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Research Article

Protection against Methamphetamine-Induced Striatal Apoptosis by Epigallocatechin Gallate (EGCG) in the Mouse Brain

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Abstract Methamphetamine (METH) is a psychostimulant abused worldwide and shown to be neurotoxic to both dopamine terminals and striatal neurons. Thus it would be advantageous to discover natural compounds that protect the brain from the METH-induced neural injury. In the present study, we tested the ability of epigallocatechin gallate (EGCG), the most abundant antioxidant in green tea, to attenuate the METH-induced striatal injury. A dose of 2 mg/kg of EGCG (IP) given 30 min prior to METH (30 mg/kg, IP) significantly reduced the METH-induced depletion of striatal tyrosine hydroxylase (a marker of the dopamine terminals) and the apoptosis of some striatal neurons. Moreover, the neuroprotective effects exerted by EGCG in the striatum were independent of the METH-induced hyperthermia. Our data are consistent with the hypothesis that active ingredients in green tea are neuroprotective in the presence of METH and may prove useful in halting the progress of some neurodegenerative diseases of the nervous system and for the treatment of drug abuse.

Keywords methamphetamine; green tea; striatum; apoptosis; neurotoxicity; neuroprotection

1. Introduction

Methamphetamine (METH) is a widely abused psychostimulant with potent sympathetic and central nervous system actions [12,59]. The estimated annual prevalence rate of METH use in the United States was 1.4% in 2006 [47]. METH use represents a burden to public health and results in reduced job productivity and the deterioration of quality of life [49]. Compounding these social costs, a considerable body of evidence indicates that METH abuse can also lead to serious and persistent cognitive, psychiatric, and neurological dysfunction in the user [53,55]. The striatum, as the major component of the basal ganglia, participates in some cognitive functions and rewarding behaviors [6,22,44] and has also been shown to undergo some neurodegeneration due to METH abuse in both the dopamine terminals and striatal neurons [18,41,42]. In rodents and humans, METH induces the reduction of reliable markers of the dopamine system such as tyrosine hydroxylase, dopamine transporter, and dopamine content [35,48,65,70]. METH also induces the apoptosis of some striatal neurons [16,17,40,68]. Although the majority of studies investigating the damaging effects of METH in the brain have focused on the striatum, other brain regions are also affected such as the hippocampus [44], cortex [19, 51], and the olfactory bulb [15].

Although there is ample evidence supporting the striatal injury induced by METH, the corresponding mechanisms are not fully understood yet. Several studies demonstrate that reactive oxygen species (ROS) can exert significant effects on METH-induced dopamine terminal toxicity and striatal neuronal apoptosis [21,37,50,57,67]. For example, transgenic mice overexpressing copper/zinc superoxide dismutase were partially resistant to the METH-induced neurotoxicity of the dopamine terminals, the activation of caspase-3 activity, and neuronal apoptosis [8,14,20, 33]. Therefore, oxidative stress has emerged as a plausible contributor to METH-induced striatal injury. Consequently, antioxidants such as calcitriol, selenium, and melatonin have been found to protect against the METH-induced striatal dopamine terminal toxicity [9,36,38].

Our laboratory has been investigating the role of endogenous striatal agents that modulate the METH-induced elevation of oxidative stress. Striatal neuropeptides are in a strategic position to modulate striatal dopaminergic responses. We have found that the striatal neuropeptide substance P synergizes with METH in elevating the production of nitric oxide [64] and consequently striatal injury [73]. Interestingly, two other striatal neuropeptides, somatostatin and neuropeptide Y, attenuate the METH-induced striatal production of nitric oxide [1,69]. In addition to endogenous

agents such as neuropeptides, it is advantageous to identify exogenous substances that ameliorate oxidative stress and thus could be neuroprotective in the presence of METH. For example, green tea extracts and polyphenols increase antioxidant capacity in humans [54] and rats [5]. EGCG is the major polyphenolic constituent extracted from green tea and displays antioxidant activity [10, 13]. EGCG reduces the deleterious effects of reactive oxygen species associated with Parkinson's and Alzheimer's diseases in vivo [28,27,29,30]. EGCG's neuroprotective properties may be independent of the mechanisms by which some neuropeptides modulate the METH-induced striatal apoptosis. In the present study, we report on the ability of EGCG to protect striatal dopamine terminals and neurons from the damaging effects of a neurotoxic single dose of METH. Moreover, EGCG protects from METH without preventing the METH-induced hyperthermia.

2. Materials and methods

2.1. Animal care and use

ICR male mice between 12 and 14 weeks old (Taconic, Germantown, NY) were housed individually on a 12-hour light/dark cycle with food and water provided ad libitum. The mice were habituated for at least two weeks prior to commencement of intraperitoneal (IP) drug administration. All procedures involving animal use were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Hunter College, The City University of New York. The Hunter College animal facility is certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

2.2. Drug preparation and treatment

(−)-Epigallocatechin gallate (EGCG) of purity greater than 95% from green tea (Sigma, St. Louis, MO, USA) was dissolved in 0.9% sodium chloride and administered to the animals intraperitoneally at doses of 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 8 mg/kg, and 16 mg/kg of body weight. Four groups of animals received drug treatments and each group contained six or more mice. Control groups received either PBS (phosphate-buffered saline, pH 7.4) or 0.9% sodium chloride. Some animals received a bolus of METH at a dose of 30 mg/kg of body weight. Animals receiving METH and EGCG were injected with the latter compound 30 min before the METH injection.

2.3. Immunoblot of tyrosine hydroxylase

Mice were sacrificed by decapitation under anesthesia by isoflurane 72 h after drug injection. The brains were dissected out of the cranium and kept at −80 °C until used. Coronal sections (2 mm in thickness) at the level of the striatum were removed using a brain blocker and quickly frozen at −80 °C for protein extraction. Striatal tissue was disrupted by sonication and protein was extracted in 200 μL of RIPA buffer. The extract was centrifuged at 5,000 rpm for 15 min and the supernatant was saved for protein determination. A 12 μg sample of protein was boiled for 7 min at 95 °C in loading buffer. The proteins were resolved by molecular weight in a 10% Tris-HCL SDS-PAGE gel (Bio-Rad, Hercules, CA, USA). After transferring proteins to the PDVF membrane with iBlot transfer system (Invitrogen, Carlsbad, CA, USA), the membrane was blocked with an Odyssey blocking buffer for 1 hour followed by incubation with polyclonal rabbit anti-TH primary antibody (1:5,000, Millipore, Temecula, CA, USA) and monoclonal mouse anti β-actin primary antibody (1:20,000, Sigma, St. Louis, MO, USA) in an Odyssey blocking buffer with 0.2% Tween 20 at 4 °C overnight. The next day, after a wash in 0.1% Tween PBS, the membrane was incubated in a mixture of Odyssey IRDye secondary antibody donkey anti-rabbit 800 CW (1:15,000) and donkey anti-mouse 680 LT (1:22500) in an Odyssey blocking buffer with 0.2% Tween 20 at room temperature for 1 hour. After final washes with 0.1% Tween PBS, the membranes were analyzed using the Odyssey infrared imager. Bands were quantified by Odyssey Imager analysis software and normalized against actin as an internal standard.

2.4. Histochemistry and quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

24 h after drug administration, the animals were fully anesthetized and perfused with 30 mL of PBS followed by 30 mL of 4% paraformaldehyde in PBS. Then, the brains were dissected out of the skull and immediately post-fixed in 4% paraformaldehyde for 18 h at 4 °C followed by cryoprotection in 30% sucrose in PBS solution at 4 °C for 48 h. Tissue sections were then stored at −80 °C until used. Coronal sections (30 μm in thickness) from each brain were transferred and serially collected in six adjacent wells between bregma 0.02 and 1.0 mm. Approximately 50 striatal sections were collected from each brain. Five sections were randomly selected from one well and mounted on Superfrost plus slides. Before heating in 0.4% Triton X-100 PBS for 40 min at 70 °C, the slides were washed three times in PBS for 10 min. After permeabilizing the cell membrane with Triton X-100, TUNEL reagents were applied to the sections and incubated at 37 °C for 4 h. The sections were then washed with PBS to remove TUNEL reagents and the slides were coverslipped with Vectashield fluorescent hardset mounting medium H1000 and No. 1.5 coverslips. Images were then obtained from both hemispheres of each section using a Nikon ET epifluorescent microscope with FITC filter. All striatal images were taken at the level of the striatum at 100× magnification.

2.5. Immunohistochemistry

Coronal sections from both saline and METH groups were washed with PBS and then incubated with Anti-NeuN (rabbit polyclonal) primary antibody (1:50, Millipore) for 2 h followed by a wash in PBS and coverslipped with Vectashield fluorescent hardset mounting medium H1000 and No. 1.5 coverslips. Images were then obtained from both hemispheres of each coronal section using Nikon ET epifluorescent microscope with TRICT filter. Striatal images were taken at 100× magnification.

2.6. Cell counts and quantification

μm-thick coronal sections were taken from bregma 0.38 ± 0.1 mm. Cells were counted from 30 μm-thick coronal sections in an area of 0.52 mm^2 for each region of the striatum (ventral, dorsal). TUNEL cell counts were averaged from five 30 μm serial sections per animal. Average neuronal cell counts were done using the same procedure.

2.7. Body temperature detection

Core body temperature was determined using a BAT-12 thermometer coupled to RET-3 mouse rectal probe (Physitemp Instruments, Clifton, NJ, USA). Ambient room temperature was maintained at 20–22 °C.

2.8. Statistical analysis

Analysis was performed from mean ±SEM. Differences between groups were analyzed by analysis of variance (ANOVA) followed by Tukey comparison. The null hypothesis was set at the 95% confidence level.

3. Results

3.1. EGCG protects the striatal dopamine terminals

To assess the potential neuroprotective effects of EGCG on the striatal dopamine terminals, we injected mice with METH (30 mg/kg) because this psychostimulant has been shown to induce the toxicity of the striatal dopamine terminals. We measured striatal tyrosine hydroxylase protein levels with Western blots because depletion of this synthetic enzyme is reliably correlated with toxicity of the dopamine terminals [35]. Our data show that METH caused a significant decrease in tyrosine hydroxylase protein levels in the striatum three days after injection, approximately 51% decrease relative to the control (Figure 1), an observation consistent with previous reports from our group [74]. Pretreatment with EGCG 30 minutes prior to the METH injection attenuates the METH-induced depletion of tyrosine hydroxylase. In the presence of EGCG at a dose of 2 mg/kg, the METH-induced depletion of tyrosine hydroxylase was significantly attenuated (88% relative to control, Figure 1). Note that EGCG alone does not affect the levels of striatal tyrosine hydroxylase (Figure 1). Higher dosages of EGCG increased the levels

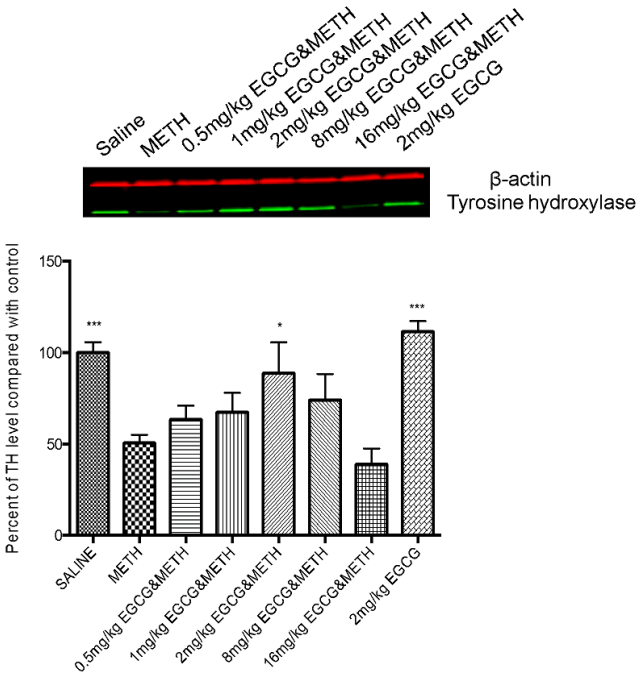

Figure 1: Pretreatment with EGCG attenuates the METH-induced depletion of tyrosine hydroxylase in a dose-dependent manner. Animals received EGCG at various dosages (IP) 30 min prior to the administration of 30 mg/kg METH. 72 h post-METH administration, we assessed the levels of tyrosine hydroxylase protein by Western blot with β-actin as internal control. Data represent Mean ±SEM. All data were analyzed by one-way ANOVA followed by Tukey. $^{*}P \leq 05$, $^{***}P \leq 001$, compared to METH-treated animals. $n \geq 6$.

of tyrosine hydroxylase protein in the striatum although no significant differences were observed relative to the METH-treated group. Also note that the highest dose of EGCG (16 mg/kg) exacerbates the METH-induced depletion of tyrosine hydroxylase, suggesting the existence of toxic drug effects at this high dose of EGCG (Figure 1).

3.2. EGCG abrogates the METH-induced striatal apoptosis

In addition to the toxicity of the striatal dopamine terminals observed three days after METH, there is also the apoptosis of some striatal neurons observed 24 h after METH. To test the hypothesis that EGCG would also protect the METH-induced apoptosis of some striatal neurons, we applied a widely used assay to detect late stages of apoptosis involving extensive breaks to the DNA strands, namely, the terminal deoxynucleotidyl transferase dUTP nick end-labeling assay (TUNEL). We injected mice with METH (30 mg/kg) and animals were sacrificed 24 h later. Figure 2 shows TUNEL-positive cells as green histofluorescence against the dark background. To estimate the amount of striatal apoptotic neurons, we counted the total number of striatal

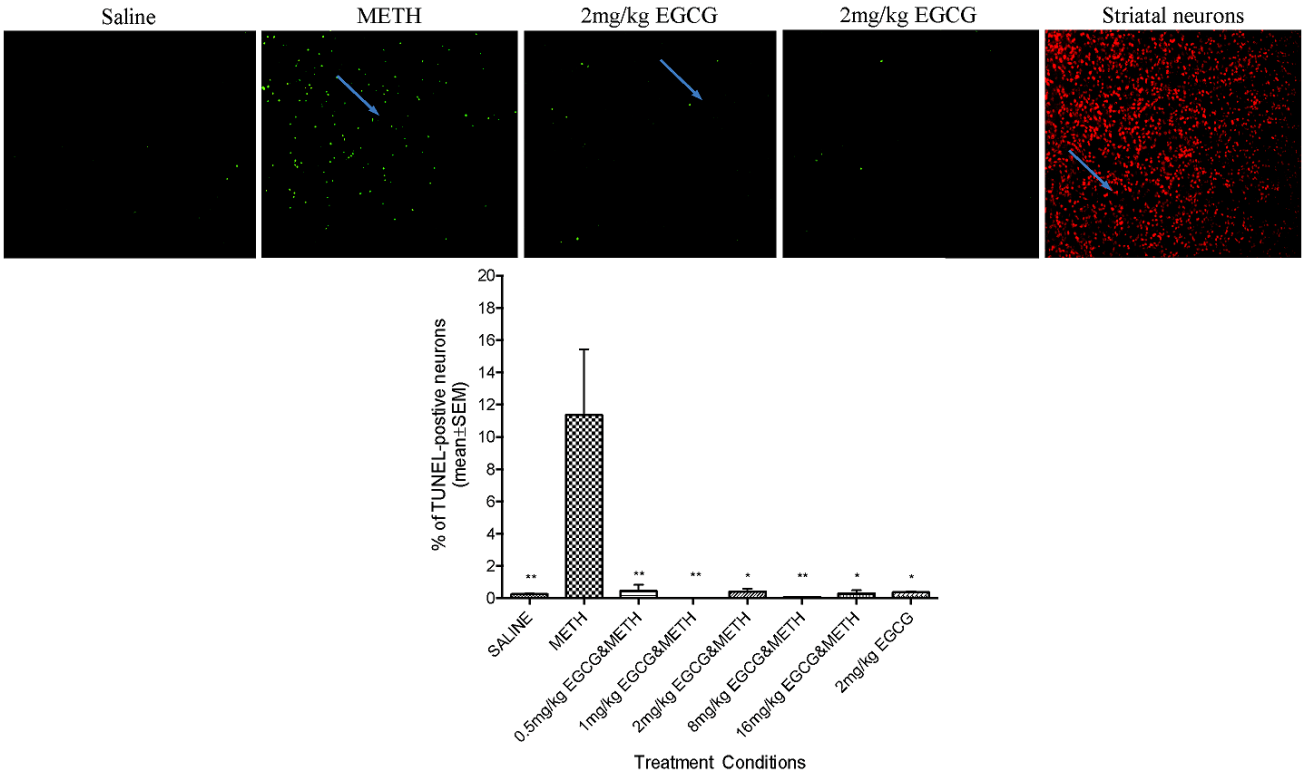

Figure 2: EGCG protects against the METH-induced striatal apoptosis. Animals received EGCG at various dosages (IP) 30 min prior to the administration of 30 mg/kg METH. 24 h post-METH administration, we assessed TUNEL and NeuN immunohistochemistry to visualize apoptotic neurons and total neurons in the striatum. Top panel: scanning fluorescent micrographs of TUNEL-positive neurons and total neurons in the striatum. Arrows indicate the apoptotic cells (green) and neurons (red). Magnification $= 100\times$. Lower panel: percentage of striatal neurons displaying TUNEL staining. Data represent Mean $\pm$SEM; all data were analyzed by one-way ANOVA followed by Tukey. $^{*}P \leq .05$, $^{**}P \leq .01$ compared with METH group.

neurons labeled with the neuronal selective marker NeuN, another nuclear marker like TUNEL. A representative photomicrograph is shown in Figure 2. The magnitude of cell death was expressed as the ratio of TUNEL/NeuN-labeled cells, as shown in Figure 2. Consistent with other studies from our group, our results demonstrate that METH induced significant apoptosis compared with vehicle-treated mice [75]; approximately 12% of all striatal neurons were apoptotic in the present study. Pretreatment with EGCG (30 min prior to METH) at doses ranging from 0.5 to 16 mg/kg abrogated the METH-induced striatal apoptosis (Figure 2).

3.3. Effect of EGCG on core body temperature

We measured core body temperature with a mouse rectal probe. Before drug administration, animals in all four groups had similar body temperatures of approximately 37 °C. METH increased the body temperature to 39.5 °C 2 h after METH administration. Pretreatment with EGCG at 0.5 mg/kg did not attenuate the METH-induced hyperthermia. At 4 h post-METH administration, the body core temperature dropped back to 38.1 °C and so did the temperature of EGCG pretreated mice. Body core temperatures returned to normal at 37 °C, for both METH and EGCG pretreated groups at 6 h. Note that there is no significant difference in temperature between METH-treated and EGCG/METH-treated mice (Figure 3).

4. Discussion

The present study investigated the neuroprotective potential of EGCG, a polyphenol found in green tea. We employed a model of apoptosis studied in our laboratory that exploits the ability of METH to induce neuronal apoptosis in the mouse striatum [74]. For example, a single high dose of METH (30 mg/kg) induces the loss of approximately 20% of striatal neurons including projection and interneurons [17, 75]. This dose of METH also induces the loss of striatal dopamine terminal markers such as dopamine transporters, tyrosine hydroxylase, and tissue dopamine content, a phenomenon referred to as dopamine terminal toxicity [19, 31, 52, 66]. In the present study, we observed that pretreatment with EGCG attenuated both the dopamine terminal

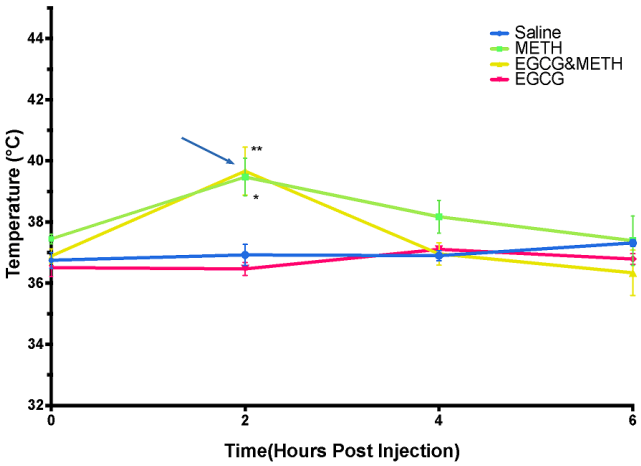

Figure 3: EGCG has no effect on METH-induced hyperthermia. Mice received injections of EGCG (0.5 mg/kg) 30 min prior to METH (30 mg/kg). Core body temperature was detected with a rodent thermometer probe at four time points after injection. Data represent Mean ±SEM; all data were analyzed by one-way ANOVA followed by Tukey. $^{*}P \leq .05$, $^{**}P \leq .01$ compared with Saline control. Arrow indicates EGCG does not affect hyperthermia induced by METH 2 h post-METH administration.

toxicity and the striatal apoptosis. This is in sharp contrast to other neuroprotective agents studied in our laboratory that prevent the striatal injury (apoptosis) or the dopamine terminal toxicity, but not both. For example, the neuropeptide substance P, which is synthesized by striatonigral projection neurons [25], exacerbates METH-induced striatal apoptosis, while a neurokinin-1 receptor (substance P receptor) antagonist protects those striatal neurons [63,71,73]. Similarly, ablation of the striatal neurokinin-1 receptor-expressing interneurons abrogates METH-induced apoptosis [73]. Moreover, substance P potentiates the METH-induced build-up of striatal nitric oxide, implicating the latter in the loss of striatal neurons [64]. The loss of striatal neurokinin-1 receptor-expressing interneurons protects the striatal neurons from METH [75] but fails to prevent or attenuate the METH-induced toxicity of the striatal dopamine terminals in mice [23,24].

Our published studies demonstrate that the striatal neuropeptides play a dynamic homeostatic role under conditions of excessive and prolonged dopamine overflow and that, unlike substance P, other neuropeptides attenuate the impact of METH. For example, neuropeptide Y (NPY) is synthesized by a population of striatal interneurons comprising less than 1% of all striatal neurons [41]. Exposure to METH increases the levels of preproNPY mRNA suggesting increased utilization of this neuropeptide [34]. In addition, genetic and pharmacological studies demonstrate that NPY counters the damaging effects of METH, protecting striatal neurons from this psychostimulant [61,69]. The same population of striatal interneurons making NPY also makes the neuropeptide somatostatin [41]. Our laboratory recently reported that somatostatin also counters the damaging effects of METH protecting the striatal neurons from the METH-induced apoptosis [1]. Thus, neuropeptides represent endogenous agents expressed and utilized in the healthy striatum that modulate dopaminergic responses under physiological homeostatic conditions as well as in aberrant states involving a surfeit of dopamine such as induced by METH.

Various studies have established that METH induces an elevation of core body temperature (hyperthermia) and this phenomenon has been associated with the neurotoxic actions of this drug [3,7,72]. In particular, experimental conditions that prevent the METH-induced hyperthermia attenuate or abrogate the neural damage induced by METH in various brain regions such as the striatum [4,60]. However, some pharmacological and genetic manipulations have been shown to prevent the METH-induced toxicity without influencing the elevation of core body temperature. For example, interleukin-6 knockout mice displayed METH-induced hyperthermia and were resistant to the METH-induced axonal degeneration of dopamine and 5-HT neurons, cell death, and microgliosis [45]. In the present study we assessed core body temperature four different times after the administration of METH. Our results show that core body temperature increased significantly at 2 h after METH and that coadministration of EGCG failed to attenuate the METH-induced hyperthermia, but EGCG was effective in attenuating the METH-induced depletion of tyrosine hydroxylase from striatal terminals and the apoptosis of some striatal neurons.

EGCG represents an exogenous natural agent that is also neuroprotective in the presence of METH and can be administered intraperitoneally. Published work has demonstrated that this compound readily enters the brain, achieving equal levels in the liver, kidney, and lungs [58]. A likely mechanism by which EGCG protects striatal tissue from METH may pertain to its antioxidant properties [46]. METH is well known to increase oxidative stress in brain tissue. For example, METH administration decreases the levels of copper-zinc superoxide dismutase, catalase, and glutathione while increasing the levels of lipid peroxidation and protein carbonyls [11,26,32,39,43,56]. Thus EGCG may achieve its neuroprotective effects by attenuating the METH-induced oxidative stress. But in addition to this, EGCG may be beneficial to brain tissue by elevating the levels of growth factors. For example, intrathecal EGCG infusion improved functional recovery from spinal cord injury by elevating brain- and glial-derived neurotrophic factors [62]. In a different study, EGCG was neuroprotective because it attenuated the release of mediators of inflammation [2]. The ability of EGCG to

protect from METH-induced damage will most likely involve various pathways leading to neurodegeneration.

In summary, our data demonstrate that EGCG confers protection to striatal dopamine terminals and striatal neurons from the METH-induced striatal apoptosis reported by our group and several other laboratories. Moreover, EGCG does not prevent the METH-induced elevation of body core temperature, a phenomenon that has been implicated in the neural injury in the striatum. Thus EGCG is a good candidate to be investigated as a neuroprotective agent in brain tissue. More studies are needed to elucidate the mechanism by which EGCG protects striatal dopamine terminals and neurons from METH.

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