasma membrane damages. Cocaine-induced cytoskeletal damage was reported in neurons [64], but there are no studies of cocaine’s effects on astrocytic cytoskeleton. This is the first report to show rapid damage to F-actin filaments of cytoskeleton in astroglia-like cells upon cocaine exposure (Fig. 4). The dose-dependent LDH release (Fig. 3D) further supports the view that cocaine-induced ROS acts at the level of cytoskeleton and membrane to cause cell death (Fig. 3A). The presence of intact nuclei as indicated by PI staining (Fig. 4) suggests that cocaine-induced cell death is not mediated through apoptosis. Rather, our data collectively suggest that the mechanism of cytotoxicity in C6 astroglia-like cells is through the early response changes (Fig. 8). Pharmacological interventions that inhibit these early response changes of cocaine-induced toxicity in astrocytes may not only prevent astrocytic cell death but neuronal cell death as well owing to their interdependency [12, 13].

**NAC Offers Protection**

Astrocytes contain higher levels of GSH compared to other cell types in the brain [65]. GSH provides both extracellular protection and serves as a precursor for intra neuronal GSH synthesis. Thus, depletion of GSH levels in astrocytes not only makes them prone to oxidative injury but also causes imbalances in the redox potential of neighboring neurons. In our study, given that early response changes underlie loss of astroglia-like cells upon acute exposure to cocaine, we sought ways to counteract and/or prevent them to boost survival rates. Based on the observation that some early response changes such as increased ROS and decreased GSH levels (Figs. 3B, C) could contribute to the cell death, we reasoned that pretreatment with NAC—a well-known antioxidant and therapeutic agent for several oxidant-related CNS diseases [66]—could surmount cocaine toxicity in astroglia-like cells. Recently this compound has been viewed as a potential pharmacological drug for treating cocaine dependence [67].

We observed that NAC pretreatment clearly enabled astroglia-like cells to retain their morphology upon cocaine exposure (Fig. 5) while maintaining 100% viability (Fig. 6A). Astroglia-like cells pretreated with NAC showed no signs of vacuolization even at high levels of GSH (Fig. 5B, D). Interestingly, NAC pretreated cells when subjected to repeated cocaine exposure,
maintained a high cell viability (81–100%), suggesting that NAC could provide protection against recurring drug abuse in addicts. Further research, however, is warranted to ascertain if this result is also applicable to cases in which cocaine addicts have already sought medical treatment following a prolonged exposure. The results of our study mimicking this situation clearly indicate that post NAC exposure significantly rescues cells from cocaine toxicity, albeit to a lesser degree (6–25%, Fig. 6B) – a novel observation.

The NAC-mediated protection did not appear to result from NAC-cocaine complex formation because GSH levels were significantly elevated upon cocaine exposure (Figs. 6C, 8) and may have played a major protective role against cocaine toxicity. Cell viability and GSH production were also unaffected when NAC was withdrawn following pretreatment [21] and, as can be seen from the present study, GSH levels remained invariant (Fig. 6C) between NAC pretreated control cells (no cocaine) and NAC pretreated cells that were exposed to 2–4 mM cocaine. These data suggest that GSH is most likely synthesized during the pretreatment period to render protection during subsequent exposure of the cells to cocaine. NAC’s protection also appears to extend to cocaine-induced methylation in astroglia-like cells as the increased methylation at H3-K27 observed in this study (Fig. 7B) was abrogated by pretreatment with NAC. Studies have shown that histone methylation is associated with neurodegenerative diseases such as Parkinson’s, schizophrenia, Alzheimer’s disease, and other cognitive defects [68] and increased methylation may put cocaine addicts at a risk for these diseases early in life.

**Conclusion**

We identified several early response changes such as alterations in mitochondrial membrane potential [7], mitochondrial respiratory status [8], vacuolation (Fig. 2B), ROS production...
(Fig. 3B), cellular GSH levels (Fig. 3C), F-actin cytoskeleton (Fig. 4), and histone methylation (Fig. 7B) in C6 astroglia-like cells following acute exposure to cocaine. Our data provide compelling evidence to support the hypothesis that inhibition of the early response changes by NAC enhances cell survival through increased GSH levels. Compounds which support GSH synthesis could therefore mitigate toxicity of early response events in cells exposed to cocaine [22]. The recapitulation of cocaine-induced changes observed in our model (e.g. vacuolization [58]) lends credibility to its use for studying cocaine induced toxicity in vivo.

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Author Contributions
Wrote the paper: RBB SSK. Conceived, designed and performed most of the research: RBB. Provided overall support and advice and served as the person-in-charge of the project: CBG. Conducted fluorescent microscopy studies: EM JRA MWD. Performed the methylation study: RDH. Helped interpret the data and write portions of the paper: CAF.

References
1. Fumagalli F, Bedogni F, Frasca A, Di Pasquale L, Racagni G, et al. (2006) Corticostriatal up-regulation of activity-regulated cytoskeletal-associated protein expression after repeated exposure to cocaine. Mol Pharmacol 70: 1726–1734. PMID: 16908598
2. Covington HE 3rd, Maze I, Sun H, Bomze HM, DeMaio KD, et al. (2011) A role for repressive histone methylation in cocaine-induced vulnerability to stress. Neuron 71: 656–670. doi: 10.1016/j.neuron.2011.06.007 PMID: 21867882
3. Maze I, Covington HE 3rd, Dietz DM, LaPlant Q, Renthal W, et al. (2010) Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science 327: 213–216. doi: 10.1126/science.1179438 PMID: 20056891
4. Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ (1987) Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237: 1219–1223. PMID: 2820058
5. Pixley SK (1992) CNS glial cells support in vitro survival, division, and differentiation of dissociated olfactory neuronal progenitor cells. Neuron 8: 1191–1204. PMID: 1610570
6. Attwell D (1994) Glia and neurons in dialogue. Nature 369: 707–708. PMID: 8008062
7. Badisa RB, Darling-Reed SF, Goodman CB (2010) Cocaine induces alterations in mitochondrial membrane potential and dual cell cycle arrest in rat C6 astroglia cells. Neurochem Res 35: 288–297. doi: 10.1007/s11064-009-0053-2 PMID: 19757036
8. Badisa RB, Goodman CB (2012) Effects of chronic cocaine in rat C6 astroglial cells. Int J Mol Med 30: 687–692. doi: 10.3892/ijmm.2012.1038 PMID: 22735768
9. Tsacopoulos M, Magistretti PJ (1996) Metabolic coupling between glia and neurons. J Neurosci 16: 877–885. PMID: 8558256
10. Abbott NJ, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7: 41–53. PMID: 16371949
11. Abbott NJ (2013) Blood-brain barrier structure and function and the challenges for CNS drug delivery. J Inherit Metab Dis 36: 437–449. doi: 10.1007/s10545-013-9608-0 PMID: 23609350
12. Chiu SY, Kriegler S (1994) Neurotransmitter-mediated signaling between axons and glial cells. Glia 11: 191–200. PMID: 7927647
13. Araque A (2006) Astrocyte-neuron signaling in the brain—implications for disease. Curr Opin Investig Drugs 7: 619–624. PMID: 16869114
14. Little KY, Ramssen E, Welcho R, Volberg V, Roland CJ, et al. (2009) Decreased brain dopamine cell numbers in human cocaine users. Psychiatry Res 168: 173–180. doi: 10.1016/j.psychres.2008.10.034 PMID: 19233481
15. Sibenaller ZA, Etame AB, Ali MM, Barua M, Braun TA, et al. (2005) Genetic characterization of commonly used glioma cell lines in the rat animal model system. Neurosurg Focus 19: E1. PMID: 16398474
16. Zimmer DB, d Van Eldik LJ (1989) Analysis of the calcium-modulated proteins, S100 and calmodulin, and their target proteins during C6 glioma cell differentiation. J Cell Biol 108: 141–151. PMID: 2910876

17. Bissell MG, Rubinstein LJ, Bignami A, Herman MM (1974) Characteristics of the rat C-6 glioma maintained in organ culture systems. Production of glial fibrillary acidic protein in the absence of glialfibrillogenesis. Brain Res 82: 77–89. PMID: 4373142

18. Shao Z, Dyck LE, Wang H, Li XM (2006) Antipsychotic drugs cause glial cell line-derived neurotrophic factor secretion from C6 glioma cells. J Psychiatry Neurosci 31: 32–37. PMID: 16496033

19. Garg UC, Turmdorf H, Bansinath M (1993) Effect of cocaine on macromolecular syntheses and cell proliferation in cultured glial cells. Neuroscience 57: 467–472. PMID: 7509470

20. Gu J, Yassini P, Goldberg G, Zhu W, Konat GW, et al. (1993) Cocaine cytotoxicity in serum-free environment: C6 glioma cell culture. Neurotoxicology 14: 19–22. PMID: 8361674

21. Badisa RB, Goodman Carl B, Fitch-Pye Cheryl A (2013) Attenuating effect of N-acetyl-L-cysteine against acute cocaine toxicity in rat C6 astroglial cells. Int J Mol Med, 32(2): 497–502. doi: 10.3892/ijmm.2013.1391 PMID: 23708443

22. Badisa RB, Fitch-Pye CA, Agharahimi M, Palm DE, Latinwo LM, et al. (2014) Milk thistle seed-extract prevents acute cocaine toxicity in rat C6 astroglial cells. Mol Med Reports 10: 2287–2292. doi: 10.3892/mmr.2014.2524 PMID: 25174449

23. Mangoura D, Sakellaridis N, Jones J, Vernadakis A (1989) Early and late passage C-6 glial cell growth: similarities with primary glial cells in culture. Neurochem Res 14: 941–7. PMID: 2575233

24. Feng Z, Zhang JT (2004) Protective effect of melatonin on beta-amyloid-induced apoptosis in rat astroglia C6 cells and its mechanism. Free Radic Biol Med 37:1790–801. PMID: 1528038

25. Cechin SR, Dunkley PR, Rodnight R (2005) Signal transduction mechanisms involved in the proliferation of C6 glioma cells induced by lysophosphatidic acid. Neurochem Res 30:603–11. PMID: 16176093

26. Funchal C, Dos Santos AQ, Jacques-Silva MC, Zamoner A, Gottfried C, et al. (2005) Branched-chain alpha-keto acids accumulating in maple syrup urine disease induce reorganization of phosphorylated GFAP in C6-glioma cells. Metab Brain Dis 20: 205–17. PMID: 16187198

27. Chen YC, Chow JM, Lin CW, Wu CY, Shen SC (2006) Bicarbonate inhibition of oxidative-stress-induced apoptosis via modulation of ERKs activation and induction of HO-1 gene expression in rat glioma cells C6. Toxicol Appl Pharmacol 2006a; 216:263–73. PMID: 16814338

28. Chen TJ, Jeng JY, Lin CW, Wu CY, Chen YC (2006)b Quercetin inhibition of ROS-dependent and DNA damage in C6 glioma cells. Neurochem Res 31: 867–872. PMID: 16176093

29. Quincozes-Santos A, Bobermin LD, Latini A, Wajner M, Souza DO, et al. (2013) Resveratrol protects C6 astrocyte cell line against hydrogen peroxide-induced oxidative stress through heme oxygenase 1. PLoS ONE 8(5): e64372. doi: 10.1371/journal.pone.0064372 PMID: 23691207

30. Quincozes-Santos A, Andreazza AC, Nardin P, Funchal C, Gonçalves C, et al. (2007) Resveratrol attenuates oxidative-induced DNA damage in C6 glioma cells. NeuroToxicology 28: 886–891. PMID: 17498806

31. Chow MJ, Ambre JJ, Ruo TI, Atkinson AJ Jr., Bowsher DJ, et al. (1985) Kinetics of cocaine distribution, elimination, and chronotropic effects. Clin Pharmacol Ther 38: 318–324. PMID: 20828628

32. Barnett G, Hawks R, Resnick R (1981) Cocaine pharmacokinetics in humans. J Ethnopharmacol 3: 353–366. PMID: 7242115

33. Odewumi CO, Badisa VL, Le UT, Latinwo LM, Ikediobi CO, et al. (2011) Protective effects of N-acetylcysteine against cadmium-induced damage in cultured rat normal liver cells. Int J Mol Med 27: 243–248. doi: 10.3892/ijmm.2010.564 PMID: 21125209

34. Badisa RB, Tzakou O, Couladis M, Pilarinou E (2003) Cytotoxic activities of some Greek Labiatae herbs. Phytother Res 17: 472–476. PMID: 12748981

35. Lopez-Erauskin J, Galino J, Ruiz M, Cvezua JM, Fabregat I, et al. (2013) Impaired mitochondrial oxidative phosphorylation in the peroxisomal disease X-linked adrenoleukodystrophy. Hum Mol Genet 22: 3296–3305. doi: 10.1093/hmg/ddt186 PMID: 23604518

36. Smith IK, Vierheller TL, Thorne CA (1988) Assay of glutathione reductase in crude tissue homogenates using 5,5’-dithiobis(2-nitrobenzoic acid). Anal Biochem 175: 408–412. PMID: 3239770

37. Ippen J, Feingl P (1970) Bancroft’s introduction to biostatistics. New York: Harper & Row. PMID: 17043231

38. Benda P, Lightbody J, Sato G, Levine L, Sweet W (1968) Differentiated rat glial cell strain in tissue culture. Science 161: 370–371. PMID: 4873531
40. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, et al. (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9: 519–525. PMID: 16501568

41. National Institute of Drug Abuse, Research Report Series, March 2010, Bethesda, Maryland, USA.

42. Phillips K, Luk A, Soor GS, Abraham JR, Leong S, et al. (2009) Cocaine cardiotoxicity: a review of the pathophysiology, pathology, and treatment options. Am J Cardiovasc Drugs 9: 177–196. doi: 10.2165/00129784-200909030-00005 PMID: 19463023

43. Park SJ, Lee JH, Kim HY, Choi YH, Park JS, et al. (2002) Astrocytes, but not microglia, rapidly sense H(2)O(2) via STAT6 phosphorylation, resulting in cyclooxygenase-2 expression and prostaglandin release. J Immunol 188: 5132–5141. doi: 10.4049/jimmunol.1101600 PMID: 22504638

44. Fattore L, Puddu MC, Picciau S, Cappai A, Fratta W, et al. (2002) Astroglial in vivo response to cocaine in mouse dentate gyrus: a quantitative and qualitative analysis by confocal microscopy. Neurosci 188: 5132–5141. PMID: 11882367

45. Pearson J, Richter RW (1979) Addiction to opiates: neurologic aspects. In Handbook of Clinical Neurology. Intoxications of the Nervous System, Part II. Eds Vinken PJ, Bruyn GW. Amsterdam: North-Holland Publishing Company, 365–400. PMID: 583334

46. Büttner A, Weis S (2006) Neuropathological alterations in drug abusers, The involvement of neurons, glial, and vascular systems. Forensic Sci Med Pathol 2: 115–126.

47. Zheng F, Zhan CG (2012) Modeling of pharmacokinetics of cocaine in human reveals the feasibility for development of enzyme therapies for drugs of abuse. PloS Comput Biol 8: e1002610. doi:10.1371/journal.pcbi.1002610 PMID: 22844238

48. Repetto G, Del Peso A, Garfia A, Gonzalez-Munoz MJ, Salguero M, et al. (1997) Morphological, biochemical and molecular effects of cocaine on mouse neuroblastoma cells culture in vitro. Toxicol In Vitro 11: 519–525. PMID: 9491492

49. Oliveira MT, Rego AC, Morgadinho MT, Macedo TR, Oliveira CR (2002) Toxic effects of opioid and stimulant drugs on undifferentiated PC12 cells. Ann N Y Acad Sci 969: 487–496. PMID: 12105124

50. Cunha-Oliveira T, Rego AC, Cardoso SM, Borges F, Swedlow RH, et al. (2006) Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or ampheta-mine. Brain Res 1089: 44–54. PMID: 16638611

51. Cunha-Oliveira T, Rego AC, Morgadinho MT, Macedo T, Oliveira CR (2006) Differential cytotoxic responses of PC12 cells chronically exposed to psychostimulants or to hydrogen peroxide. Toxicology 217: 54–62. PMID: 21242230

52. Kugelmass AD, Oda A, Monahan K, Cabral C, Ware JA (1993) Activation of human platelets by cocaine. Circulation 88: 876–883. PMID: 7649042

53. Stewart A, Heaton ND, Hogbin B (1990) Body packing—a case report and review of the literature. Postgrad Med J 66: 659–661. PMID: 2217036

54. Stichensehr M, Stelwag-Carion C, Klupp N, Honigschnabl S, Vychudilik W, et al. (2000) Suicide of a body packer. Forensic Sci Int 108: 61–66. PMID: 10697780

55. Fraker TD Jr., Temesy-Armos PN, Brewster PS, Wilkinson RD (1990) Mechanism of cocaine-induced myocardial depression in dogs. Circulation 81: 1012–1016. PMID: 2306831

56. Smart RG, Anglin L (1987) Do we know the lethal dose of cocaine? J Forensic Sci 32: 303–312. PMID: 3572327

57. Vitullo JC, Karam R, Mekhail N, Wicker P, Engelmann GL, et al. (1988) Cocaine induced small vessel spasm in isolated rat hearts. Am J Pathol 135: 85–91. PMID: 2774060

58. Finol HJ, Mondragon DD, Gonzalez YM, Marquez A, Gonzalez N, et al. (2000) Hepatocyte ultrastructural alterations in cocaine users. J Submicrosc Cytol Pathol 32: 111–116. PMID: 10877709

59. Bahar M, Cole G, Rosen M, Vickers MD (1984) Histopathology of the spinal cord after intrathecal cocaine, bupivacaine, lignocaine and adrenaline in the rat. Eur J Anaesthesiol 1: 293–297. PMID: 6549556

60. Yu CT, Li JH, Lee TC, Lin LF (2008) Characterization of cocaine-elicited cell vacuolation: the involvement of calcium/calmodulin in organelle deregulation. J Biomed Sci 15: 225–226. PMID: 17922255

61. Cubells JF, Rayport S, Rajendran G, Sulzer D (1994) Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. J Neurosci 14: 2260–2271. PMID: 8158268

62. Lotharius J, Dugan LL, O’Malley KL (1999) Distinct mechanisms underlying neurotoxin-mediated cell death in cultured dopaminergic neurons. J Neurosci 19: 1284–1293. PMID: 9952406
63. Bragin DE, Zhou B, Ramamoorthy P, Muller WS, Connor JA, et al. (2010) Differential changes of glutathione levels in astrocytes and neurons in ischemic brains by two-photon imaging. J Cereb Blood Flow Metab 30: 734–738. doi: 10.1038/jcbfm.2010.9 PMID: 20104233

64. Su TP, Hayashi T (2001) Cocaine affects the dynamics of cytoskeletal proteins via sigma(1) receptors. Trends Pharmacol Sci 22: 456–458. PMID: 11543872

65. Dringen R (2000) Metabolism and functions of glutathione in brain. Prog Neurobiol 62: 649–671. PMID: 10880854

66. Deigner HP, Haberkorn U, Kinscherf R (2000) Apoptosis modulators in the therapy of neurodegenerative diseases. Expert Opin Investig Drugs 9: 747–764. PMID: 11060707

67. LaRowe SD, Mardikian P, Malcolm R, Myrick H, Kalivas P, et al. (2006) Safety and tolerability of N-acetylcysteine in cocaine-dependent individuals. Am J Addict 15: 105–110. PMID: 16449100

68. Kramer JM (2012) Epigenetic regulation of memory: implications in human cognitive disorders. Biomedical concepts 4: 1–12.