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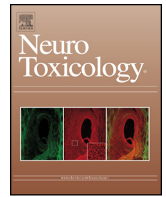

Methamphetamine-induced neuronal necrosis: the role of electrographic seizure discharges

Denson G. Fujikawa[a,b,c,*], Emil S. Pais[a], Ernesto R. Aviles Jr.[a], Kung-Chiao Hsieh[a], Muhammad Tariq Bashir[a]

[a] Experimental Neurology Laboratory, Sepulveda VA Ambulatory Care Center and Nursing Home, VA Greater Los Angeles Healthcare System, North Hills, CA, USA
[b] Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[c] Brain Research Institute, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

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We have evidence that methamphetamine (METH)-induced neuronal death is morphologically necrotic, not apoptotic, as is currently believed, and that electrographic seizures may be responsible. We administered 40 mg/kg i.p. to 12 male C57BL/6 mice and monitored EEGs continuously and rectal temperatures every 15 min, keeping rectal temperatures <41.0 °C. Seven of the 12 mice had repetitive electrographic seizure discharges (RESDs) and 5 did not. The RESDs were often not accompanied by behavioral signs of seizures—i.e., they were often not accompanied by clonic forelimb movements. The 7 mice with RESDs had acidophilic neurons (the H&E light-microscopic equivalent of necrotic neurons by ultrastructural examination) in all of 7 brain regions (hippocampal CA1, CA2, CA3 and hilus, amygdala, piriform cortex and entorhinal cortex), the same brain regions damaged following generalized seizures, 24 h after METH administration. The 5 mice without RESDs had a few acidophilic neurons in 4 of the 7 brain regions, but those with RESDs had significantly more in 6 of the 7 brain regions. Maximum rectal temperatures were comparable in mice with and without RESDs, so that cannot explain the difference between the two groups with respect to METH-induced neuronal death. Our data show that METH-induced neuronal death is morphologically necrotic, that EEGs must be recorded to detect electrographic seizure activity in rodents without behavioral evidence of seizures, and that RESDs may be responsible for METH-induced neuronal death.

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1. Introduction

D-methamphetamine (METH) abuse is a major health problem in this country (Cho and Melega, 2002; Rawson et al., 2002). Until relatively recently, in animal studies the toxic effect of METH on brain was focused on damage to nerve terminals in striatum (Ricaurte et al., 1982; Ricaurte et al., 1984) and not on directly assessing in which brain regions and by what mechanism or mechanisms METH kills neurons. METH can produce neuronal death in brain regions other than striatum (Deng et al., 2001; Schmued and Bowyer, 1997), and METH-induced neuronal death involves excitotoxicity (Cadet et al., 2007).

The morphology of acutely injured neurons in cerebral ischemia (Colbourne et al., 1999), hypoglycemia (Auer et al., 1985a,b) and prolonged epileptic seizures (Fujikawa et al., 1999, 2000) is necrotic, not apoptotic. Cadet and colleagues have suggested that METH-induced neuronal death is apoptotic, based on low-magnification photomicrographs of cells with pyknotic, TUNEL-positive nuclei (Deng et al., 2001). This is precisely the morphology of seizure-induced necrotic neurons (Fujikawa et al., 2002, 1999, 2000, 2007, 2010). We and others have shown that apoptotic neurons have large, usually round chromatin clumps, with relatively early disruption of nuclear membranes (Fujikawa et al., 2000; Ikonomidou et al., 2000, 1999; Ishimaru et al., 1999), so that fragmented DNA mingles with cytoplasm, producing apoptotic neurons with TUNEL-positive cell bodies and TUNEL-negative chromatin clumps (Fujikawa et al., 1999, 2000, 2010) (see also Fig. 3E).

It is recognized that epileptic seizures can occur following METH administration (Bowyer and Ali, 2006; Cadet et al., 2007),

* Corresponding author at: VA Greater Los Angeles Healthcare System, Mail Code 151B4, 16111 Plummer Street, North Hills, California 91343-2036, USA.
Tel.: +1 818 895 9441; fax: +1 818 895 9368.
E-mail address: dfujikaw@ucla.edu (D.G. Fujikawa).

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but this has relied on behavioral observations alone. In this study we show that seizure-like behavior is not a reliable indication of electrographic seizure activity, that recording EEG activity should be done to determine if seizure discharges are present, and that widespread METH-induced neuronal necrosis only occurs in mice with repetitive electrographic seizure discharges (RESDs).

2. Materials and methods

2.1. Materials

D-methamphetamine HCl was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Eosin Y was purchased from J.T. Baker Company (Philipsburg, NJ, U.S.A.). Hematoxylin and Hemo-De were purchased from Fisher Scientific Company (Pittsburgh, PA, U.S.A.). Paraformaldehyde was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Pentobarbital sodium (50 mg/ml) and ketamine HCl (50 mg/ml) were obtained from the Pharmacy Service at Sepulveda VA Ambulatory Care Center and Nursing Home.

2.2. Experimental protocol

Our experimental protocol was approved by the Animal Studies Subcommittee of the Research and Development Committee of the VA Greater Los Angeles Healthcare System. The guidelines published in the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996) were followed. Every attempt was made to minimize the number of mice used and to minimize their pain and distress.

Male C57BL/6J mice (24–28 g, 8–12 weeks of age, The Jackson Laboratory, Bar Harbor, ME, U.S.A.) were anesthetized with 45 mg/kg of pentobarbital and 25 mg/kg of ketamine i.p. Four stainless steel screws (three for EEG recording) were implanted into mouse skulls and secured with dental acrylic. Three electrode wires attached to a 3-strand wire connector and 3-channel cable were wound around the anterior two and one of the posterior skull screws and secured with dental acrylic. The 3-channel cable was connected to a commutator that in turn was connected to a differential amplifier (World Precision Instruments, Sarasota, FL, U.S.A.), A-D converter (Biopac Systems, Inc., Goleta, CA, U.S.A.) and PC with an AqKnowledge EEG acquisition program (Biopac Systems, Inc.).

Seven days later mice were given 40 mg/kg of free-base D-methamphetamine HCl (METH) or normal saline i.p. EEGs were recorded continuously; behavioral observations and rectal temperatures were recorded every 15 min. Rectal temperatures were measured for at least 4 h after METH or normal saline injection with rectal temperature probes connected to a YSI 73A temperature controller, and were kept below 41.0 °C in mice given METH by placing them on cloth-covered ice packs as needed. Mice were killed with an overdose of pentobarbital (200 mg/kg i.p.) 24 h after METH or normal saline injection. They then underwent transcardiac perfusion with 4% phosphate-buffered paraformaldehyde.

2.3. Tissue processing and hematoxylin and eosin (H&E) staining of brain sections

Brains were kept *in situ* at 4 °C overnight, after which they were removed and placed in the same perfusate. Brains were placed in a Kopf Brain Blocker and cut in the coronal plane so that the dorsal hippocampus (1.94 mm posterior to bregma) and ventral hippocampus (3.16 mm posterior to bregma) were included in the brain blocks (Paxinos and Watson, 1998). Brain slices were dehydrated, embedded in paraffin, cut into 6-μm-thick coronal sections, rehydrated, and stained with hematoxylin and eosin (H&E). The apoptotic neurons in Fig. 3D and E are unpublished photomicrographs from the retrosplenial cortex of a postnatal day 8 (P8) rat pup, stained with H&E and with TUNEL (terminal deoxynucleotidyl transferase [TdT], biotinylated dUTP nick-end labeling) and methyl green counterstain, respectively. TUNEL labels double-stranded DNA fragments (Gavrielli et al., 1992). Our protocol of obtaining neonatal cortical tissue for staining of apoptotic neurons with H & E and TUNEL and the TUNEL staining procedure itself has previously been published (Fujikawa et al., 2000).

2.4. Semi-quantitative assessment of normal and acidophilic neurons

The semi-quantitative assessment of normal and acidophilic (necrotic) neurons in four hippocampal subregions (CA1, CA2, CA3 and hilus) and in amygdala, piriform cortex and entorhinal cortex) was performed as we have described previously for rats (Fujikawa, 1995, 1996, 1994, 2002, 1999, 2000, 2007, 2010; Zhao et al., 2010). We estimated the numbers of acidophilic neurons on a 0–3 grading scale, 0 = none, 0.5 = slight (<10%), 1.0 = mild (10–25%), 1.5 = mild-to-moderate (26–45%), 2.0 = moderate (46–54%), 2.5 = moderate-to-severe (55–75%), and 3.0 = severe (>75%), as previously published (Fujikawa, 1995, 1996, 1994, 2002, 1999, 2000, 2007, 2010; Zhao et al., 2010).

2.5. Statistical analysis

The damage score data conformed to a Poisson distribution rather than a normal curve, so in consultation with our longtime statistical consultant, Dr. Jeffrey Gornbein (see Acknowledgments), we performed a two-factor (group and brain region) analysis of deviance, with *post-hoc t*-tests using pooled S.D.s and $\alpha = 0.05$, as we did in a recently published article (Fujikawa et al., 2010). The maximal rectal temperature (T_{max}) data were analyzed with a one-factor (group) ANOVA, with *post-hoc t*-tests using the pooled standard deviation. In addition, Spearmann and Pearson correlation analyses were used to compare T_{max} to maximal damage scores and the number of brain regions damaged.

3. Results

3.1. Methamphetamine-treated mice can develop repetitive electrographic seizure discharges (RESDs)

Of 12 methamphetamine (METH)-treated mice, 7 developed repetitive electrographic seizure discharges (RESDs) (Fig. 1). The latency to the first seizure, the total duration of RESDs and the total time RESDs were present are summarized in Table 1. Four of the 7 mice with RESDs had no behavioral evidence of seizures, whereas 3 of the 5 mice without RESDs had clonic forelimb movements (Racine seizure stage 3) (Racine, 1972). Thus, clonic forelimb movements are not a reliable indicator of electrographic seizure activity.

3.2. Methamphetamine-treated mice with RESDs show irreversibly damaged acidophilic neurons

Acidophilic neurons are shrunken, with pyknotic nuclei and eosinophilic cytoplasm by hematoxylin and eosin (H&E) stain, and are the light-microscopic equivalent of necrotic neurons shown by electron microscopy (Fujikawa et al., 1999, 2000, 2010). Fig. 2A, D and G, show normal neurons in the hippocampal CA3, CA1 and hilus of control mice, METH-treated mice without RESDs (B, E and H) and acidophilic neurons in METH-treated mice with RESDs (C, F and I). The acidophilic neurons in C, F and I are shrunken, with pyknotic (shrunken), condensed nuclei and eosinophilic cytoplasm.

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