Methamphetamine Administration Causes Death of Dopaminergic Neurons in the Mouse Olfactory Bulb

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Background: Methamphetamine (METH) is an addictive drug that can cause neurological and psychiatric disorders. In the rodent brain, toxic doses of METH cause damage of dopaminergic terminals and apoptosis of nondopaminergic neurons. The olfactory bulb (OB) is a brain region that is rich with dopaminergic neurons and terminals.

Methods: Rats were given a single injection of METH (40 mg/kg) and sacrificed at various time points afterward. The toxic effects of this injection on the OB were assessed by measuring monoamine levels, tyrosine hydroxylase (TH) immunocytochemistry, terminal deoxynucleotidyl transferase-mediated deoxyribonucleotide triphosphate (dNTP) nick end labeling (TUNEL) histochemistry, and caspase-3 immunochemistry.

Results: Methamphetamine administration caused marked decreases in dopamine (DA) levels and TH-like immunostaining in the mouse OB. The drug also caused increases in TUNEL-labeled OB neurons, some of which were also positive for TH expression. Moreover, there was METH-induced expression of activated caspase-3 in TH-positive cells. Finally, the METH injection was associated with increased expression of the proapoptotic proteins, Bax and Bid, but with decreased expression of the antideath protein, Bcl2.

Conclusions: These observations show, for the first time, that METH can cause loss of OB DA terminals and death of DA neurons, in part, via mechanisms that are akin to an apoptotic process.

Key Words: Caspase-3, dopamine, neurodegeneration, neuronal apoptosis, olfactory bulb

Methamphetamine (METH) is an illicit drug that causes psychiatric symptoms. These include euphoria, excessive agitation, hallucinations, and mood disturbances, as well as long-term cognitive and psychomotor impairments (Freese *et al.* 2000; Iwanami *et al.* 1994; Urbina and Jones 2004). The neuropsychiatric abnormalities are thought to be dependent, in part, on the neurotoxic effects of the drug on the human brain. Methamphetamine-induced neuropathological changes include striatal dopaminergic terminal degeneration (Volkow *et al.* 2001a, 2001b) and nondopaminergic striatal pathologies (Ernst *et al.* 2000). Abnormalities in other brain regions, including the orbitofrontal, lateral prefrontal, cingulate, insular cortices, and the amygdala, have also been reported in imaging studies of METH-abusing patients (Thompson *et al.* 2004). Findings similar to those observed in human brains have been reported in rodents treated with METH. These include damage to dopaminergic terminals and neuronal cell death (Cadet *et al.* 2005; Davidson *et al.* 2001; Commins and Seiden 1986; Eisch *et al.* 1998; Matsuzaki *et al.* 2004; O'Callaghan and Miller 1994; Schmued and Bowyer 1997; Zhu *et al.* 2006a, 2006b).

The mechanisms involved in METH-induced destruction of monoaminergic terminals have long been investigated (Cadet *et al.* 2003; Davidson *et al.* 2001; Itzhak and Achat-Mendes 2004). In contrast, although METH-induced cell death was reported in the brain as early as 1967 (Zalis *et al.* 1967), it is only recently that a number of groups have begun to try to identify pathways that might be involved in METH-induced cell death (Eisch *et al.* 1998; Langford *et al.* 2004; Matsuzaki *et al.* 2004; O'Dell and Marshall 2000; Pu *et al.* 1996; Schmued and Bowyer 1997; Sonsalla *et al.* 1996; Stumm *et al.* 1999; Zhu *et al.* 2006a, 2006b). It has indeed been reported that METH can cause cell death both in vitro (Cadet *et al.* 1997; Choi *et al.* 2002; Deng *et al.* 2002; Genc *et al.* 2003; Stumm *et al.* 1999) and in vivo (Deng *et al.* 1999; Eisch *et al.* 1998; Jayanthi *et al.* 2001, 2004, 2005; O'Dell and Marshall 2000; Zhu *et al.* 2005, 2006a, 2006b). Methamphetamine-induced cell death has been observed in the frontal cortex (Deng *et al.* 1999), the parietal cortex (Eisch and Marshall 1998), somatosensory cortex (Commins and Seiden 1986; O'Dell and Marshall 2000), the striatum (Deng *et al.* 1999; Zhu *et al.* 2006a, 2006b), and in hippocampal remnants (Schmued and Bowyer 1997). Mechanistic studies have suggested that METH-induced cell death might be secondary to the activation of multiple apoptotic pathways in the rodent brain (Cadet *et al.* 2003, 2005). These pathways include mitochondria-dependent (Jayanthi *et al.* 2001; Jimenez *et al.* 2004), endoplasmic reticulum-mediated (Jayanthi *et al.* 2004), and FasL/Fas-activated (Jayanthi *et al.* 2005) death pathways in the frontal cortex and striatum of the rodent brain.

Similar to the frontal cortex and the striatum, the olfactory bulb (OB) is a brain region that is involved in psychological performance in both normal and pathological states (Moberg and Turetsky 2003; Song and Leonard 2005). This is due, in part, to its role as part of the limbic system, which participates in affective and mnestic components of behaviors (Cecchi *et al.* 2001). For example, olfactory function is very important in animal courtship, mating, and reproduction (Keverne 2005; Yoon *et al.* 2005). Interestingly, olfactory bulbectomy has often been used as an animal model of depression, because these animals show cognitive and neurovegetative signs that are attenuated by drugs that treat depression in humans (Song and Leonard 2005). Also of interest are reports that the OB is reduced in size in schizophrenic patients (Turetsky *et al.* 2003). Pathological changes in the OB are also thought to underlie hyposmia in patients who suffer from Parkinson disease (PD) (Huisman *et al.* 2004; Katzenschlager *et al.* 2004).

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Structurally, the OB is divided into five layers that include the subependymal (SEL), the combined mitral and granule cell (MCL/GCL), the external plexiform (EPL), and the glomerular (GLM) layers (Fiske and Brunjes 2001). These layers contain neurons that are undergoing different phases of neurogenesis (Belluzzi *et al.* 2003; Liu and Martin 2003). Cells in the SEL are mostly neuronal progenitors that originate from the anterior subventricular zone and migrate to the OB along with the rostral migratory stream (Liu and Martin 2003). The newly differentiated cells then move away from SEL to GCL. Maturing neurons express different neuronal receptors and ion channels in a sequential fashion (Carleton *et al.* 2003; Usrey 2002). As occurred during embryonic development, both neurogenesis and programmed cell death coexist in the adult OB (Belluzzi *et al.* 2003; Fiske and Brunjes 2001), with the GCL exhibiting high apoptotic indices (Fiske and Brunjes 2001). In contrast, the GLM, an OB layer where dopaminergic neurons are abundantly expressed, shows no apoptotic indices in the normal adult brain (Fiske and Brunjes 2001). Newly generated neurons are believed to respond preferentially to the plastic modulation initiated by sensory stimulations and are thought to act to increase the accuracy of olfactory memory (Cecchi *et al.* 2001) and odor discrimination (Cecchi *et al.* 2001; Gheusi *et al.* 2000).

Given the relative abundance of dopamine (DA) in the OB (Ase *et al.* 2000; Davila *et al.* 2003; Philpot *et al.* 1998), we wanted to know if METH administration would cause DA depletion in that structure in a fashion similar to the striatum. We also sought to determine if METH would cause neuronal apoptosis similar to that observed in the cortex and striatum (Jayanthi *et al.* 2005). Our results show that METH does indeed cause neurodegenerative processes in the OB. These include increased neuronal cell death and damage to dopaminergic terminals.

Methods and Materials

Animals and METH Injection

Male ICR mice, 10 to 14 weeks old and weighing 33 g to 38 g, were obtained from Taconic Labs (Germantown, New York). They were given a single injection of METH (40 mg/kg) or saline, based on findings reported in our previous papers (Jayanthi *et al.* 2005). Other investigators have used similar dosing paradigms (Zhu *et al.* 2006a, 2006b). Mice were then sacrificed at various times after the METH injection and used in various analyses as described below. All animal-use procedures were according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

High-Performance Liquid Chromatography Analysis

High-performance liquid chromatography (HPLC) analyses were carried out as previously reported (Jayanthi *et al.* 2005). Mice were sacrificed at 1 week by cervical dislocation. Brains were removed and placed on an ice-cooled plate. Olfactory bulbs were dissected and immediately frozen on dry ice and stored at -80°C until extraction. Each OB was weighed, ultrasonicated in 1% perchloric acid, and centrifuged at 25,000g for 12 min. Concentrations of DA, 3, 4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in supernatants of OB extracts were measured by HPLC with electrochemical detection. The analytical column was a Symmetry C-18 (3.5 μm, 4.6 × 150.0 mm; Waters, Milford, Massachusetts); the mobile phase consisted of .01 mol/L sodium dihydrogenphosphate, .01 mol/L citric acid, 2 mmol/L sodium ethylenediaminetetraacetic acid (EDTA), 1 mmol/L sodium octylsulfate, 10% methanol, and pH 3.5 and was used at flow rate .9 mL/min and temperature 25°C. The installation consisted of a Waters 717 Plus automated injection system, a Waters 1525 Binary pump, and Coulochem III detector (ESA, Chelmsford, Massachusetts). Waters Breeze system was used for data collection and analysis. Contents of DOPAC, DA, 5-HIAA, and 5-HT were calculated as pg/mg of tissue weight.

Autoradiographic Assays

Binding assays for dopamine transporters (DAT) were performed according to methods previously described by this laboratory (Asanuma *et al.* 1998; Deng *et al.* 1999). Briefly, animals (six per group) were sacrificed at 1 week after METH or saline treatment. Thereafter, 60-μm OB sections and 20-μm coronal striatal sections were cut using a cryostat. Slide-mounted sections were incubated for 60 min at room temperature with 100,000 cpm/mL of radioiodinated 3β-[4-(trimethylstannyl)phenyl]-tropan-2β-carboxylic acid isopropyl ester ([^{r125}I]RTI-121) (specific activity, 2200 Ci/mmol; PerKinElmer, Boston, Massachusetts), using a binding buffer consisting of 137.0 mmol/L sodium chloride (NaCl), 2.7 mmol/L potassium chloride (KCl), 10.14 mmol/L sodium phosphate dibasic (Na_2HPO_4), 1.76 mmol/L potassium phosphate monobasic (KH_2PO_4), and 10.0 mmol/L sodium iodide (NaI). Specific binding was determined in the presence of 10 μm 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylprophyl)piperazine dihydrochloride (GBR-12909) (Research Biochemicals International, Natick, Massachusetts) and represented greater than 90% of total binding. At the end of the incubation, the slides were washed twice in fresh binding buffer for 20 min at room temperature, dipped in ice-cold distilled water, and dried under cool air stream. The slides were then apposed to radiosensitive films (Biomax MS, Kodak, New Haven, Connecticut) with a plastic standard (^{125}I microscales, Amersham, United Kingdom) for 3 days at 4°C. The films were then developed and images were acquired and scanned at 1200 dpi. Mean densities representing [^{125}I]RTI-121 binding in the OB and striatum were quantified by using software NIH 1.62 (NIH, Bethesda, Maryland).

Tyrosine Hydroxylase Immunohistochemistry

To further evaluate the toxic effects of METH on dopaminergic systems, we use tyrosine hydroxylase (TH) immunohistochemistry (Deng *et al.* 2002) using animals killed at 1 week after drug or saline treatment. Briefly, the animals (five for each group) were perfused transcardially, under deep pentobarbital anesthesia, first with saline followed by 40 mL of 4% paraformaldehyde in .1 mol/L phosphate buffer at 4°C. The brains were removed, postfixed overnight in 4% paraformaldehyde, and then allowed to equilibrate in 30% sucrose for 24 hours. Coronal OB sections (30 μm) were then cut in a cryostat. Free-floating sections were used for TH immunostaining. Briefly, sections were exposed to 1% hydrogen peroxide for 20 min and then incubated for 30 min in 1% bovine serum albumin and .3% Triton X-100, followed by incubation with polyclonal TH (Calbiochem, La Jolla, California) primary antibody. Subsequent processing with biotinylated secondary antibody and ABC complex was performed according to the manufacturer's procedures described in the ABC kit (Vector Laboratories, Burlingame, California). The free-floating sections were then reacted with 3,3′-diaminobenzidine and hydrogen peroxide to visualize the peroxidase reaction.