Published in final edited form as:

Am J Neuroprot Neuroregen. 2009 June ; 1(1): 52–59. doi:10.1166/ajnn.2009.1004.

Methamphetamine Dysregulates Redox Status in Primary Rat Astrocyte and Mesencephalic Neuronal Cultures

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Abstract

Astrocytes provide structural, metabolic and trophic support to neurons. They are directly involved in the regulation of neuronal transmission and synaptic activity and respond to the synaptic release and remove neurotransmitters from the extracellular fluid. The dysfunction of astrocytes has been implicated in multiple neurotoxicities, including those associated with drugs of abuse.

Methamphetamine (METH) has long-lasting neurotoxic effects, yet little is known about the mechanisms that govern METH-induced neural dysfunction, and especially the astrocytic control over the extracellular milieu. The purpose of this study was to clarify the response of astrocytes and neurons treated with METH and determine their relative sensitivity to this drug of abuse.

Confluent rat primary astrocyte and mesencephalic neuron cultures were treated for 24 hrs with 0, 0.1, 0.5 or 1 mM METH, and the initial rate of glutamate and glutamine uptake was measured over a 5 min period. Additional studies examined the effect of METH (24 hr exposure at similar concentrations) on oxidative endpoints, namely glutathione (GSH) levels, lactate dehydrogenase (LDH) release and isoprostane (IsoP) levels, considered to be the most accurate biomarker of lipid peroxidation. There was no effect of METH on the rates of glutamate and glutamine uptake, and these were indistinguishable from controls. However, METH concentration-dependently affected astrocytic and neuronal GSH levels, leading to a significant decrease in redox potential at all of the tested concentrations ($p < 0.05$). METH also significantly enhanced astrocytic LDH release at the 0.5 and 1.0 mM exposures. Consistent with the changes in IsoPs, METH (0.5 and 1.0 mM) also increased the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor with a key role in regulating oxidative stress responses. However, this Nrf2 increased in expression was observed only in astrocytes and no effect was noted in neurons. Taken together, this study establishes that METH affects both astrocyte and neuronal functions, and that oxidative stress is a proximate mechanism for METH's-induced neurotoxicity on both cell types. Furthermore, in response to oxidative stress astrocytes efficiently upregulated Nrf2 nuclear translocation and transcription. These effects were absent in neurons. Combined with their lower content of GSH, these characteristics may account for the greater sensitivity of neurons to METH-induced toxicity.

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Keywords

Methamphetamine; Astrocytes; mesencephalic neurons; Oxidative Stress; LDH; Nrf2; Glutamate; Neurotoxicity

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Introduction

Methamphetamine (METH) is one of the most widely used drug of abuse; its highly addictive properties are known to produce a variety of neuropsychiatric complications (Lan et al., 1998; Anglin, et al., 2000; Freese et al., 2002) characterized by neurodegenerative effects in various animal models (Deng et al., 1999, 2002, Cadet et al., 2003; 2007). Acute and chronic exposure of METH can produce depletion of dopamine (DA) and its metabolites (O'Callaghan and Miller, 1994; Imam et al., 1999; Ali and Itzhak, 2000), and decrease the number of DA transporter (DAT) binding sites (Itzhak and Ali, 1996), as well as the activity of tyrosine hydroxylase (TH) in the striatum (Kogan et al., 1976). Corroborating these observations, METH has been reported to deplete DA and DAT binding in the postmortem brain of chronic METH users (Wilson et al., 1996). Recently, we and others have also reported that METH can produce neurotoxicity by generating reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Itzhak et al, 1998; Imam et al, 2001; Imam and Ali, 2001; for review see Itzhak and Ali, 2006). Furthermore, a high dose of METH and the DA D-1 like receptor agonist SKF-822958 increased striatal nNOS mRNA expression in mice (Wang and Lau, 2001) as well as striatal nNOS enzymatic activity in rats (Lin et al., 1999).

There is ample evidence in support of METH-induced DAergic-nitergic interactions, likely via glutamatergic activity. In the striatum, prefrontal cortex, hippocampus and amygdala, DA can facilitate or attenuate glutamatergic transmission depending on the type of DA glutamate receptor stimulation (Bowyer et al., 2008). Although glutamate is an ubiquitous neurotransmitter, certain glutamatergic projections are thought to be critically impacted by addictive drugs (Kalivas et al., 2008; Kalivas and O'Brien, 2008; Wolf, 1998).

The function of other CNS cell types (astrocytes, microglia, and oligodendrocytes) in METH-induced neurotoxicity has yet to be fully understood. Although astrocytes do not conduct action potentials, nor do they form synapses with neuronal cells, they interact amongst themselves and with neurons in a complex fashion, assuring optimal structural, metabolic and trophic support throughout life. With the advent of novel technologies, recent breakthroughs in astrocyte physiology have established dynamic and reciprocal signaling networks between astrocytes and neurons (Aschner and Kimelberg, 1991; Aschner and LoPachin, 1993; Aschner et al., 2002). Astrocytes are the most abundant of the mammalian CNS cell types, playing a role in wide variety of CNS pathologies, including trauma, ischemia and neurodegeneration (Pekny and Pekny, 2004) and possibly drug addiction. One of the most important links between neurons and astrocytes is the glutamate-glutamine shuttle. Astrocytes transport the vast majority of extracellular glutamate (especially neuronal pools) and convert to glutamine (Pekny and Pekny, 2004). This glutamine is shuttled back to presynaptic terminals, and is critical for the synthesis of neurotransmitter glutamate.

The abundance of polyunsaturated fatty acids in the CNS can lead to lipid peroxidation as a major consequence of free radical-mediated injury. Lipid peroxidation proceeds until the oxidizable substrate is consumed, making this fundamentally different from many other forms of free radical injury in that the self-sustaining nature of the process results in extensive damage. Because of their chemical instability and their rapid and extensive metabolism (Moore and Roberts, 1998), the use of *in vivo* reactive products of lipid peroxidation as biomarkers is limited. For these reasons, more stable lipid products of oxidative damage have generated interest, including the F₂-isoprostanes (F₂-IsoPs), which are formed by free radical-mediated peroxidation of arachidonic acid (AA) (Watts et al., 1997; Reich et al., 2000; 2001). Sources of free radicals that contribute to IsoP formation include the mitochondrial electron transport system, the P450 family of drug-metabolizing enzymes, the NADPH oxidases, the lipoxygenases, and transition metal-catalyzed formation of ROS.

Nrf2 is commonly involved in the induction of genes encoding antioxidant proteins. It heterodimerizes with members of the small Maf family of transcription factors and binds to the cis-acting antioxidant response element (ARE) sequence in the promoter regions of various phase II detoxification genes. The latter encode for a group of enzymes, such as glutamate-cysteine ligase (GCL), glutathione transferase (GST) A1 and A2, glutathione peroxidase (GPx), heme oxygenase, NAD(P)H:quinone reductase, and the glutamate-cysteine exchanger (Xc⁻) that are essential in the detoxification of xenobiotics and endogenous reactive electrophilic compounds (Kensler et al., 2007). As expected, Nrf2 knockout mice show increased sensitivity to a variety of pharmacological and environmental toxicants (Kensler et al., 2007).

Taken together, and given the lack of apparent reports which have assessed the direct effects of METH on astrocytes, we investigated astrocytic responses to this drug of abuse in both astrocytes and mesencephalic dopamine neurons. We measured different biomarkers, namely glutamate/glutamine uptake (in astrocytes), GSH, LDH and F₂-IsoPs and the transcription factors, Nrf-2 to determine the comparative sensitivity of cultured astrocytes and mesencephalic neurons to METH exposure.

Materials and Methods

Primary astrocyte and mesencephalic neuron culture:

The procedure for isolation and culturing of astrocytes from cerebral cortices of newborn (1-day-old) Sprague–Dawley rats (Aschner et al., 1994) was approved by the institutional committee for animal research and was performed in accordance with the guidelines of the National Institutes of Health (NIH) for the care and use of laboratory animals. Briefly, rat pups were decapitated, and the cerebral cortices were removed. After removal of the meninges, the cerebral cortices were digested with bacterial neutral protease (Dispase, Invitrogen, Carlsbad, CA) and astrocytes were recovered by repeated removal of dissociated cells from brain tissues. Twenty-four hours after the initial plating in 6- and 12-well plates, the media were changed to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air / 5% CO₂ incubator for 3 – 4 weeks in minimal essential medium (MEM) with Earle's salts supplemented with

10% heat-inactivated horse serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin. The media were changed twice per week. The surface-adhering monolayer cultures were >95% positive for the astrocytic marker, glial fibrillary acidic protein (GFAP).

Neuronal cultures were prepared from the cortices of embryonic day 17 rats and seeded on poly-D-lysine coated plates. Neurons were grown in Neurobasal medium (NBM, Invitrogen) supplemented with B27, 5 U/ml penicillin, 5 mg/ml streptomycin, 0.5 mM glutamine and 25 µM glutamate (Gibco, Rockville, MD), at 37°C in 5% CO₂. Cytosine arabinoside (20 µM) was added after 24 h to prevent glial proliferation. Cells were used for experiments after 1 week in culture.

Analysis of ³H-glutamine ³H-glutamate uptake in astrocytes:

³H-glutamine uptake was assayed as previously described (Allen et al., 2001). Astrocytes were studied at 3 weeks when the monolayers were confluent. Cells in 6-well plates were washed three times with 2 ml of fresh sodium-HEPES buffer consisting of the following: 122 mM of NaCl, 3.3 mM of KCl, 0.4 mM of MgSO₄, 1.3 mM of CaCl₂, 1.2 mM of KH₂PO₄, 10 mM of glucose and 25 mM of HEPES (N-2-hydroxy-ethylpiperazine N'-2-ethansulfonic acid) adjusted to a pH of 7.4 with 10 M of NaOH. In preincubation experiments, cells were pretreated in Na-HEPES buffer only, or with Na-HEPES buffer containing Mn (100 µM, 500 µM or 1 mM) for 30 min in a 37°C, 95% air/5% CO₂ incubator. Cells were then washed three times with 2 ml of Na-HEPES buffer. Thereafter, 1 ml of pre-warmed buffer containing 1 µCi/ml of L-[G-³H]-glutamine in the presence of unlabelled glutamine (at a final concentration of 50 µM) was added to each well, and glutamine uptake was measured at 1 min and 5 min at room temperature. At each time point, the reactions were terminated by aspirating the buffer from the well, followed by 5 washes with 2 ml of ice-cold mannitol buffer [290 mM of mannitol, 10 mM of Tris-nitrate, 0.5 mM of Ca(NO₃)₂; pH adjusted to 7.4 with KOH]. At the end of the experiment, cells were lysed in 2 ml of 1 M NaOH. An aliquot of 25 µl was used for protein determination by the BCA protein assay (Pierce, Rockford, IL). An aliquot of 750 µl was used for radioactivity measurement. Uptake of glutamine was expressed as cpm/mg of protein.

Measurement of GSH:

GSH concentrations were measured using HPLC (Ha et al., 2006). After treatments, cells or tissues were washed once with ice-cold PBS and precipitated in 500 µl of perchloric acid solution (5% v/v), containing 0.2 M boric acid/10 mM γ-glutamylglutamate (γGG) as internal standard. After centrifugation at 12,000 g for 5 minutes, the pellet was lysed in 100 µl of NaOH for protein analysis with the BCA method (Pierce, Rockford, IL); 300 µl of the supernatant (acid soluble extracts) was derivatized with 60 µl of 20 mM iodoacetic acid. After adjusting the pH to 9.0 ± 0.2 with KOH/potassium tetraborate, 300 µl of dansyl chloride in acetone (20 mg/ml) was added and the samples were kept at 25°C in the dark for 24 hrs. Thiol metabolites in the samples were analyzed with a Waters 2695 HPLC system using an YMC Pack NH2 (Amino) column (Waters; Milford, MA). Data were presented as percent of control corrected for protein concentration.

Lactate dehydrogenase (LDH):

LDH-leakage into the medium was measured as described by Bergmeyer (1972) using a Cobas Mira S analyzer (Bergmeyer, 1972). Leakage of LDH to the medium was expressed as a percentage of the total cellular LDH-activity as determined by disrupting the cells by incubation with 50 μ l 1% sodium azide. Generally, 40 μ l samples of medium was assayed spectrophotometrically (490:630nm), according to the manufacturer's protocol, to obtain a measure of cytoplasmic LDH released from dead and dying cells (Dobson et al., 2006; Morken et al., 2005).

F₂-isoprostanes Assay:

Total F₂-IsoPs were measured in mesencephalic neuronal and primary astrocyte cultures exposed to 100, 500 μ M or 1 mM of METH for 24 hours by using gas chromatography/mass spectrometry (GC/MS) with selective ion monitoring (Morrow and Roberts, 1999; Yin et al., 2007). Briefly, cells were resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated and then subjected to chemical saponification using 15% KOH to hydrolyze bound F₂-IsoPs. The cell lysates were adjusted to a pH of 3, followed by the addition of 0.1 ng of ⁴H₂-labeled 15-F₂-IsoP internal standard. F₂-IsoPs were subsequently purified by C₁₈ and silica Sep-Pak extraction and by thin layer chromatography. They were then analyzed by pentafluorobenzyl ester, a trimethylsilyl ether derivative, via gas chromatography, negative ion chemical ionization mass spectrometry. Results were expressed as pg/mg protein.

Nrf2 confocal assay:

Primary cerebral cortical astrocytes and mesencephalic neurons were grown on microscope cover glasses (Glaswarenfabrik Kari Hecht KG, Germany). For immunostaining, the cells were fixed in 100% methanol for 15 min and washed three times with PBS, Incubate the cells for 10 min with PBS containing 0.10% Triton X-100 and washed three times with PBS. After blocking in 10% bovine serum albumin in PBS for 30 min at room temperature, the cells were incubated for 1 h with polyclonal rabbit anti-Nrf2 antibody (1:300) in PBS containing 0.5% bovine serum albumin. The cells were incubated with Fluoresceins 488-conjugated goat anti-rabbit IgG antibody (Chemicon) (1:1000) 1 hour after serial washings with PBS. Stained cells were washed and examined using a laser scanning confocal microscope (Zeiss Upright LSM 510 META).

Statistical analysis:

The mean and standard error of the mean (S.E.M.) were determined for each set of data. One-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used for statistical analysis. A probability of 0.05 or less was considered statistically significant.

Results

Figure 1 establishes that METH produced concentration-dependent decrease in GSH levels 24 hrs after METH exposure in cultured astrocytes. Significant ($p<0.05$) effects were observed at the lowest concentration of METH (15% compared to control) and as the METH concentration increased the depletion of GSH was more pronounced (30 % at 0.5 mM and

40 % at 1mM METH) In cultured mesencephalic DAergic neurons, METH produced a similar concentration-dependent inhibition in neuronal GSH levels; however, statistical significances *vs.* controls were attained only at the high concentrations of METH (0.5 and 1.0 mM; 25 % and 30 % depletion respectively; see Figure 2).

METH exposure in primary astrocyte cultures also produced a concentration-dependent increase in LDH release into the culture media, indicating a loss of membrane integrity (Figure 3). This effect was concentration-dependent and statistically significant *vs.* controls at the two highest METH exposure concentrations (0.5 and 1.0 mM). Determinations of F-2-isoprostanes (IsoPs) levels were performed in both mesencephalic DAergic neuron and astrocyte cultures. Results established that METH produced a concentration-dependent effect on IsoP levels in neurons whilst there were no apparent effects on IsoP levels in astrocytes (Figure 4 A & B). The effects in neurons were statistically significant *vs.* controls at the two highest concentrations of METH (0.5 and 1.0 mM).

Confocal microscopy showed that METH produced accumulation of Nrf-2 in primary astrocytes after METH administration. Nuclei were sytox stained. Phase contract microscopic evaluation showed clear accumulation of translocated Nrf-2 in the nucleus (Figure 5). Significant nuclear localization of Nrf-2 was observed after treatment with as low as 0.05 mM METH. With increased METH concentrations, Nrf-2 nuclear accumulation further increased (as measured by both Nrf-2 and sytox co-localization). Notably, translocation of Nrf-2 into the nucleus was absent in neurons treated in a similar fashion (data not shown).

Glutamate and glutamine uptake in primary cultures of astrocytes was not significantly changed by METH exposure for 24 hrs (data not shown) and was indistinguishable from controls.

Discussion:

In the present study, METH produced neurotoxicity as verified by biochemical and morphological evaluations. METH exposure in cultured astrocytes and mesencephalic DAergic neurons led to GSH depletion, increased LDH release, increased IsoPs levels and increased translocation of nuclear factor Nrf-2 in astrocytes, but not in neurons. However, we didn't find any significant changes in glutamate/glutamine uptake in astrocytes, suggesting that alterations in astrocytic control over extracellular glutamate or glutamine levels are not mediating neuronal sensitivity to METH. Furthermore, decreased GSH levels suggest that METH affects both neurons and astrocytes and that oxidative stress is an important mediator of neuronal toxicity, predominantly since neurons were unable to mount an effective response to increased intracellular ROS levels. This is also evident by the lack of translocation of Nrf-2 to the neuronal nucleus, where it acts as a transcription factor that regulates the synthesis of a plethora of gene products associated with anti-oxidant responses. In contrast, in astrocytes treated with METH, Nrf-2 nuclear translocation was noted even at the lowest METH concentration (see Figure 5).

Recent studies have suggested that neurons are completely dependent-upon astrocytes to provide GSH precursor molecules (Sagara et al., 1993 and Dringen et al., 1999). Astrocytes are more resistant to oxidative stress than neurons largely due to their greater use of anaerobic glycolysis and their higher levels of GSH (Dringen et al., 1999; Sagara et al., 1993). Astrocytes also express the higher levels of the metallothionein (MT) isoforms I and II compared to neurons (Aschner, 1996; Philbert and Aschner, in press). These metal-binding proteins can directly scavenge ROS. Astrocytes possess a more efficient GSH antioxidant system compared to neurons, with intracellular GSH levels approximating 1–5 mM vs. neurons where the concentration of this important antioxidant is in the 0.1 mM range (Aschner and LoPachin, 1995; Philbert and Aschner, in press). Oxygen glucose deprivation resembling an ischemic injury has a lesser effect on mitochondrial function in astrocytes compared to neurons. Astrocytes also maintain higher levels of GSH compared to neurons under these conditions and generate lower amounts of ROS. The ability of astrocytes to use glycolysis allows these cells to continuously produce ATP even in the face of mitochondrial inhibition, whereas in neurons GSH levels fall precipitously. Although METH caused in our hands significant effects on GSH levels both in neurons and astrocytes, we posit that given the relatively low levels of this antioxidant in normal unstressed neurons, the reduction in GSH upon METH treatment leads to insurmountable and greater consequences in neurons vs. astrocytes (on a relative scale, astrocytes should still have mM GSH concentrations even in the face of 50% reduction in its levels).

Several studies have evaluated the responses of culture models to METH-induced neurotoxicity. Bennett et al (1993) and Cubella et al (1994) reported that METH caused cell death in both DAergic as well as non-DAergic cells and these effects were mediated by generation of free radicals and oxidative stress. Report by Sheng et al. (1996) implied that excessive generation of nitric oxide in primary cultures of mesencephalic neurons played a role in METH-induced DAergic neurotoxicity. Another study by Hom et al., (1997) showed that elevated level of glutathione peroxidase (Gpx) in PC12 cells afforded protection against METH-induced neurotoxicity. In a similar context, we have shown that selenium protected against METH-induced neurotoxicity both in PC-12 cells as well as *in vivo* (Imam et al., 1999; Imam and Ali, 2000). Exposure to METH led to a concomitant increase in free radical generation and oxidative stress, corroborating our findings that METH produced neurotoxicity by generating ROS, especially nitric oxide and peroxynitrite (Itzhak, et al., 1998; Imam et al. 2001; Imam and Ali, 2001 and for review see Itzhak and Ali, 2006). We have also reported that an antioxidant melatonin also protects METH neurotoxicity (Itzhak et al., 2003). Findings herein on decreased GSH levels, both in neuronal and astrocyte cultures, corroborate that oxidative stress and cellular redox are key mediators of METH-induced toxicity. Therefore, the significant depletion of GSH in both cell type, astrocytes and neurons, clearly supports the notion that GSH is one of the important antioxidants which might play a role in METH-induced DAergic neurotoxicity *in vitro* as well as *in vivo*.

METH exposure affected the astrocytic membrane integrity leading to the release of cytoplasmic LDH into the media, an effect which was concentration-dependent. L-CAR is a naturally diet supplement, antioxidant and a co-factor for the mitochondrial oxidation of fatty acids. Part of the action of L-CAR is to restore the membrane transport and reduced oxidation of lipids. Recently Binienda et al. (2006) reported that pretreatment of L-CAR

partially protect against METH induced DA toxicity in rats and suggested that L-CAR reduced oxidative stress. These data further support the notion that METH-generated free radicals affect membrane integrity as corroborated by increased LDH release (Figure 3).

The abundance of polyunsaturated fatty acids in the CNS can lead to lipid peroxidation as a major consequence of free radical-mediated injury. Lipid peroxidation proceeds until the oxidizable substrate is consumed, making this fundamentally different from many other forms of free radical injury in that the self-sustaining nature of the process results in extensive damage. Because of their chemical instability and their rapid and extensive metabolism (Moore and Roberts, 1998), the use of *in vivo* reactive products of lipid peroxidation as biomarkers is limited. For these reasons, more stable lipid products of oxidative damage have generated interest, including the F₂-isoprostanes (F₂-IsoPs), which are formed by free radical-mediated peroxidation of arachidonic acid (AA) (Watts et al., 1997; Reich et al., 2000; 2001). Sources of free radicals that contribute to IsoP formation include the mitochondrial electron transport system, the P450 family of drug-metabolizing enzymes, the NADPH oxidases, the lipoxygenases, and transition metal-catalyzed formation of ROS. In the present study we found a significant increased of this oxidative stress biomarker F₂-Iso-Ps in neurons, however, no significant effects were found in astrocytes.

When bound to its inhibitory protein Kelch-like ECH-associating protein 1 (Keap1, also known as Nrf2), Nrf2 is targeted for ubiquitination and thus has relatively low protein stability. Upon exposure to stimuli of oxidative stress or electrophilic compounds, as corroborated in the present study by both decreased GSH levels and increased IsoP levels (Figures 1 and 4, respectively), the Nrf2 protein is liberated from Keap1 and translocates into the nucleus. A common mechanism for regulating Nrf2 function is to control its subcellular distribution. Phosphorylation at serine 40 by protein kinase C promotes dissociation from Nrf2 and nuclear translocation (Huang et al., 2002; Bloom and Jaiswal, 2003). In the present study, we found that METH exposure increased the translocation and accumulation of Nrf2 and increased the intensity of Nrf2 and SyTox staining. Phase contrast evaluation showed a dose dependent increased of staining after METH exposure (figure 4).

In summary, our results demonstrated that METH exposure to astrocytes and neuronal culture produced a decreased in GSH, increased LDH in astrocytes and increased F₂-IsoPs in neurons and Nrf2 staining in astrocytes suggest an increased in oxidative stress and dysregulation of astrocytic and neuronal function.

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Figure 1:

Effects of METH (0.1 – 1 mM) on GSH levels in cultured astrocytes. A significant decrease was observed 24 hrs after 0.1 mM exposure to METH and GSH levels increased as METH concentration was increased. Each value represents the Mean $\pm$ SEM and is expressed as percent of control. All values were derived from three sets of independent experiments.

* $p < 0.05$ significantly different from control.

Figure 2:

Effects of METH (0.1 – 1 mM) on GSH levels in cultured mesencephalic dopaminergic neurons. A significant decrease was observed 24 hrs after 0.1 mM exposure to METH and GSH levels were increased as METH concentration increased. Each value represents the Mean $\pm$ SEM and is expressed as percent of control. All values were derived from three sets of independent experiments. * $p < 0.05$; ** $p < 0.01$ significantly different from control.

Figure 3:

Effects of METH (0.1 – 1 mM) on LDH release in cultured astrocytes. A concentration-dependent and significant increase was observed 24 hrs after METH (0.1–1mM). Each value represents the Mean $\pm$ SEM and is expressed as percent of control. All values were derived from three sets of independent experiments. *** $p < 0.001$ significantly different from control.

Figure 4:

Effects of METH (0.1 – 1 mM) on F₂-isoP levels in cultured primary mesencephalic neurons (A) and astrocytes (B). A significant increase was observed in F₂-isoP level after 24 hrs exposure to METH in neurons, whereas no significant effects were observed in astrocytes. Each value represents the Mean $\pm$ SEM and is expressed as percent of control. All values were derived from three sets of independent experiments. *p<0.05 significantly different from control.

Figure 5:
Confocal microscopic evaluation of astrocytes after METH (0.05 – 1 mM) exposure. Nrf2 and SyTox staining were observed in primary astrocytes cultured cells, A significant increase in staining was found at the lowest concentration of METH and staining intensity was increased with increased METH concentrations.