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Methamphetamine and lentivirus interactions: Reciprocal enhancement of central nervous system disease

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Use of methamphetamine is increasingly a significant factor for the spread of human immunodeficiency virus type 1, for in certain populations, there is a convergence of methamphetamine abuse with human immunodeficiency virus type 1 infection. Methamphetamine and human immunodeficiency virus type 1 are both individually neuropathogenic, and the neuropathology caused by these two agents occurs in overlapping brain regions. However, the biological interaction of methamphetamine with lentiviruses remains unknown. Here, we investigate the effects of simultaneous exposure of these two agents on disease progression using the feline immunodeficiency virus model. The study models the bingeing methamphetamine user with sequential and repeatedepisodes of use, which were interrupted by periods of abstinence. Methamphetamine exposure significantly accelerated and enhanced the severity of the feline immunodeficiency virus model–induced central nervous system functional pathology, as measured in delays in brainstem auditory evoked potentials. Reciprocally, feline immunodeficiency virus enhanced the severity of the methamphetamine-induced effects on brain monoamine neurotransmitter and dopamine transporter levels. The results of this study indicate that a dual potentiation occurred. That is, methamphetamine enhanced feline immunodeficiency virus model–induced central nervous system disease and feline immunodeficiency virus model enhanced the toxic effects of methamphetamine, heralding a significant concern for those individuals that are exposed to both agents. *Journal of NeuroVirology* (2010) **16,** 268–278.

Keywords: AIDS; drug abuse; FIV; HIV; neuropathology; synergism

Introduction

In certain populations, there is a convergence of methamphetamine (METH) abuse with human immunodeficiency virus type 1 (HIV-1) infection (Corsi and Booth, 2008). The sharing of needles and syringes combined with METH's perceived enhancement of sexual pleasure and the association of its use with unsafe sexual practices greatly enhances the likelihood of contracting an HIV-1 infection during METH use (Rotheram-Borus *et al,* 1994; Kipke *et al,* 1995; Molitor *et al,* 1998). Lentiviruses (Abbruzzese *et al,* 1990; Heaton *et al,* 1995; Sahakian *et al,* 1995; Meeker *et al,* 1997; Pardo *et al,* 1998; Semple *et al,* 2009) and METH (Ricaurte *et al,* 1982; Commins *et al,* 1987; Molliver *et al,* 1990; Broening *et al,* 1997; Hanson *et al,* 2009) produces toxic cellular changes in the deep gray structures.

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The neurotoxic effects of METH on the brain monoamine systems are well characterized (Ellinwood and Escalante, 1970; Woolverton *et al,* 1989; Ricaurte and McCann, 1992; Callahan *et al,* 1998; Ferris *et al,* 2008). Following METH administration to laboratory animals, striatal levels of dopamine and its metabolites have remained low for prolonged periods of time (Kogan *et al,* 1976; Wagner *et al,* 1980; Cass and Manning, 1999), tyrosine hydroxylase is persistently decreased (Kogan *et al,* 1976), and release of evoked dopamine is reduced (Cass and Manning, 1999; Ferris *et al,* 2008). HIV-1 infection is associated with neuronal loss, dendritic vacuolization, loss of dendritic processes, and decreases in synaptic densities (Wiley *et al,* 1991; Masliah *et al,* 1992, 1997; Everall *et al,* 1993; Power *et al,* 1993; Moore *et al,* 2006; Crews *et al,* 2009). Striatal (caudate) atrophy (Berger and Nath, 1997) and loss of dopaminergic neurons in the substantia nigra (Reyes *et al,* 1991) have been reported in HIV-1–infected people. In the caudate nucleus, decreased levels of dopamine (DA) and homovanillic acid have been found in acquired immunodeficiency virus (AIDS) patients (Sardar *et al,* 1996). Thus, both METH and HIV-1 are neurotoxic; and these toxic effects occur in overlapping brain regions. However, very little is known about the potential interactions of HIV-1 and METH and the impact that the simultaneous or sequential exposure of these two agents may have on disease progression.

Animal models could be extremely valuable in providing insights into early events of lentiviral infection and drug abuse. Employing the well-established feline model of neuroAIDS (Phillips *et al,* 1994, 1996, 2000; Prospero-Garcia *et al,* 1994a, 1994b, 1999; Henriksen *et al,* 1995; Gruol *et al,* 1998; Yu *et al,* 1998; Barr *et al,* 2000a), we investigated, herein, neurological alterations induced by the simultaneous exposure of feline immunodeficiency virus (FIV) and METH. In this *in vivo* study, we modeled the "bingeing methamphetamine user with repeated periods of abstinence."

Results

The effects of METH on FIV disease progression were studied on four groups of cats, Control, METH, FIV, and FIV/METH. One of the first signs of an FIV infection is the development of lymphadenopathy. As shown in Table 1, the FIV/METH group developed lymphadenopathy more rapidly than the FIV group, indicating that METH was accelerating the peripheral disease course of FIV. Similarly, although not significant, the FIV/METH-treated animals tended to have lower relative CD4/CD8 ratios than the FIV-infected animals (data not shown). There was no significant difference between the FIV and FIV/METH groups in the kinetics of the FIV antibody response (data not shown). The plasma viral load levels, as measured by real-time polymerase chain reaction (PCR), did not significantly differ between the FIV and FIV/METH groups (Table 2). Because FIV is generally more cell associated than HIV-1, detectable plasma viremia levels tend to be limited to the early time points of infection. The duration of detectable viremia was even shorter in those animals that received the METH treatment (Table 2). We also examined the number of peripheral blood mononuclear cells that were infected, using a virus isolation approach. Although not significant, at week 17 post infection, the FIV/METH cats trended to have less peripheral blood mononuclear cells (PBMCs) infected than the animals that were only infected with FIV (data not shown).

Table 1 Kinetics of lymphadenopathy onset

	Number of cats with onset of lymphadenopathy	
Time post infection	Virus only	Virus/methamphetamine
2 weeks	2	5
4 weeks	3	1
8 weeks	1	

The FIV-infected cats showed significant delays in the latency of wave component P6 ($P < .01$) as early as 25 days after infection (Figure 1). The METH alone had no effect on the brain auditory evoked potentials (BAEPs), as demonstrated by the data from METH group (Figure 1). However, when animals were simultaneously exposed to both METH and FIV, the FIV-induced alteration of the BAEPs appeared earlier in the disease course and had larger latency values (Figure 1). These results clearly show that METH accelerated and increased the FIV functional pathology in auditory pathways, demonstrating a synergistic effect of METH and FIV on altered brain function.

On postmortem examination at the light microscope level of examination, general histological analysis was uneventful, most likely due to the early time of sacrifice. The caudate nucleus from each animal was harvested and monoamine levels were examined. Neither the METH treatment with this specific administration schedule nor FIV infection alone significantly decreased the levels of dopamine (DA) in the feline caudate (Figure 2). However, in the FIV/METH group, DA levels were significantly decreased ($P < .01$), demonstrating a synergistic effect between the virus and METH on the caudate DA levels. This effect also occurred on the DA metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) (Figure 2). Although FIV had no significant effect on caudate DOPAC levels, METH significantly ($P < .05$) decreased the level of this monoamine in the feline caudate. However, the FIV/METH group had even lower DOPAC levels ($P < .001$). No significant

Table 2 Influence of methamphetamine on plasma viremia levels in FIV-infected cats

Treatment	Cat no.	Plasma viral RNA (copies/ml)					
		Week 0	Week 2	Week 5	Week 8	Week 11	Week 17
FIV	171	ND	67,574	290	1318	796	ND
	177	ND	61,365	ND	646	1047	ND
	179	ND	752,113	1,039	1103	1170	ND
	137	ND	57,404	ND	5637	3507	ND
	138	ND	ND	ND	851	ND	ND
	161	ND	103,115	376	2746	2350	ND
	Mean	—	208,314	284	2050	1479*	—
	SE	—	136,191	165	779	510	—
Meth/FIV	181	ND	471,436	4753	2198	ND	ND
	182	ND	90,904	661	4649	ND	ND
	184	ND	17,016	ND	1721	ND	ND
	140	ND	54,500	ND	696	ND	ND
	141	ND	18,738	463	453	ND	ND
	146	ND	39,622	1981	5850	ND	ND
	Mean	—	115,369	1310	2595	—	—
	SE	—	72,073	750	893	—	—

Note. FIV = FIV-infected cats; METH/FIV = METH-treated = FIV-infected cats. ND = not detected.*Significant difference one-sample *t* test, $P < .05$.

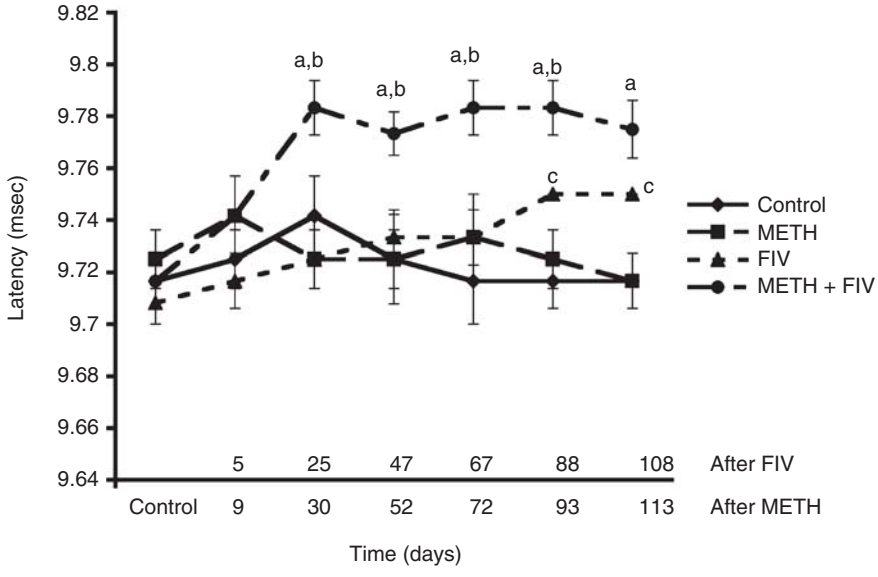

Figure 1 Brainstem auditory evoked potentials. Averaged waveform latencies of P6 of the different groups of cats of this study. a = significant delays in the METH/FIV group compared withuninfected control cats ($P < .01$). b = significant delays in the METH/FIV group compared with FIV cats ($P < .01$). c = significant delays in the FIV group compared with uninfected controls ($P < .01$). $n = 6$ cats in each group.

alterations in the downstream DA metabolite, HVA, were demonstrated. Nor were any significant effects demonstrated on the levels of serotonin (5-HT) or its metabolite, 5-HIAA, (data not shown). However, when the caudate ratio of 5-HIAA/5-HT (Figure 2) was examined, a significant increase in the ratio occurred only in the FIV/METH group, indicating, once again, a synergism (potentiation) between the METH and FIV.

Examination of the caudate, using an immunohistochemical assay for dopamine transporter (DAT), revealed that FIV alone had no demonstrable effect on the DAT levels (Table 3 and Figure 3). This is in contrast to the effects of METH. Although METH treatment substantially depleted the caudate of DAT immunoreactivity, reactivity was still present in five of the six animals examined (Table 3). When the animals from the FIV/METH group were

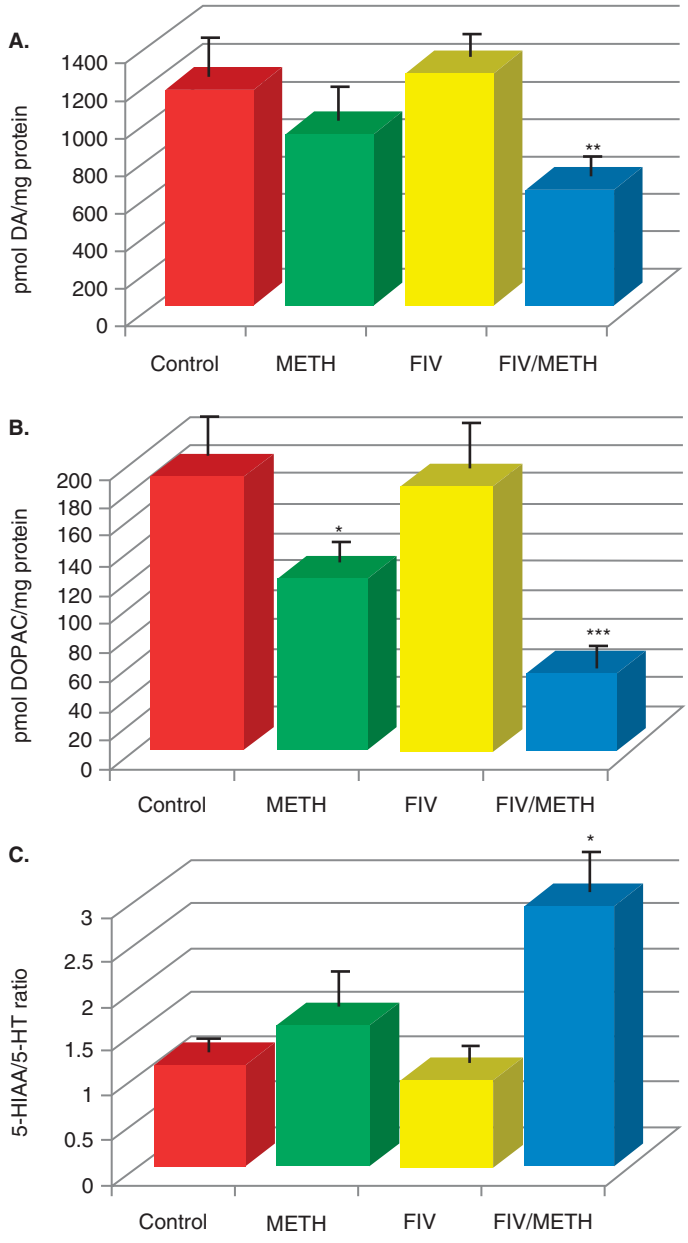

Figure 2 Synergistic effects of METH and FIV infection on monoamine levels in the caudate nucleus. (**A**) DA levels; (**B**) DOPAC, a metabolite of dopamine; (**C**) 5-HIAA/5-HT ratio. Significant differences: *Control vs. METH ($P < .05$); **significant decrease in the METH/FIV group compared with uninfected control cats ($P < .01$); ***significant decrease in the METH/FIV group compared with uninfected control cats ($P < .001$).

examined, DAT immunoreactivity was not detected in four animals and in the remaining two animals only a minimal amount of staining was detected (Table 3 and Figure 3).

Using glial fibrillary acidic protein (GFAP) immunoreactivity assay, the level of astrocyte activation was evaluated. There was no difference among the various treatment groups in the total number of astrocytes found. However, differences in the size and amount of GFAP were found. The appearance of the cells and the level of activation in both the control and FIV-infected animals were indistinguishable from each other (Table 4 and Figure 4). Thus, FIV, at this early stage of infection, had no effect on the level of astrocyte activation. Although the number of astrocytes in the METH-treated animals were not increased over the controls, the astrocytes from the METH-treated cats were enlarged, as both the cell bodies and processes were increased relative to the control animals (Figure 4). The astrocytes in the caudate samples from the FIV/METH-treated cats appeared nearly identical to the astrocytes from the METH-treated animals.

Table 3 DAT Immunohistochemistry[a] staining density scale 0 to ++++

Control cats	FIV only	METH only	FIV and METH
Cat 145 ++++	Cat 137 ++++	Cat 142 −/+	Cat 140 −/+
Cat 147 ++++	Cat 138 ++++	Cat 143 ++	Cat 141 −
Cat 148 ++++	Cat 161 ++++	Cat 144 −/+	Cat 146 −
Cat 169 ++++	Cat 171 ++++	Cat 173 −	Cat 181 −
Cat 172 ++++	Cat 177 ++++	Cat 174 −/+	Cat 182 −
	Cat 179 ++++	Cat 175 ++	Cat 184 −/+

[a]Staining density scale of − to ++++.

Table 4 GFAP Immunohistochemistry, cat caudate[a,b]

Control cats	FIV only	METH only	FIV and METH
Cat 145 −/+	Cat 137 −/+	Cat 142 ++++	Cat 140 ++++
Cat 147 −/+	Cat 138 +	Cat 143 ++	Cat 141 +++
Cat 148 −/+	Cat 161 −/+	Cat 144 ++	Cat 146 ++
Cat 169 −/+	Cat 171 −/+	Cat 173 +++	Cat 181 ++
Cat 172 −/+	Cat 177 −/+	Cat 174 ++++	Cat 182 ++++
	Cat 179 −/+	Cat 175 ++	Cat 184 +++

[a]No obvious differences in total number of cells at low magnification.[b]Evidence of astrocyte activation, ratings based on observations at 40× (cell size, degree of engorgement, and thickening of processes). Scale: − to ++++.

Discussion

This is the first report of an enhancement of METH-associated toxicity by a concurrent FIV infection and the potentiation of FIV-induced neurological disease through exposure to METH. Such synergism may have implications for those HIV-infected patients who also use and abuse METH.

The results of this study clearly demonstrated the METH exposure enhanced the virus-induced alterations in BAEPs. Disruptions in the middle components of BAEPs have been observed in asymptomatic HIV-1 patients (Smith *et al,* 1988; Cazzullo *et al,* 1990; Hausler *et al,* 1991; Ollo *et al,* 1991; Vigliano *et al,* 1997, 2000; Castello *et al,* 1998; Christensen *et al,* 1998; Reyes-Contreras *et al,* 2002), simian immunodeficiency virus (SIV)-infected monkeys (Prospero-Garcia *et al,* 1996; Raymond *et al,* 1998; Fox *et al,* 2000), and FIV-infected cats (Phillips *et al,* 1994; Henriksen *et al,* 1995; Barr *et al,* 2000a), suggesting that these alterations could be considered as a sign of an incipient impairment of nervous system function. In fact, the alterations in BAEPs have been the most consistent and reliable sign of FIV-induced central nervous system (CNS) disease (Phillips *et al,* 1994; Henriksen *et al,* 1995; Barr *et al,* 2000a). As expected, the FIV-infected cats showed significant delay in the latency of P6. This effect was demonstrated as early as 25 days after infection, whereas METH alone had no effect on the BAEPs. However, when animals were simultaneously exposed to both METH and FIV, the FIV-induced alteration of the BAEPs appeared earlier in

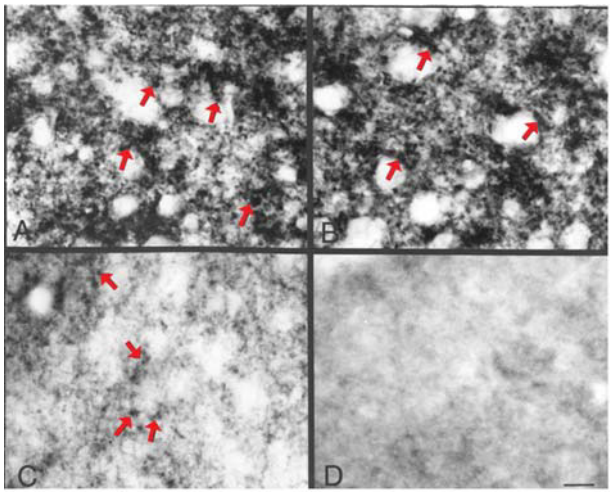

Figure 3 DAT immunoreactivity in cat caudate. In each of the panels (**A–D**) representative photomicrographs were chosen for the indicated treatment group. **A** is from a control animal; **B** is from an FIV-infected cat; **C** is representative of the METH-treated cats; **D** is from an animal that the received METH and was infected with FIV. Calibration bar = 10 μ. Arrows marking clusters of immunoreactivity. In **A** and **B,** note the dense plexus of DAT immunoreactivity in the control and FIV cats. In **C,** note the depletion of DAT immunoreactivity in the METH-treated cat, with only a few remaining immunoreactivity sites (*arrows*) scattered throughout the caudate. In **D,** there is a near total depletion of DAT immunoreactivity in the FIV/METH cat.

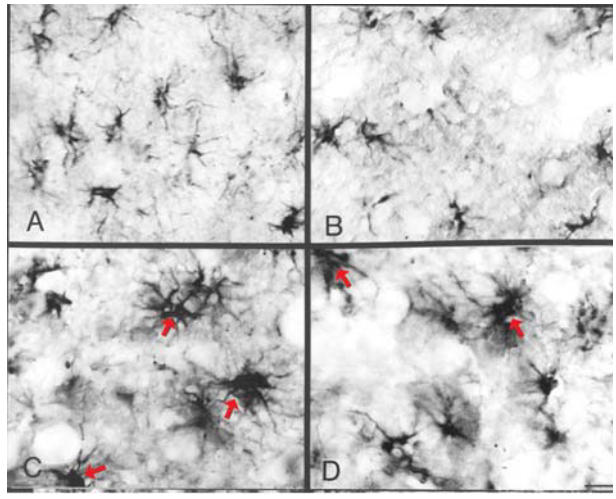

Figure 4 GFAP immunoreactivity in cat caudate. In each of the panels (**A–D**) representative photomicrographs were chosen for the indicated treatment group. **A** is from a control animal; **B** is from an FIV-infected cat; **C** is representative of the METH-treated cats; **D** is from an animal that received METH and was infected with FIV. Calibration bar = 10 μ. **A** and **B** both exhibit nearly identical GFAP immunoreactivity in terms of number, size, and distribution of cells. **C** and **D** both exhibit enlarged glial cells (enlarged cell bodies and processes).

the disease course and had larger latency delays. These results clearly show that METH accelerated and increased the FIV functional pathology in auditory pathways, demonstrating a synergistic effect of METH and FIV on altered brain function.

This study demonstrates that the synergism was bidirectional, in that FIV enhanced the toxic effects of METH. Neither the METH treatment with this specific administration schedule nor FIV infection alone significantly decreased the levels of dopamine (DA) in the feline caudate. However, in those animals that were exposed to both FIV and METH, DA levels were significantly decreased, demonstrating a synergistic effect between the virus and METH on the caudate DA levels. This effect was also very striking on the DA metabolite, DOPAC. No significant alterations were found on the levels of serotonin (5-HT) or its metabolite, 5-HIAA (data not shown). However, when the caudate ratio of 5-HIAA/5-HT was examined, a significant increase in the ratio was demonstrated only in the FIV/METH group, indicating, once again, a synergism (potentiation) between the METH and FIV. This alteration in the ratio may indicate an increase in the turnover rate of 5-HT in the FIV-infected METH-exposed animals.

The synergistic effect of FIV on METH-induced toxicity was also demonstrated when dopamine transporter (DAT) levels were examined. The caudate DAT levels in animals infected with FIV were similar to the levels found in the controls. METH treatment alone substantially depleted the caudate of DAT immunoreactivity, but in the METH/FIV group, an even greater reduction was demonstrated in DAT immunoreactivity. These data also demonstrate a potentiation effect occurred between FIV and METH, regarding the amount of DAT detected in the feline caudate. Though the METH depletion of DAT was substantial, animals exposed to both METH and FIV resulted in even greater DAT depletion, indicating an important alteration in DA neurochemical homeostasis. DA in the striatum is involved in both behavioral and cognitive functions, therefore widespread motor and cognitive dysfunction could accompany disruptions in the neurochemistry of dopamine homeostasis.

This study demonstrated that reciprocal enhancement of CNS dysfunction occurred, with METH exposure increasing the FIV-induced functional pathology of the CNS, and FIV increasing the METH-induced CNS toxicity. The observed effects of these two agents on CNS cannot be explained by mere additive effects of METH and FIV, but rather the results are highly suggestive of synergistic interactions occurring between these two agents. The precise nature of these interactionsis currently unknown. Further investigations are needed to determine the underlying synergistic mechanisms.

Our results are consistent withthe findings that HIV-1–infected patients with a history of METH abuse generally have a more rapid neurologic disease progression when compared to HIV-1–infected patients who have not abused METH (Bell *et al,* 1998; Bouwman *et al,* 1998; Nath *et al,* 2001, 2002; Langford *et al,* 2003; Chang *et al,* 2005; Theodore *et al,* 2007). In addition, the data from this present study support previous studies where methamphetamine and HIV-1 Tat protein, injected directly into the striatum of mice, synergistically impaired striatal dopaminergic function (Maragos *et al,* 2002; Cass *et al,* 2003; Theodore *et al,* 2006). In the present study, we also found synergistic interactions between METH and FIV. However, the lentivirus exposure in the present study was dependent upon a peripheral lentivirus infection and a productive virus replication in its natural host, and thus, likely represents a more natural lentivirus exposure. The fact that two disparate animal models are showing similar results enhances the likelihood that these findings are functionally valid and are also occurring in HIV-1–infected METH abusers. Both of these animal models are needed to study the pathogenic mechanisms of the METH/lentivirus interactions in the CNS. The precise role of FIV Tat and other potential pathogenic mechanisms needs further elucidation.

The results in this study with METH treatment differ from the results we have previously described in two similarly designed studies with morphine (Barr *et al,* 2000b, 2003). In these studies, FIV disease progression was delayed, prevented, or decreased by morphine exposure (Barr *et al,* 2000b, 2003). Thus, exposure to drugs of abuse may have different effects on lentivirus pathogenesis and the outcome of simultaneous exposure of an individual to multiple drugs of abuse could be difficult to predict.

Though the main purpose of this study was to examine the interactions of METH and FIV on the CNS, we also examined non-CNS parameters. Because FIV is generally more cell-associated than HIV-1, detectable plasma viremia levels tend to be limited to the early time points of infection. Yet, the duration of detectable viremia was even shorter in those animals that received the METH treatment, becoming statistically different in the 11th week post infection. The data from the present *in vivo* study appear to contradict our own *in vitro* data (Phillips *et al,* 2000; Nath *et al,* 2001), as well the *in vitro* data of others, where the addition of METH enhanced virus replication with both FIV (Gavrilin *et al,* 2002) and HIV-1 (Liang *et al,* 2008) in cell culture. The differing results among these studies point out the necessity to conduct *in vivo* studies to confirm *in vitro* findings. However, FIV/METH-treated animals did develop lymphadenopathy more rapidly than those animals that were infected with FIV but not treated with METH. Also, the FIV/METH group tended to have lower CD4/CD8 ratios, although significant differences were not detected. From these data, it is clear that the effects of METH on FIV peripheral disease

progression are complex and somewhat contradictory and are in need of further investigation.

The results of this study indicate that a dual potentiation occurred. That is, METH enhanced FIV-induced CNS disease and FIV enhanced the toxic effects of METH. The mechanism(s) of these effects need further investigation. Although HIV-1 and METH are very different disease-producing agents, they both cause neurotoxic effects in overlapping brain regions (mesostriatal and corticostriatal). Also, it is clear that simultaneous exposure to lentiviruses and methamphetamine is occurring in certain subject populations. Thus, future studies using the FIV cat model to investigate the underlying mechanism(s) of this dual potentiation are being planned.

Materials and methods

Animals

All animal experiments were formally approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Twenty-four 6-month-old female specific pathogen–free cats, obtained from Liberty Laboratories (Liberty Corners, NJ), were randomly placed into four groups of six cats each. Group 1 was not infected with FIV nor METH treated, group 2 was METH treated but not FIV infected, group 3 was FIV infected but not exposed to METH, and Group 4 was FIV infected and METH treated. METH was given per os to the appropriate cats (all animals not given METH received empty gel capsules). The dose of METH given to each individual animal was titrated to physiological effect (ranging from 0.3 to 1.5 mg/kg), as the individual differences in pharmacological response to METH exposure varied. Dosing was reduced, in more sensitive animals, to prevent their temperature from rising more than 1°F to 1.5°F above normal core body temperature. The rational for this dosing approach are twofold: (1) on the street METH abusers titrate their initial doses based on the emergence of "unpleasant" side effects (Jaffe, 1990; Angrist, 1994); and (2) METH-induced hyperthermia correlates with neurotoxicity, but can have fatal consequences if not controlled (Albers and Sonsalla, 1995; Cappon *et al,* 1997). The study consisted of 5 consecutive days of a single METH dose and a 3-week period of no drug treatment between each of the 5-day dose cycles. This cycle of dosing was repeated six times. The appropriate animals were intravenously infected with 10,000 $TCID_{50}$ units of FIV-PPR on the fifth day of the first dosing cycle. Those animals not infected received a sham intravenous saline injection. Animals receive a blinded physical and neurological examination at weeks 0, 2, 4, 8 12, 16, and 18, by a veterinarian with 5 years of clinical experience. Lymph node enlargement was determined as part of the physical examination. At week 18 in the study, 3 weeks after the last METH exposure, the study was terminated by placing the animals into a deep plane of anesthesia using isoflurane. To clear the blood from the brain and reduce postmortem changes, the heads were perfused with 2 L of ice-cold phosphate-buffered saline (PBS) at a pH of 7.4, containing 1 U heparin/ml. The brains were quickly removed, dissected onice, and frozen at −70°C.

Real-time quantitative PCR

Viral RNA was prepared from 280 μl of EDTA-anticoagulated, cell-free plasma using the Viral RNA Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted from the silica-columns in 30 μl of nuclease-free water and analyzed immediately.

Oligonucleotides for the real-time TaqMan PCR system were chosen within the FIV *gag* gene. Probe and primer sequences are adapted from a previously described TaqMan system (Klein *et al,* 2001) to FIV subtype A pPPR (GenBank accession no. M36968) and were selected according to parameters defined from the Primer Express software (Applied Biosystems, Foster City, CA).

FIV *gag*-specific primers and probe used in FIV TaqMan assay:

Primer or probe	Sequence	Fragment (bp)
Forward primer FIV-526f	GCC TTC TCT GCA AAT TTA ACA CCT	
Reverse primer FIV-646r	GAT CAT ATT CTG CTG TCA ATT GCT TT	311
Probe FIV-555p	(b)CAT GGC CAC ATT AAT AAT GGC CGC A(a)	

Sequences of primers and probes are given in 5′→3′ orientation. Numbers represent nucleotides beginning at the start codon of the gag sequence of M36968. (a) Nucleotide to which the reporter dye 6-FAM is coupled; (b) nucleotide to which the quencher dye TAMRA is coupled. The probe was designed to bind against the reverse strand.

The real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was run, as previously described (Klein *et al,* 2001). Briefly, all reactions were carried out in a 7700 ABI PRISM Sequence Detector (Applied Biosystems). Real-time fluorescence measurements were performed, and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline, as determined between cycles 3 and 15). Efficiencies of amplification were determined by running standard curves with diluted samples.

Brain auditory evoked potentials (BAEPs)

BAEPs were conducted as previously described (Phillips *et al,* 1994; Henriksen *et al,* 1995; Barr *et al,* 2000a, 2000b). Briefly, subcutaneous monopolar needle electrodes were placed aseptically in the scalp and grounded in the nuchal muscles. Plastic bilateral

ear tubes placed in the external auditory canal delivered the auditory stimuli, and evoked events were recorded and analyzed by use of LabView software (National Instruments, Austin, TX). Averaged peak latencies and amplitudes were calculated.

Caudate monoamine levels

Caudate monoamine levels were determined by a modification of previously published method (Taffe *et al,* 2002). Briefly, preweighed (approximately 200 μg each) tissue samples punched from a mid rostral-caudal slice were homogenized by ultrasonic disruption in 250 μl of chilled 0.1 N perchloric acid containing 100 nM N^{ω}-methyl-5-hydroxytryptamine (*N*-methylserotonin) as an internal standard. Following centrifugation, 30 μl of the supernatant was injected onto a high-performance liquid chromatography (HPLC) column (2 × 150 mm BetaBasic C18, 3 μm particles, 150 Å pore size; Keystone Scientific, Bellefonte PA) and monoamines and their metabolites were eluted using a mobile phase consisting of 150 mM citric acid, 15 mM sodium acetate, 1.4 mM sodium octyl sulfate, 100 μM EDTA, 29 mM triethylamine and 5% (*v/v*) methanol (apparent pH of 2.5) delivered at 0.1 ml/min by an 1100 series HPLC pump from Agilent Technologies (Wilmington, DE). The column eluent was delivered directly to a standard electrochemical cell containing two glassy carbon working electrodes (model MF-1000; BioAnalytical Systems, Lafayette IN) arranged in series and maintained at +700 mV against a Ag/AgCl reference electrode (Model RE4; BioAnalytical Systems, Lafayette, IN). The electrode potential and current analyses were controlled by a LC-4B amperometric detector (BioAnalytical Systems). External calibration curves were generated daily from fresh standard solutions and the limit of detection was approximately 4 nM for all analytes.

Immunohistochemistry for dopamine transporter (DAT) and glial fibrillary acidic protein (GFAP)

The contralateral cat caudate nuclei were removed, immersion-fixed in 4% parafomaldehyde in phosphate-buffered saline (PBS) for 1 week, and cryoprotected in graded sucroses (12%, 16%, and 20%) in PBS. 50um frozen sections of each caudate nucleus were prepared and every section was counted and saved in a cryopreservative solution (phosphate buffer containing ethylene glycol) and stored at −80°C. Prior to immunohistochemistry, sections were removed from the cryopreservative, thawed, and rinsed several times in PBS. Sections were then incubated in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 for one hour room temperature.

Sections were then incubated with the primary antibodies diluted in PBS containing bovine serum albumin (BSA) (1 mg/1 ml) and O.3% Triton X-100 for 24 to 48 h at 4°C. Rabbit anti-cow glial fibrillary acidic protein (GFAP) polyclonal antibody (Dako) was used diluted 1:1000 and rat anti-dopamine transporter monoclonal antibody (DAT) (Chemicon, Temecula, CA) was diluted 1:500. After sections were rinsed 3 × 10 min in PBS, they were processed with an ABC kit (Vector, Burlingame, CA). Sections were incubated in a 1:200 dilution of the corresponding biotinylated secondary antibody for 2 h. Sections were rinsed in PBS (as above) and incubated in a 1:100 dilution of avidin-biotinylated horseradish peroxidase for 2 h. Sections were rinsed and the peroxidase reaction was developed with 0.05% 3,3-diaminobenzidine-4HCl (DAB) and 0.003% hydrogen peroxide. Following completion of the reaction, the sections were rinsed in PBS, mounted on coated slides, air-dried, dehydrated, and permanently coverslipped. All material was blindly analyzed by an experienced histologist. The staining density was graded on a scale of − (none) to ++++ (maximum density). Representative fields were photographed under bright-field using a Zeiss Axiophot microscope.

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