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Methamphetamine augments concurrent astrocyte mitochondrial stress, oxidative burden and antioxidant capacity: tipping the balance in HIV-associated neurodegeneration

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Abstract

Methamphetamine (METH) use, with and without human immunodeficiency virus (HIV)-1 comorbidity, exacerbates neurocognitive decline. Oxidative stress is a probable neurotoxic mechanism during HIV-1 central nervous system infection and METH abuse; as viral proteins, antiretroviral therapy and METH have each been shown to induce mitochondrial dysfunction. However, the mechanisms regulating mitochondrial homeostasis and overall oxidative burden in astrocytes are not well understood in the context of HIV-1 infection and METH abuse. Here we report METH-mediated dysregulation of astrocyte mitochondrial morphology and function during prolonged exposure to low levels of METH. Mitochondria became larger and more rod shaped with METH when assessed by machine learning, segmentation analyses. These changes may be mediated by elevated mitofusin expression coupled with inhibitory phosphorylation of dynamin-related protein-1, which regulate mitochondrial fusion and fission, respectively. While METH decreased oxygen consumption and ATP levels during acute exposure, chronic treatment of one to two weeks significantly enhanced both when tested in the absence of METH. Together, these changes significantly increased expression of antioxidant proteins, augmenting the astrocyte's oxidative capacity, but also oxidative damage. We propose that targeting astrocytes to reduce their overall oxidative burden and expand their antioxidant capacity could ultimately tip the balance from neurotoxicity towards neuroprotection.

Keywords

Astroglia; neurotoxicity; mitochondria; oxidative stress; machine learning; extracellular flux; dynamin-related protein; mitofusin

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Compliance with Ethical Standards

Conflict of interest

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Introduction

As a popular psychostimulant, methamphetamine (METH) use leads to long-lasting, strong euphoric effects and is highly addictive. Data collected by the National Institute on Drug Abuse state that 25% of diagnosed human immunodeficiency virus (HIV)-1-infected individuals report treatment for the use of drugs and alcohol (CDC 2007; NIDA 2012). In a study of 1168 HIV-1+ gay and bisexual men in New York and San Francisco, approximately 10–15% of the men reported METH use (Purcell et al. 2005). This is a staggering comorbidity, as METH use exacerbates HIV-1 infection, accelerating the severity and onset of HIV-associated neurocognitive disorders (HAND), along with immune dysfunction and resistance to therapy (Salamanca et al. 2014; Var et al. 2016). METH abuse results in neurotoxic outcomes including deficits in memory and executive function; increasing anxiety, depression and psychosis; and functional dependence (Blackstone et al. 2013; Cadet and Krasnova 2009; Gupta et al. 2011; Nagai and Yamada 2010; Rippeth et al. 2004; Rusyniak 2013). Studies report that 53–58% of HIV-1+ METH users exhibit neurocognitive impairment compared to 40% in either HIV-1+ or METH+ alone; although, their interaction is not well understood (Gupta et al. 2011; Rippeth et al. 2004). Both METH and HAND neuropathogenesis mechanistically concur with neuroinflammation, astrocyte activation, excitotoxicity and oxidative stress; however, how METH alters astrocyte contributions is less clear.

Astrocyte activation is an important commonality of METH abuse and HIV-1 CNS infection. It is multifaceted and can range from upregulation of glial fibrillary acidic protein (GFAP), morphological changes, cellular proliferation, calcium signaling imbalance, oxidative and endoplasmic reticulum (ER) stress, to mitochondrial dysfunction and excitotoxicity (Chaboub and Deneen 2013; Ojeda et al. 2014; Richardson et al. 1999; Valsecchi et al. 2013). The effect of METH on neurons is well studied. It directly binds the neuronal trace amine associated receptor 1 (TAAR1), which reverses the action of the dopamine transporter leading to secretion of dopamine into the synapse (Miller 2011; Xie and Miller 2009). The dopamine transporter is normally responsible for dopamine reuptake, and without this function, the person experiences an extreme, long-lived euphoria. Prolonged use leads to tolerance and the amount of METH needed to achieve a comparable effect increases significantly (Cho and Melega 2002; Segal and Kuczenski 2006). Two receptors have been implicated in mediating astrocyte responses to METH, TAAR1 (Cisneros and Ghorpade 2014) and sigma receptors (Robson et al. 2014; Zhang et al. 2015).

Investigations in human and rodent brains have shown that astrocytes are activated during METH exposure (Silva et al. 2014; Tong et al. 2014). METH is found in rodent brain within minutes of injection at a 13:1 brain to plasma ratio. In rats, brain METH levels achieved a peak level of ~1000 ng/g tissue (6 μ M) within minutes when injected intravenously with a METH dose comparable to those used by humans (1 mg/kg). While pregnant mice brain METH levels reached 294–335 ng/mg (2 mM) one hour post injection with injected subcutaneously with a much higher dose of 40 mg/kg. Brain levels remained elevated (60–600 nM) through 4–6 hours in both studies (Riviere et al. 2000; Won et al. 2001). The euphoria provoked by METH is long compared to many drugs of abuse, lasting 8–24 hours depending on delivery method and tolerance (NIDA 1998). Since METH is often repeatedly

abused by addicts, it remains in the central nervous system (CNS) for prolonged periods at low levels.

Several studies suggest METH-induced neurotoxicity involves the production of reactive oxygen species (ROS) *via* mitochondrial dysfunction and increased energy, thereby mediating oxidative pathways (Mashayekhi et al. 2014; Riddle et al. 2006; Sanchez-Alavez et al. 2013). Methamphetamine is known to directly impair mitochondrial function as it can inhibit complexes II and III of the electron transport chain, induce mitochondrial permeability transition pore (mPTP) opening, and loss of the mitochondrial membrane potential, which facilitates oxidative phosphorylation and ATP production (Thrash et al. 2009). HIV-associated proteins and therapies also dysregulate mitochondrial dynamics, function and axonal trafficking (Avdoshina et al. 2016; Cote 2007; Fields et al. 2016). Mitochondrial homeostasis, in terms of biogenesis, size and recycling, is maintained by a dynamic set of proteins. Mitofusin (MFN) 1 and 2 mediate mitochondrial fusion to salvage healthy areas and segregate damaged ones. Dynamin-related protein-1 (Drp-1) regulates mitochondrial fission during both mitochondrial division and recycling by mitophagy. When oxidative damage accumulates, Drp-1 fragments damaged mitochondria, which are enveloped by microtubule associated protein light chain 3 (LC3) with the help of other autophagy mediators. The autophagosome then fuses with a lysosome to degrade the damaged mitochondrial bud. When mitochondrial homeostasis is disrupted during drug abuse and HIV-1 CNS infection the oxidative burden can become neuropathological oxidative stress (Fields et al. 2016; Lau et al. 2000; Mashayekhi et al. 2014; Shah et al. 2013).

Reactive nitrogenous species (RNS) and ROS participate in signaling and metabolic pathways during physiological conditions (Ray et al. 2012). During homeostasis, antioxidant enzymes, including glutathione, glutathione reductase, super oxide dismutase (SOD), and catalase, tightly regulate and neutralize reactive molecules such as superoxide, hydrogen peroxide (H₂O₂) and hydrogen radicals. Excessive ROS induced by a variety of mechanisms, including inflammatory cytokines, mitochondrial respiration, ischemia, and infection, are implicated in aging, cardiovascular disease, diabetes, stroke and neurodegeneration (Cobb and Cole 2015; Raz et al. 2015; Salisbury and Bronas 2015). Unchecked oxidative and nitrosative modifications to cellular components, such as the mitochondria, often augment oxidative stress and induce apoptosis (Cossarizza et al. 2002; Indo et al. 2015; Jou 2008). Neurons intrinsically have low antioxidant defenses and rely heavily on support from astrocytes (Baxter and Hardingham 2016). Thus, an astrocyte under undue oxidative burden may fail to provide sufficient neuronal support, as it attempts to restore homeostasis within, leading to neurotoxicity.

Here we evaluate the effects of acute and chronic METH exposure on mitochondrial morphology, function and antioxidant capacity in human astrocytes. As expected, short-term HIV-1 mediated mPTP opening, while acute METH increased oxidative damage, and decreased oxygen consumption rate (OCR) and ATP levels. However, by using an innovative low dose, prolonged exposure *in vitro* paradigm, which mimics astrocyte METH exposure during chronic abuse, we were able to detect and describe a phenomenon never before measured in human astrocytes. Mitochondria became enlarged when exposed to METH at

levels comparable to those measured *in vivo*. Further, these changes were accompanied by dysregulation of mitochondrial regulatory proteins, promoting fusion and decreasing fission. Interestingly, prolonged METH treatment defied expectation, significantly *elevating* OCR, ATP and antioxidant expression in human astrocytes. Understanding of astrocyte response to METH-mediated dysfunction will highlight new ways to therapeutically target oxidative stress during neurodegenerative disease.

Methods

Isolation, cultivation and activation of primary human astrocytes

Human astrocytes were isolated from first and early second trimester elected fetal brain specimens as previously described (Gardner et al. 2006). Tissues were procured in full compliance with the ethical guidelines of the National Institutes of Health, Universities of Washington and North Texas Health Science Center. Cell suspensions were mechanically dissociated by filtering through a Nitex mesh and trituration. When a single cell suspension was achieved, cells were washed with several low speed centrifugation/trituration steps and cultured initially at a density of 50×10^6 cells/ 150 cm^2 at 37°C and 5% CO_2 . Every 7–10 days, adherent astrocytes were passaged with trypsin-EDTA and cultured at 20×10^6 cells/ 150 cm^2 to enhance the purity of replicating astroglial cells until the preparations were >99% pure as measured by immunocytochemistry staining for GFAP. Astrocytes were treated with METH (50 nM, 5 μM or 500 μM , Sigma-Aldrich Inc., St. Louis, MO) for 24 hours to 16 days, with passaging every 6–10 days. Astrocytes were plated in the presence of METH.

METH exposure and dosing

By back calculating the concentration of METH in the brain, the peak (6 μM –2 mM) and prolonged ranges (60–600 nM) were determined for *in vitro* investigations (Riviere et al. 2000; Won et al. 2001). In this context, astrocytes were treated with METH (50 nM, 5 μM , 100 μM or 500 μM , Sigma-Aldrich Inc.,) for 24 hours to 16 days, with passaging every 6–10 days. Astrocytes were plated for experimental assays in 3–6 replicates in the presence of METH.

Mitochondrial morphological assessment by trainable Weka segmentation

Astrocytes were cultured in T150 flasks in the presence of METH (50 nM, 5 μM or 500 μM) for seven days. Cells were passaged and plated into 48 well plates at 1×10^5 cells/well in multiple replicates in control or METH containing media and allowed to recover overnight. Astrocytes were then loaded with Mitotracker Red® (MTR, 1:1000 Thermo Fisher Scientific, Waltham, MA) and Hoechst 33342 (nuclear blue, 1:1000) fluorescent dyes for 15–20 min. Cultures were washed with Hank's balanced salt solution (HBSS) and then imaged in 100 μl phenol-free ASM with ProLong® Live anti-fade reagent (1:1000, Thermo Fisher Scientific). Live cell micrographs (10 or more) were taken of each treatment at 400X original magnification using an Eclipse Ti-300 (Nikon Instruments, Inc, Melville, NY) equipped with a black and white Luca R (Andor Technology, Belfast, Ireland). Images were then pseudocolored with NIS-Elements software (Nikon). Mitochondrial morphology was assessed using Fiji ImageJ software; Version: 2.0.0-rc-41/1.5d and machine learning

“Trainable Weka Segmentation” (version 3.211, Weka V3.9.0) as developed by Arganda-Carreras *et al.* (2017). The Weka was trained based on mitochondrial morphology (punctate, rod shaped, large spots and networked mitochondria) as previously described (Collins *et al.* 2002; Dagda *et al.* 2009; Wang *et al.* 2008; Yu *et al.* 2006). It was then used to quantify the average area occupied by each morphological category in individual micrographs and the fold change in mitochondrial size across treatments.

Briefly, the mitochondrial layer from each micrograph was extracted and contrast was enhanced using the software process “enhance contrast” (saturated pixels set to 0.3%). The images were then segmented by the trained Weka described above. The probability image stack was then separated into five individual layers (punctate, rod, large spot, network and background) (supplementary Fig. 1). Each layer was inverted to show mitochondria-specific fluorescence as black pixels, and then the threshold was adjusted to optimally resolve individual mitochondria. A macro was developed to quantify each image with the process “analyze particles” and saved the number of particles, size of particles, and percent image area occupied to an Excel worksheet. To calculate the percent area occupied by each mitochondrial morphology within a micrograph, the percent of total image area for each layer (punctate, rods, large spots, and networks) was totaled and taken as total mitochondrial area. The percent of total mitochondrial area was then calculated by dividing the image area for each morphological type by the total mitochondrial area. The average size across untreated control (0 nM) images was calculated for each mitochondrial type (punctate, rods, large spots, and networks) and used to compute fold change in average size across treatment groups (METH 50 nM, 5 μ M and 500 μ M).

Mitochondrial morphology and permeability transition pore assay

The Image-iT® LIVE Mitochondrial Transition Pore Assay Kit (Thermo Fisher Scientific) was used according to a modified protocol. Briefly, astrocytes were plated at 5×10^4 or 1×10^5 in 96 or 48 well plates, respectively and treated for 24 hours with METH (500 μ M) alone and in combination with HIV-1_{JR-FL} (HIV-1 p24, 10 ng/ml). The cells were then loaded with the mPTP indicators: MTR (mitochondria, 1:1000), calcein-AM (green, 1:1000), Hoechst (nucleus, 1:1000), and cobalt chloride (cytosolic calcein quencher, 1:250) for 15–20 min in phenol free-HBSS. Cultures were washed with HBSS and mitochondrial depolarization was detected when cobalt chloride quenched green calcein fluorescence in the cytoplasm leaving predominantly red fluorescence (MTR) in the mitochondria. Live cell micrographs (5 or more) were taken of each treatment at 200X original magnification using an Eclipse Ti-300 (Nikon) equipped with a black and white Luca R (Andor). Images were then pseudocolored with NIS-Elements software (Nikon).

Real-time gene expression analyses

Astrocyte RNA was isolated by the Trizol method (Thermo Fisher Scientific) followed by DNA digestion and precipitation. A Nanodrop fluorospectrometer (Thermo Fisher Scientific) was used to assess RNA purity and to quantify total RNA levels. Transcripts were made into cDNA with the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) at 50–100 μ g/ml. Expression levels were measured by real-time polymerase chain reaction (PCR) using Taqman® gene expression assays and the StepOnePlus detection

system (Thermo Fisher Scientific). Assay identifiers used in human cells are compiled in Table 1. The 10 µl reactions with 50–100 ng cDNA were carried out at 50°C for 2 min, 95°C for 20 sec, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec in 96-well optical, real-time PCR plates. Transcript levels were normalized to GAPDH quantified in a duplex PCR reaction. Astrocyte expression levels are represented as fold changes to respective controls as calculated by the comparative CT method (Livak and Schmittgen 2001).

Protein isolation, identification and analysis

Total cellular proteins were isolated by lysing cells directly with mammalian protein extraction reagent (MPER, Thermo Fisher Scientific). Protease and phosphatase inhibitors were used in all lysates (Sigma). Protein levels were determined by Precision Red advanced protein reagent (Cytoskeleton, Inc, Denver, CO) or the BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. The levels of specific proteins were determined by western blot using the Bolt electrophoresis system, the iblot transfer system (Thermo Fisher Scientific) and imaged in a Fluorochem HD2 (ProteinSimple, San Jose CA) or by the WES capillary protein detection system (ProteinSimple) according to manufacturer's directions. Between 20–40 µg of protein was loaded in Bolt gels and 0.04 – 0.4 mg/ml protein was loaded into WES capillaries. Blots or columns were probed with antibodies for cytochrome c oxidase (COX) IV-HRP (mouse, Cell Signaling, Danvers, MA) (WB 1:1000, WES 1:50), MFN 1/2 (rabbit, Cell Signaling) (WB 1:1000, WES 1:200), Drp-1 (rabbit, Cell Signaling) (WB 1:000), phosphorylated (p) serine 637-Drp-1 (mouse, Abcam, Cambridge, MA) (WB 1:500), LC3 A/B (rabbit, Cell Signaling) (WB 1:1000), malondialdehyde (MDA) (rabbit, Cell Biolabs, San Diego, CA) (WB 1:1000) and GAPDH (mouse, Santa Cruz, Dallas, TX) (WB 1:1000, WES 1:50) antibodies. Secondary detection was achieved with goat anti-rabbit-HRP and anti-mouse-HRP. Chemiluminescence was detected with SuperSignal West Femto (Thermo Fisher Scientific) on the FluorochemHD or WES column based protein detection. Densitometry for the HD2 or WES was calculated using AlphaView (SA) and Compass for SW, respectively.

Mitochondrial oxygen consumption and ATP levels

Astrocytes were plated at 7.5×10^4 /well in seahorse assay plates or at 5×10^4 /well in 96 well plates overnight with their respective treatments at 37°C and 5% CO₂. Mitochondrial OCR was measured by extracellular flux (XF) assay (Seahorse XFp analyzer, Agilent Technologies, Santa Clara, CA). Levels of ATP were determined by ATP bioluminescence assay (Sigma) according to manufacturer's procedures. Briefly, XF cartridges were hydrated overnight with XF calibrant at 37°C in the absence of CO₂. In parallel, XF assay media (pH 7.4) was warmed to 37°C in the absence of CO₂. One hour prior to the assay, culture treatments were removed and XF media was added with and without the TAAR1 selective antagonist EPPTB [*N*-(3-ethoxyphenyl)-4-(1-pyrrolidinyl)-3-(trifluoromethyl) benzamide] (20 µM, Bio-Techne, Minneapolis, MN) (Bradaia et al. 2009; Stalder et al. 2011). Cultures were then placed in a CO₂-free incubator to acclimate for up to an hour. The XF sensor cartridge was loaded with oligomycin (final concentration 2 µM) as a positive control for oxygen consumption inhibition and METH doses, if applicable. Micro changes in pH, as a measure of oxygen consumption, were assayed in three minute intervals repeated three times per well. In acute experiments, wells were sequentially analyzed over three hours as control,

100 μ M METH, 500 μ M METH and finally oligomycin (OligM). Treatment OCR were normalized to initial control readings specific to each well. In chronic experiments, no METH was injected and separate wells were assayed as control (0 nM), 50 nM and 5 μ M METH prolonged exposure. In parallel, astrocytes in 96 well plates were lysed with 50 μ l nucleotide releasing buffer and the equivalent of 5×10^4 cells were used to assess ATP levels in control and METH-treated astrocytes in a GloMax luminometer (Promega, Madison, WI). Percent changes were calculated control verses METH-exposed astrocytes in 6–8 wells per treatment per donor.

Statistical Analyses

Prism V7 (GraphPad Software, La Jolla CA) was used to determine statistical significance ($P < 0.05$). For comparisons with more than one variable, a one-way ANOVA followed by Dunnett's multiple comparisons test was used, while a student's t-test was used in single variable analyses.

Results

METH alters astrocyte mitochondrial morphology and size

To investigate how mitochondrial morphology would be affected by prolonged METH exposure cultured human astrocytes were treated with increasing doses of METH continuously for one week. Live mitochondrial morphology was visualized with MTR (red) and nuclei with Hoechst (blue) (Fig. 1). Astrocyte mitochondria appeared longer when treated with 50 nM, 5 μ M and 500 μ M as compared to untreated controls (0 nM) (Fig. 1b–d and a, respectively). To quantify METH-mediated changes in mitochondrial morphology, area and size; micrographs were assessed by trainable Weka segmentation (Arganda-Carreras et al. 2017). Examples of the mitochondrial morphological categories: punctate, rods, large spots, and network are highlighted in Fig. 1, and morphological layers as identified by the Weka from a representative live imaging micrograph are shown in supplementary Fig. 1a–f. Stacked layers from at least ten representative images were used to calculate the area occupied by each mitochondrial type. The area of punctate, large spots and networked mitochondria did not change significantly with METH (Fig. 1e, g, h). However, the area occupied by rod shaped mitochondria increased significantly with 50 nM and 5 μ M METH (Fig. 1f, $P < 0.05$ and $P < 0.01$, respectively). Since the shape and size of mitochondria can affect mitochondrial function and recycling (Westermann 2010), the average size of each morphological type was compared across METH concentrations. The average size of punctate mitochondria in untreated control astrocytes was $87 \pm 52 \mu\text{m}^2$, and increased approximately 1.5-fold with METH exposure, regardless of concentration (Fig. 1i, $137 \pm 33 \mu\text{m}^2$ $P < 0.05$ and $P < 0.01$). Changes in rod shaped mitochondria were the most visually noticeable in MTR-labeled astrocytes. The average size of rod shaped mitochondria increased significantly with higher METH concentrations (Fig. 1j, 5 μ M, $725 \pm 312 \mu\text{m}^2$ $P < 0.01$; 500 μ M, $851 \pm 206 \mu\text{m}^2$ $P < 0.001$) as compared to controls (average $430 \pm 300 \mu\text{m}^2$). While the area of large spots and networks did not change, the average size of each increased with exposure to 500 μ M METH (Fig. 1k, l, $P < 0.05$). Thus, METH significantly affected the size and shape of mitochondria. Given that several regulatory proteins tightly regulate mitochondrial shape and size, we next examined key players in these mechanisms.

METH dysregulates mitochondrial regulatory proteins

In parallel to morphological experiments, levels of key mitochondrial proteins were assessed by real-time PCR and immunoblotting to decipher the regulatory process underlying METH-mediated changes. As a key member of the electron transport chain, cytochrome c oxidase (COX) IV is often used to identify and normalize expression of mitochondrial proteins. Levels of COX IV were elevated by 10 to 20% after METH exposure for 1 week (Fig. 2a, e, $P < 0.05$). Next, the levels of proteins regulating mitochondrial fusion (MFN) and fission (Drp-1) were assayed by real-time PCR. Mitofusin was elevated by approximately 20%, regardless of METH concentration (Fig. 2b, $P < 0.05$), while Drp-1 increased by $17 \pm 0.06\%$ with 5 μM METH only (Fig. 2c, $P < 0.05$). Despite the overall modest changes in mRNA levels, the data suggest a proportional rise in both fusion and fission proteins, which would theoretically maintain, rather than alter, mitochondrial morphology. To investigate further, the protein levels of MFN 1/2 and Drp-1 were examined. METH did not statistically alter MFN protein levels; however, MFN changes were variable across astrocyte donors ($n=6$) and levels were either unchanged or elevated, not decreased (Fig. 2f). Furthermore, the ratio of phosphorylated-Drp-1 to total Drp-1 protein increased by $196 \pm 56\%$ (Fig. 2g, $P < 0.01$). Phosphorylation at serine 637 blocks the mitochondrial fission activities of Drp-1. To evaluate the induction of autophagy in METH-treated astrocytes LC3 expression and protein levels were assessed. Although overall expression of LC3 was not altered by METH as compared to controls (Fig. 2d), total LC3 levels and cleavage of LC3I to LC3II increased significantly (Fig. 2h, $P < 0.05$).

METH and HAND-relevant stimuli induce mitochondrial dysfunction

As discussed above, METH abuse is a prevalent comorbidity in the US HIV-1+ population, and both are known inducers of mitochondria dysfunction. To assess the effects of this on human astrocytes, astrocytes were acutely exposed to HIV-1_{JR-FL} (10 ng/ml HIV-1 p24) alone and in combination with METH (500 μM) for 24 hours. Cultures were then loaded with mitochondrial marker MTR (red) and nuclear marker Hoechst (blue). The state of the mPTP was visualized with calcein-AM (green) and the quencher cobalt chloride, which selectively quenches green fluorescence in the cytoplasm and not the mitochondria. Colocalization of green and red fluorescence indicates closed mPTP, while red only indicates mPTP opening. Control astrocytes possessed primarily red/green mitochondria (Fig. 3a, a1 arrow). HIV-1 treatment decreased green mitochondrial fluorescence, indicating mPTP opening and depolarization (Fig. 3b1, arrowheads). Treatment with METH alone and in combination with HIV-1 showed little effect on mPTP opening (Fig. 3c1–d1).

Mitochondrial oxidative consumption and ATP levels in METH-treated astrocytes

METH is known to directly interfere with the mitochondrial electron transport chain, which maintains an electrochemical potential and ultimately permits oxidative phosphorylation and ATP synthesis. To investigate the effect of METH on astrocyte OCR and ATP generation, astrocytes were treated with an augmenting dose of 100 μM and 500 μM METH over 2–3 hours during an XF Seahorse assay. Oligomycin, a known inhibitor of ATP synthase, was used as a positive control for OCR changes. Not surprisingly, 500 μM METH significantly decreased oxygen consumption as compared to controls (Fig. 4a, $P < 0.001$). Since TAAR1

has been identified as a METH receptor in neurons and astrocytes (Cisneros and Ghorpade 2014; Xie and Miller 2009), astrocytes were treated in parallel with the TAAR1 selective antagonist EPPTB for one hour prior to the XF assay. Contrary to our expectations, EPPTB failed to block METH-mediated decreases in OCR (Fig. 4a, $P < 0.001$). To determine if OCR correlated with available ATP, astrocytes were treated for three hours with 0 nM, 50 nM, 5 μ M and 500 μ M, and ATP was measured by luciferase assay. Levels of ATP decreased significantly with all METH doses (Fig. 4b, $P < 0.01$, $P < 0.001$) as compared to control (0 nM). To assess this phenomenon during low dose, prolonged exposure, astrocytes were treated with 50 nM for one week prior to testing OCR and ATP levels in the absence of METH. Surprisingly, prolonged METH exposure augmented OCR by $250 \pm 7\%$ (Fig. 4c, $P < 0.001$). However, the METH-mediated increase in OCR did not directly correlate with ATP levels, which rose by only $20 \pm 4\%$ (Fig. 4d, $P < 0.001$). In this context, we next investigated METH-induced changes in antioxidant expression and oxidative damage.

METH enhances the antioxidant capacity and oxidative burden in chronically treated human astrocytes

As mitochondrial dysfunction is directly implicated in oxidative stress, we investigated antioxidant expression during prolonged METH exposure. Acute high-level METH (500 μ M) exposure induced oxidative stress as demonstrated MDA modification of cellular proteins in 24 hours (data not shown). To assess whether astrocytes could maintain antioxidant responses during prolonged METH-mediated mitochondrial stress, astrocytes were treated with 50 nM, 5 μ M and 500 μ M for two weeks. Cultures were passaged weekly and untreated astrocytes (0 nM) were maintained in parallel. After two weeks OCR remained significantly elevated in cultures exposed to lower doses of METH (Fig. 5a, 50 nM $P < 0.01$), and appeared to decrease at the highest dose (500 μ M). Levels of mRNA of key antioxidant proteins were assessed by real-time PCR in parallel. Glutathione reductase expression, an enzyme that maintains the ROS scavenging capacity of glutathione, was significantly augmented by 5 μ M and 500 μ M METH (Fig. 5b, $P < 0.001$). Levels of SOD1, which convert super oxide anions into H_2O_2 , were also elevated by prolonged METH exposure, regardless of concentration (Fig. 5c, $P < 0.01$, $P < 0.001$). Similar increases were not evident in SOD2, which is expressed specifically in mitochondria (Fig. 5d). Catalase is capable of hydrolyzing H_2O_2 into water, which is the final step in ending the action of ROS such as those produced in the mitochondria. METH significantly augmented catalase levels in chronically treated astrocytes (Fig. 5e, $P < 0.01$, $P < 0.001$). However, MDA-modification of cellular proteins (Fig. 5f), a marker of oxidative damage, increased in parallel with antioxidant protein levels such as SOD1 (Fig. 5f) in total cell lysates following two weeks of METH exposure.

Discussion

Mitochondrial dysfunction and oxidative stress are mechanistically implicated in aging, cardiovascular disease, diabetes, stroke, neurodegeneration, drug abuse and HAND (Avdoshina et al. 2016; Cobb and Cole 2015; Mashayekhi et al. 2014; Raz et al. 2015; Salisbury and Bronas 2015). A variety of mechanisms including inflammatory cytokines, mitochondrial respiration, ischemia, and infection generate ROS, which damage cellular

DNA, proteins and lipids to mediate pathology during disease. During HIV-associated neurodegeneration, mitochondrial stress occurs in most neural cells types [as reviewed by (Rozzi et al. 2017)]. As oxidative stress is a common neurotoxic mechanism during HIV-1 CNS infection and METH abuse, we investigated the effect of METH on astrocyte mitochondrial function and oxidative capacity.

Our study demonstrates METH-induced dysregulation of mitochondrial morphology by inactivation of fission proteins and concurrent increase of fusion transcripts in human astrocytes. Acute treatments with HIV-1 led to mPTP opening, and METH decreased ATP availability. Contrary to our expectations, chronic METH augments mitochondrial oxygen consumption and ATP levels during breaks in METH exposure. However, these were not proportional, suggesting that METH also elevates oxidative stress in astrocytes. In response, astrocytes enhance antioxidant expression, yet oxidative damage remains evident. As key determinants of neuronal survival, the antioxidant capacity of astrocytes is a critical therapeutic target for HIV- and METH-induced oxidative stress. Further investigations are needed to determine if the astrocytic oxidative burden during HIV-1 and METH coexposure exceeds their antioxidant capacity leading to direct or indirect toxicity to neurons (Fig. 6). Targeting astrocytes to decrease their oxidative burden, while promoting their antioxidant capacity, should be considered in development of future therapies in HAND and METH abuse.

Since the discovery of HIV-1, viral proteins and antiretroviral therapy are recognized as potent inducers of neuronal apoptosis *via* calcium overload, caspase activation, oxidative stress and apoptosis, often mediated through mitochondrial dysfunction (Blas-Garcia et al. 2011; Kallianpur et al. 2016; Kruman et al. 1998; Perry et al. 2005; Roumier et al. 2002; Singh et al. 2004). HIV-1 glycoprotein (gp)120 and viral protein R (Vpr) decrease oxygen consumption, impair axonal mitochondrial trafficking, and promotes mitochondrial depolarization in neurons (Avdoshina et al. 2016; Fields et al. 2016; Saxena et al. 2016; Wang et al. 2017). Our data show that acute HIV-1-treatment induces mPTP opening. Other studies also demonstrate HIV-1 gp120 mediated ER stress and apoptosis in simian virus 40 transformed astrocytes (SVGA) (Shah et al. 2016). HIV-1 transactivator of transcription (Tat) increases mitophagy, as measured by LC3 cleavage, and is associated antioxidant depletion, a sign of oxidative stress (De Simone et al. 2016; Porntadavity et al. 2005). Further, several antiretroviral drugs interfere with physiological mitochondrial function, contributing to oxidative stress. Nucleotide/side reverse transcriptase inhibitors (NRTI) interfere with mitochondrial polymerase gamma leading to mitochondrial DNA damage, ribonucleotide deficiency and dysfunction of the electron transport chain (Cote 2007; Selvaraj et al. 2014). Alternatively, protease inhibitors have anti-apoptotic effects by inhibiting HIV-1 protease-mediated loss of the mitochondrial membrane potential and caspase processing (Phenix et al. 2001). Together, these data indicate that HIV-1 and METH induce comparable mitochondrial impairment, thus, their comorbidity would likely exacerbate the oxidative burden in neural cells.

Recently, impaired mitochondrial recycling, including enlarged and elongated mitochondria in the soma of damaged neurons, was observed in the frontal cortex tissues of gp120-transgenic mice and HIV-1+ individuals (Fields et al. 2016). HIV-1 gp120 alters the

physiological balance between mitochondrial fission proteins, including Drp-1, and fusion proteins such as MFNs (Avdoshina et al. 2016; Fields et al. 2016). Together these proteins regulated mitochondrial division, segregation, and axonal transport in neurons. A cohort of 27 HIV-1+ individuals with HAND showed higher MFN protein levels and diminished Drp-1 activation, as measured by serine 616 phosphorylation in frontal cortex lysates (Fields et al. 2016). An imbalance in the function of these two proteins may mediate increases in mitochondrial size. Our studies in astrocytes showed that prolonged METH exposure induced analogous dysregulation in astrocytes, which could be exacerbated with HIV-1 coexposure.

TAAR1 is upregulated by HIV-associated activation in astrocytes (Cisneros and Ghorpade 2014). As a stimulatory G-protein coupled receptor, TAAR1-mediated cAMP signaling may modulate the activity of mitochondrial morphology regulators including Drp-1 and MFN. Protein kinase A (PKA) phosphorylates Drp-1 at serine 637 and 656, which in turn, downregulates GTPase activity, ultimately resulting in reduced mitochondrial fission (Chang and Blackstone 2007; Cribbs and Strack 2007; Hyun et al. 2017). PKA activity depends on cAMP, suggesting that METH signaling through TAAR1 could downregulate or inhibit Drp-1 function. Normally during mitochondrial depolarization, sustained cytosolic calcium activates the cytosolic phosphatase calcineurin, which dephosphorylates Drp-1 and permits Drp-1 recruitment to mitochondria (Cereghetti et al. 2008). However, in the context of HIV-1 in the brain and the prolonged METH exposure in astrocytes, cAMP-dependent PKA activity appears to be the predominant regulator of Drp-1 phosphorylation. Mitochondrial fragmentation is often observed during apoptosis, which facilitates mitophagy, and plays a role in cytochrome c release, cristae remodeling and Bcl-2-associated X protein (BAX) accumulation (Ko et al. 2016) [as reviewed by (Westermann 2010)]. Conversely, hypermitofusion may be a survival strategy during starvation and cellular stress. Mitofusin 1 primarily regulates mitochondrial fusion; and MFN 2 tethers mitochondria to ER membranes. Both MFN 1/2 mediate their function through interaction with the Bcl-2 family of proteins. Moreover, MFN activation is regulated by reversible ubiquitination. Deubiquitylases stabilize MFN levels to promote mitochondrial fusion and network formation, while ubiquitin ligases facilitate MFN degradation, permitting fission (Anton et al. 2013). MFN is also regulated by extracellular signal-regulated kinase (ERK) 1/2-mediated phosphorylation at tyrosine 562, which helps promote Bcl-2 homologous antagonist/killer (BAK) oligomerization and apoptosis (Pyakurel et al. 2015). Together these data suggest that dysregulation of mitochondrial regulatory proteins may be targeted in astrocytes by attenuating METH-mediated secondary messenger signaling downstream of METH receptors such as sigma receptors or TAAR1.

Several groups have shown that METH induces loss of the mitochondrial membrane potential and/or mPTP opening in neurons and striatal cells (Deng et al. 2002; Langford et al. 2004; Turchan et al. 2001). Methamphetamine (between 2.5–20 μ M) directly mediates mitochondrial dysfunction through complexes II and III in isolated liver mitochondria, which also rapidly elevates ROS in parallel (Mashayekhi et al. 2014). Despite degradation of ROS by antioxidants, oxidative damage can accumulate and necessitate mitochondrial turnover by mitophagy. Our data show activation of LC3 cleavage indicating that METH stimulates autophagy in astrocytes; although not that mitophagy is occurring, because

increased mitochondrial size can impede autophagosome development [as reviewed by (Gomes and Scorrano 2013)]. Our results also demonstrate that METH dysregulates mitochondrial morphology, inducing larger rod shaped mitochondria. This together with elevated COX IV levels and augmented OCR/ATP may indicate that METH-exposed astrocytes have more mitochondria, perhaps due to impaired mitophagy or to compensatory mechanisms in an effort to synthesize sufficient ATP.

In SVGA, METH (500 μ M) initiates the unfolded protein response and ER stress (Shah and Kumar 2016); which are linked to mitochondrial dysfunction *via* calcium imbalance. Shah's work builds upon previous studies implicating METH in astrocyte neuroinflammatory responses and oxidative stress (Shah et al. 2013; Shah et al. 2012). Much of their work results in HIV-1 gp120- and METH-mediated astrocyte apoptosis (Shah and Kumar 2016; Shah et al. 2016). In our studies acute METH treatment failed to trigger mPTP opening, and overwhelming apoptosis was not provoked in primary human astrocytes, as evidenced by survival during prolonged METH treatment for 1–2 weeks. It would be a worthwhile endeavor to study ER stress, mPTP opening, and apoptosis induction in the prolonged exposure model as inadequate astrocyte antioxidant support would contribute to neuronal toxicity during METH and/or HIV-1 CNS insult. Interestingly, a single low dose METH exposure is proposed as a protective therapy, improving functional behavior and cognition, in a traumatic brain injury rat model (Rau et al. 2016). Our results show that astrocytes have the capacity to significantly increase antioxidant levels, presumably to protect themselves and the neurons they support.

As terminally differentiated cells, neurons have weak antioxidant capacity and rely heavily on astrocytes for expression of antioxidant response element (ARE)-regulated genes (Baxter and Hardingham 2016). Genes in this family include glutathione reductase and synthase, SODs and catalase. Our data demonstrate that the expression of key antioxidants, including glutathione reductase, SOD1 and catalase, in response to a severe insult such as 500 μ M METH, can be robustly enhanced in astrocytes. However, similar increases were not evident in SOD2, signifying that the antioxidant response is regulated and not indiscriminate. Importantly, prolonged exposure to a physiological, low METH level (50 nM) also modestly elevated (20%) antioxidant levels. This suggests that the antioxidant response is tapered to the insult and that astrocytes have the capacity to promote survival whilst undergoing mitochondrial stress, dysfunction and oxidative damage.

While METH can directly mediate oxidative stress in neurons and astrocytes by inhibiting the electron transport chain, this is not the only pathway to ROS. Exogenous application of dopamine to astrocytes, mimicking METH-induced dopamine release into the synapse by neurons, produces a dose-dependent increase in intracellular calcium concentrations ($[Ca^{+2}]_i$) stimulating transient calcium uptake in mitochondria and mitochondrial dysfunction (Vaarmann et al. 2010). Astrocyte METH receptors such as TAAR1 or sigma receptors modulate cAMP and calcium signaling that may in turn dysregulate mPTP opening and mitochondrial potential (Cisneros and Ghorpade 2014; Zhang et al. 2015). Further, dopamine metabolism generates aldehydes and H_2O_2 that induce lipid peroxidation, phospholipase C activation and $[Ca^{+2}]_i$ by IP_3 -dependent and independent mechanisms. Astrocyte sensitivity to dopamine- and glutamate-induced $[Ca^{+2}]_i$ flux is responsible for

deficiencies in cellular redox homeostasis (Narita et al. 2008). Unchecked ROS and RNS can damage cellular components, such as proteins, lipids, and DNA; often amplifying stress and triggering cell death (Cossarizza et al. 2002; Indo et al. 2015; Jou 2008). Since excessive oxidative burden is implicated in aging, cardiovascular disease, diabetes, stroke and neurodegeneration (Cobb and Cole 2015; Raz et al. 2015; Salisbury and Bronas 2015), the mechanisms regulating the antioxidant capacity of astrocytes in the brain are an important therapeutic target.

The balance between oxidative stress and antioxidant expression are tightly linked in astrocytes. Both endogenous and exogenous stimuli induce oxidative stress and antioxidant responses in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, stroke and HAND [reviewed by (Barnham et al. 2004)]. Mitochondrial dysfunction *via* dysregulation of mitochondrial turnover during METH and HIV-1 comorbidity may be targeted in astrocytes, as it is feasible that TAAR1-mediated inhibition of Drp-1 fission activity could be blocked with a selective antagonist, such as EPPTB. Restoration of physiological mitochondrial turnover would reduce oxidative stress and tip the scale towards antioxidant capacity. Alternatively, catalase delivery to mitochondria can extend the life span of mice and decrease age-related heart disease (Dai et al. 2010; Dai et al. 2009; Schriener et al. 2005). While targeting the brain with such a strategy poses challenges, the implications for neurodegenerative disease are thereby exciting. Given that neurons rely upon astrocytes for antioxidants in the brain, targeting astrocytic mitochondria would increase antioxidant levels in the immediate neuronal milieu without altering physiological gene expression in neurons. Since astrocytes processes extend from the blood brain barrier to multiple neuronal synapses, they will be excellent targets for oxidative stress combating therapeutic strategies.

Conclusion

Our data clearly demonstrate that METH dysregulates astrocyte mitochondrial morphology and function, which in turn, concurrently increase both their oxidative burden and antioxidant capacity. This Yin-Yang of oxidative stress and antioxidant capacity in astrocytes reflects a delicate balance in brain redox homeostasis (Fig. 6). Similar dichotomous dysregulation is seen in neurons and astrocytes alike in the context of HIV-associated neurological disease providing common mechanistic links in neurodegeneration. In the brain microenvironment, neurons are ultimately dependent upon astrocytes for antioxidant support, thus acutely sensitive to astrocyte oxidative burden. Our results reveal that therapeutically targeting the source of oxidative stress in the astrocytes, while promoting expression of antioxidant responsive genes in unison, will help tip the balance towards robust antioxidant capacity and reduced oxidative burden. Such a therapeutic approach will be novel on multiple fronts. First, it will target astrocytes, a paramount target destination in the brain to spare neurons from unnecessary functional intervention [as reviewed in (Joshi et al. 2017)]. Second, it will focus on restoring mitochondrial health, an intracellular target, facilitating development of novel drug delivery approaches. Lastly, given the ubiquitous presence of mitochondria and the roles of mitochondrial function and oxidative stress in disease mechanisms, these data will have far-reaching applications beyond preserving astrocytes in HIV-associated neurodegeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Astrocyte mitochondria become enlarged with prolonged exposure to METH
Human astrocytes were exposed to 0 nM (a, Control), 50 nM (b), 5 μM (c), or 500 μM (d) METH for one week. Mitochondria were then fluorescently labeled with Mitotracker red (MTR, red) and nuclei with Hoechst (blue). Live cultures were imaged at an original magnification of 400X. Total mitochondria were segmented into morphological types including punctate (e, i), rod (f, j) large spots (g, k) and networks (h, l) with Weka machine learning in Fiji, ImageJ (see supplementary Fig. 1 for example layers). The area occupied by mitochondria of each type (e, f, g, h) and fold change in average size (i, j, k, l) were calculated based on the analysis of segmented layers in each of 10–15 micrographs per condition. Representative images of each mitochondrial morphology are shown, below representative images from each treatment. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test with GraphPad Prism (V7) and error bars are standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Fig. 2. METH increases mitochondrial regulatory protein levels and dysregulates their activity Primary human astrocyte cultures were treated with METH (50 nM and 5 μM) for one week. Levels of cytochrome c oxidase (COX) IV (a, e), mitofusin (MFN) (b, f), dynamin-related protein 1 (Drp-1) (c, g) and microtubule associated protein light chain 3 (LC3) (d, h) were evaluated by real-time PCR and immunoblotting or WES, respectively. Graphs are cumulative average expression level in 3 to 6 separate astrocyte donors and representative blots are shown. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test (a–d) or student's test (e–g) with GraphPad Prism (V7). Error bars are SEM. Hatched lines indicate blot cropping of non-adjacent bands. *P<0.05, **P<0.01

Fig. 3. Mitochondrial permeability transition pores (mPTP) open during HIV-1 exposure
Astrocyte cultures were treated with HIV-1_{JR-FL} (10 ng/ml HIV-1 p24, b/b1), METH (500 μ M, c/c1), or both (d/d1) for 24 hours. Control (a/a1) and treated astrocytes were then fluorescently labeled with Mitotracker Red® (MTR, red), calcein-AM (green, when sequestered in mitochondria) and the nuclear stain Hoechst (blue). Examples of astrocytes with closed mPTP are indicated by red/green (yellow) mitochondrial fluorescence (arrows). Open mPTP are visualized by decreased mitochondrial green fluorescence (arrowheads), as calcein is quenched when released into the cytoplasm by cobalt chloride, suggesting mitochondrial dysfunction. Original magnification 200X

Fig. 4. Prolonged METH increases oxygen consumption and ATP levels in primary human astrocytes

Since TAAR1 is a known astrocyte receptor for METH, astrocytes were pretreated with the selective TAAR1 antagonist EPPTB (20 μ M) for one hour (a). Oxygen consumption was measured by extracellular flux assay (Seahorse) with the sequential injection of 100 μ M and 500 μ M METH over the course of three hours. Oxygen consumption rates (OCR) were normalized to control (0 μ M) and EPPTB pretreated rates, respectively on a per well basis. Oligomycin, an ATP synthase inhibitor, was used as a positive control for decreased OCR. Bars represent the average OCR in seven separate astrocyte cultures $\pm$ SEM. In a separate experiment ATP levels were measured three hours post-METH treatment (50 nM-500 μ M) by luciferase assay and compared to control (0 nM) levels (b). Bars represent the average fold change in relative light units (RLU) in three separate astrocyte cultures $\pm$ SEM. In another paradigm, astrocytes were treated with 50 nM METH for one week and OCR (c) and ATP levels (d) were assayed following METH withdrawal. Bars represent the average OCR or fold change in ATP in two independent astrocyte cultures $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test (a-b) or student's test (c-d) with GraphPad Prism (V7). ** P <0.01, *** P <0.001

Fig. 5. Chronic METH exposure increases the antioxidant capacity and oxidative burden of human astrocytes

To examine the effects of prolonged METH exposure, astrocytes were treated with METH between 50 nM and 500 μ M for two weeks. Untreated control (0 nM) astrocytes were maintained in parallel. Oxygen consumption rate (OCR) was measured in the absence of METH by extracellular flux Seahorse assay (a). Antioxidant protein expression was measured by real-time PCR. The average fold change in glutathione reductase (b), superoxide dismutase (SOD) 1(c), SOD2 (d) and catalase (e) expression levels as compared to control (0 nM) are shown. As measure of oxidative balance, levels of malondialdehyde (MDA)-modified proteins and SOD1 were evaluated in total cell lysates by immunoblotting in parallel. GAPDH levels were evaluated as housekeeping and loading controls, respectively. Panels are representative experiments repeated in two independent astrocyte donors. Significance was determined with one-way ANOVA followed by Dunnett's multiple comparisons test with GraphPad Prism (V7). ** $P < 0.01$, *** $P < 0.001$

Fig. 6. METH disturbs the delicate Yin & Yang of oxidative burden and antioxidant capacity in human astrocytes

Prolonged METH exposure induces changes in mitochondrial morphology and size in astrocytes (Fig. 1). Mitochondria become larger and rod shaped. Direct and indirect effects of METH on intracellular signaling and the electron transport chain likely contribute to this phenomenon. METH increases astrocyte mitofusin (MFN) levels, which may promote mitochondrial fusion and networking. In parallel, METH induces inhibitory phosphorylation of dynamin-related protein-1 (Drp-1) at serine 637, which may decrease mitochondrial fission (Fig. 2). Together these changes in mitochondrial regulatory proteins may disturb mitochondrial homeostasis, morphology and oxidative stress. HIV-1 induces mitochondrial permeability transition pore (mPTP) opening (Fig. 3), a measure of mitochondrial stress and dysfunction, while acute METH exposure impairs mitochondrial respiration. Prolonged METH exposure augments oxygen consumption (O₂) and ATP availability (Fig. 4), suggesting an overall increase in astrocyte mitochondria. METH-mediated oxidative stress promotes the expression of antioxidant response element (ARE) regulated genes increasing antioxidant levels in astrocytes (Fig. 5). Since neurons are highly dependent on astrocytes for antioxidant support, neurotoxicity successfully prevented if the antioxidant capacity of METH-exposed astrocytes is greater than the oxidative burden of astrocyte and adjacent neurons combined. Astrocyte directed therapy could tip the balance towards increasing antioxidant capacity while reducing oxidative burden.

Table 1

Assay Identifiers used in human astrocytes.

Gene expression assay target	Assay number (Thermo Fisher)	Dye
CAT, catalase	Hs00156308_m1	(FAM/MGB)
Cytochrome c oxidase (COX) IV	Hs00971639_m1	(FAM/MGB)
Dynamin-related protein (Drp)-1	Hs01552605_m1	(FAM/MGB)
Glutathione reductase	Hs00167317_m1	(FAM/MGB)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	4310884E	(VIC/TAMRA)
Mitofusin (MFN) 1	Hs00966851_m1	(FAM/MGB)
Super oxide dismutase (SOD) 1	Hs00533490_m1	(FAM/MGB)
Super oxide dismutase (SOD) 2	Hs00167309_m1	(FAM/MGB)