Neuropsychopharmacology (2006) 31, 956–966

www.neuropsychopharmacology.org

Methamphetamine Causes Alterations in the MAP Kinase-Related Pathways in the Brains of Mice that Display Increased Aggressiveness

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Aggressive behaviors have been reported in patients who suffer from some psychiatric disorders, and are common in methamphetamine (METH) abusers. Herein, we report that multiple (but not single) injections of METH significantly increased aggressiveness in male CD-1 mice. This increase in aggressiveness was not secondary to METH-induced hyperactivity. Analysis of protein expression using antibody microarrays and Western blotting revealed differential changes in MAP kinase-related pathways after multiple and single METH injections. There were statistically significant ($p<0.05$) decreases in MEK1, Erk2p, GSK3α, 14-3-3e, and MEK7 in the striata of mice after multiple injections of METH. MEK1 was significantly decreased also after a single injection of METH, but to a much lesser degree than after multiple injections of METH. In the frontal cortex, there was a statistically significant decrease in GSK3α after multiple (but not single) injections of METH. These findings suggest that alterations in MAP kinase-related pathways in the prefronto-striatal circuitries might be involved in the manifestation of aggressive behaviors in mice.
Neuropsychopharmacology (2006) **31,** 956–966. doi:10.1038/sj.npp.1300891; published online 28 September 2005

Keywords: proteomics; violence; drug abuse; psychiatric disorders

INTRODUCTION

Violence and aggressive behaviors are important public health problems because of their medical and criminal consequences (Dahlberg, 1998; Golding, 1996; Lederhendler, 2003; Prothrow-Stith, 1995). These issues are compounded by the fact that increased aggressiveness is often observed in patients who suffer from bipolar disorder, major depressive disorder, and antisocial personality disorders (Posternak and Zimmerman, 2002; Swann *et al*, 2004). Aggressive behaviors are also quite common among abusers of methamphetamine (METH, speed) (Carey and Mandel, 1968; Ellinwood, 1971; Hawks *et al*, 1969; Miczek and Tidey, 1989; Szuster, 1990). As aggressive behaviors and social interactions can also be influenced by METH in rodents, cats, and non-human primates (Crowley, 1972; Maeda *et al*, 1985; Miczek and O'Donnell, 1978; Shintomi, 1975; Sokolov *et al*, 2004), understanding of neuronal adaptations that might be associated with METH-induced aggressiveness in animals should provide a window toward identifying some of the neurobiological substrates that subsume these behaviors.

Complex psychosocial behaviors, including neuropsychiatric disorders, such as bipolar and major depressive disorders, that often involve increased aggressiveness (Posternak and Zimmerman, 2002; Swann *et al*, 2004), are thought to be secondary to dysfunctions in prefronto-striatal circuitries (Strakowski *et al*, 2005). Dysfunctions in prefronto-striatal circuits might also subsume psychostimulant abuse, which is frequently associated with violent behaviors (Gold *et al*, 1989; London *et al*, 2004; Schultz, 2002; Sekine *et al*, 2001). These clinical observations suggest that the prefronto-striatal system is involved in aggressive behaviors. There is extensive literature regarding the association between frontal lobe injury and aggressiveness (Brower and Price, 2001). For example, a study of 279 Vietnam veterans, who had suffered penetrating head injuries, indicated that patients with frontal ventromedial lesions had significantly higher Aggression/Violence Scale scores than controls and patients with lesions in other brain areas (Grafman *et al*, 1996). Comparative neuropsychology has also provided evidence for a role for the striatum in certain forms of aggression. Examining human subjects with focal lesions affecting the ventral striatum demonstrated a disproportionate impairment in recognizing human signals of aggression, suggesting the role of the striatum in coding signals of aggression (Calder *et al*, 2004). Abnormalities in striatal dopamine re-uptake sites in

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Received 21 February 2005; revised 7 June 2005; accepted 5 August 2005
Online publication: 5 August 2005 at http://www.acnp.org/citations/Npp0805050114/default.pdf

habitually violent alcoholic offenders has been reported (Kuikka *et al*, 1998). Moreover, enhanced defensive aggressiveness, excessive alcohol intake, and impulsive behavior were found in rats following extensive lesions of the ventral striatum and the septal area (Johansson and Hansen, 2000). Furthermore, chemical lesions of the nucleus accumbens septi have been reported to influence apomorphine-induced aggression in rats (Pucilowski and Valzelli, 1986). These observations suggest that prefronto-striatal circuits may be involved in the regulation of aggressive behaviors. As a first step towards testing this idea, we sought to discriminate patterns of protein expression in the brains of mice that exhibited aggressive behaviors after chronic treatment with METH. Herein, we report that repeated injections of METH over a period of 8 weeks caused marked alterations in the expression of proteins involved in MAP kinase-related pathways in the striata and frontal cortex of aggressive mice.

METHODS

All animal use procedures were according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of NIDA IRP. All efforts were made to use the minimal possible number of animals to address the questions raised by the current study.

Male CD-1 mice (9–11 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were randomly assigned to the METH/METH (chronic METH), Sal/METH (acute METH), or Sal/Sal (control) groups. Before being given the injections, mice were habituated to their environment for 1 week. Mice were housed in groups of four or in a cage ($27 \times 16 \times 12\,cm^3$) with free access to food and water. Mice were single housed, starting the 6th week when they were 15–17 weeks old. They were maintained on a 12 h light/dark cycle (lights on at 0700 h) at $21 \pm 2^{\circ}C$.

The chronic METH regimen was designed to bear some degree of similarity with clinical patterns of METH abuse, which can vary significantly, but usually include gradual increases of drug intake at early stages and occasional interruptions of drug use after binges (Cadet *et al*, 2003; Kramer *et al*, 1967; Yen *et al*, 2005). METH/METH mice received intraperitoneal injections of METH in 0.5 ml of saline at 0800 and 1400 h according to the following schedule of escalating METH doses: during the first week on day 1, the mice were injected with 1 mg/kg (0800 h) and 2 mg/kg (1400 h) of METH; on day 2, they were injected with 3 mg/kg (0800 h) and 4 mg/kg (1400 h); and on day 3, they received 5 mg/kg (0800 h) and 6 mg/kg (1400 h) of METH. After these escalating METH injections, mice did not get the drug for 2 days. During the second, third, and sixth–eight weeks, they received two injections of METH (6 mg/kg) at 0800 and 1400 h for 5 days; they received no injections on the last 2 days of the week. Moreover, the animals did not get any injections on the fourth and fifth weeks.

Control (Sal/Sal) mice received repeated injections of 0.5 ml of saline on a similar schedule as the METH/METH animals. Sal/METH mice received saline in a fashion similar to the Sal/Sal mice, except for the last injection, which was one of METH (6 mg/kg). This approach is different from the administration of single dose large or multiple moderate doses of METH which cause neurotoxic damage to the rodent brain (Cadet *et al*, 2003).

Assessment of Locomotor Activity

Cages with mice were transported to the testing room 45–60 min before the start of the test session to minimize the possible effects of stress associated with the transfer. In order to examine locomotor activity immediately after METH challenge, mice chronically treated with saline or METH were placed in an activity monitor cage (Med. Associates, Inc., East Fairfield, VT) in the morning, 19 h after the last injection, and locomotor activity was recorded for 15 min as cm/min traveled. At this time point, mice were challenged with 6 mg/kg METH and recording locomotor activity was continued for another 30 min. The total observation time was 45 min.

Locomotor activity at 4 and 20 h after METH challenge was tested in other groups of mice. To test locomotor activity at 4 or 20 h after injection, each animal from each treatment group was taken from its home cage, injected with either SAL (Sal/Sal group) or 6 mg/kg METH (METH/METH and Sal/METH groups), and returned to its home cage. At 4 or 20 h point after challenge with METH, the cage was placed into an activity monitor. Mice were allowed to habituate to the activity monitor for 15 min and then activity was recorded for 15 min.

Assessment of Aggressiveness

Aggressiveness was examined as the latency time before the first bite attack using the 'resident–intruder' paradigm (Miczek and O'Donnell, 1978). Tested mice (residents) were single-housed for 3 weeks before tests and received no treatments. Intruder mice were housed in groups of four. Intruder mice were of the same age and from the same shipment as the tested mice. In order to avoid injury, intruder mice were removed immediately after attack. Bedding in cages was changed once a week. Tests for aggressiveness were performed on days 6 or 7 after changing the bedding. In order to avoid confounds caused by stress related to moving, tests were performed in the same room where the mice were housed. Each mouse was tested only once and each intruder was used in only one test. Latency before the bite attack was measured as the time between the placement of the intruder in the resident cage and the first bite attack. The latency for mice that did not initiate a bite attack was assumed to be 900 s, which corresponded to the total time of observation.

Antibody Microarray Analysis

The antibody microarrays (BD Clontech AB Microarray 380, BD, Biosciences, Palo Alto, CA) used in the current study were composed of 378 distinct monoclonal antibodies printed at high density on a glass microscope slide. Sal/Sal and METH/METH mice pairs were assigned for the analysis randomly. The Ab Mircoarray 380 contains antibodies with specificities for proteins related to signal transduction, oncogene products, cell cycle regulation, cell structure, apoptosis, and neurobiology among others.

About 95% of the antibodies are directed at intracellular proteins, while approximately 5% are cytokines and receptors. In all, 95% of the antibodies were raised against human proteins. Whenever possible, antibodies that also recognize the specific mouse and rat proteins where chosen. The specific details on the cross-species reactivities for each antibody can be found on the antibodies datasheet found on the BD Biosciences Pharmingen website (http://www.clontech.com/clontech/products/families/abarray/index.shtml). Total protein for microarray analysis was extracted from 75–100 mg of striatum (including the caudate and nucleus accumbens) or frontal cortex by homogenizing in Extraction/Labeling Buffer provided by the manufacturer of microarrays. Extracted proteins were then labeled by covalent attachment of fluorophores Cy3 or Cy5. After protein labeling, unbound dye was removed by gel exclusion chromatography using PD-10 Desalting Columns (Amersham Biosciences). The protocol was designed to effectively control for variations in labeling efficiency. Specifically, the METH/METH and SAL/SAL samples were each split into two equal portions. Each portion was then labeled with either Cy5 or Cy3 to produce four samples: METH/METH-Cy3, METH/METH-Cy5, SAL/SAL-Cy3, and SAL/SAL-Cy5. In labeling proteins, each of the dye solutions was split into equal portions so that both samples react with identical dye stocks. The labeled samples were combined to produce a mixture of Cy5- and Cy3-labeled proteins. METH/METH-Cy3 was combined with SAL/SAL-Cy5 (mixture #1), while METH/METH-Cy5 was combined with SAL/SAL-Cy3 (mixture #2). After thorough mixing, an aliquot from each mix (50 μg of total protein) was incubated with a separate array in separate chambers, washed, dried, and scanned using GenePix 4000B Axon scanner (Axon Instruments, Union City, CA). Cy3/Cy5 ratio for each antigen captured on microarray was determined from the ratio of absorbance at 552 and 650 nm, respectively. Internally normalized ratio (INR) for each antigen was then calculated as root square of the ratio between Cy5/Cy3 ratio in the mixture #2 and Cy5/Cy3 ratio in the mixture #1.

Kinetworks^{TM} KPKS 1.0 Protein Kinase Screen

Brain tissue was homogenized on ice in the following buffer: 20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, and 0.5% Triton X100. To inhibit protein–serine phosphatases, the buffer also contained 30 mM sodium fluoride, 40 mM β-glycerophosphate, and 10 mM sodium pyrophosphate. To inhibit protein–tyrosine phosphatases, the buffer contained 2 mM sodium orthovanadate. To inhibit proteases, the buffer contained 1 mM phenylmethylsulfonylfluoride, 3 mM benzamide, 5 μM pepstatin A, and 10 μM leupeptin. Tissue homogenates were diluted to a protein concentration of 1.0 mg/ml in SDS-PAGE sample buffer. The final composition of the sample buffer in the sample was 31.25 mM Tris-HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), and 0.02% bromophenol blue (w/v). The sample was boiled for 4 min at 100°C and stored at −20°C. Kinetworks^{TM} KPKS 1.0 high-throughput Western blotting analysis of the expression of 75 different protein kinases was performed by Kinexus Bioinformatics Corp. (Canada), with 800 μg of brain tissue lysate protein. Quantification of the immunoreactive bands on the Kinetwork blots with ECL detection was performed with a Bio-Rad FluroS Max Imager and Bio-Rad Quantity One software.

Western Blotting

Proteins for Western blotting were prepared as described for the Kinetworks analysis (see above). Proteins (10–15 μg/well) were separated on a 7.5 or 10% SDS-polyacrylamide gel and transferred onto nitrocellulose (Bio-Rad) membranes. The MEK1/2, phospho-Mek1/2 (Ser-217/Ser-221) antibody (rabbit polyclonal IgG, affinity purified) was obtained from Cell Signaling Technology (New England Biolabs). Anti-rabbit secondary antibody, antibiotin antibody conjugated to horseradish peroxidase, and biotinylated protein marker were also obtained from Cell Signaling Technology. Chemiluminescent detection was performed using Phototope-HRP Western Detection Kit (Cell Signaling Technology). For quantification of band density, films were analyzed using BioImaging Systems and Lab Works Imaging and Analysis densitometric software (UVP, Inc., Upland, CA).

Data Analysis

Differences between groups were examined using one-way ANOVA, followed by Dunnett's treatment *vs* control *post hoc* test (two-tailed). Dunnett's *post hoc* test treats one group (SAL/SAL) as control and compares all other groups against it. The significance of difference in AB microarray analysis was examined using one-sample *t*-test (two-tailed). Statistical analysis was carried out using SSPS 13.0 for Windows. Data are expressed as means $\pm$ SE, with the number of individual experiments presented in the figures and tables.

RESULTS

Effects of Repeated and Single METH Injections on Aggressive Behaviors

Aggressiveness was measured as time latency before the first bite attack in the resident/intruder test. Time latency before an attack measured at both 15 min and 20 h after the METH challenge was significantly shorter in mice chronically treated with METH (METH/METH group) than in mice treated with saline (SAL/SAL group). By contrast, time latency in mice that received a single injection of METH (SAL/METH group) was not different from controls (SAL/SAL group) (Figure 1).

Effects of the METH Challenge on Locomotor Activity

The METH injection caused an increase in locomotor activity in mice chronically treated with METH and mice chronically pretreated with saline, with the METH-pretreated mice showing significantly greater changes (Figure 2a). Locomotor activity remained increased 4 h after the METH challenge in mice chronically treated with METH ($p = 0.003$), but not in saline-treated mice (SAL/METH group) (Figure 2b). No significant changes in locomotion were found when the mice were tested 20 h after the METH challenge (Figure 2c). These observations are in contrast to those observed in the test of aggressive

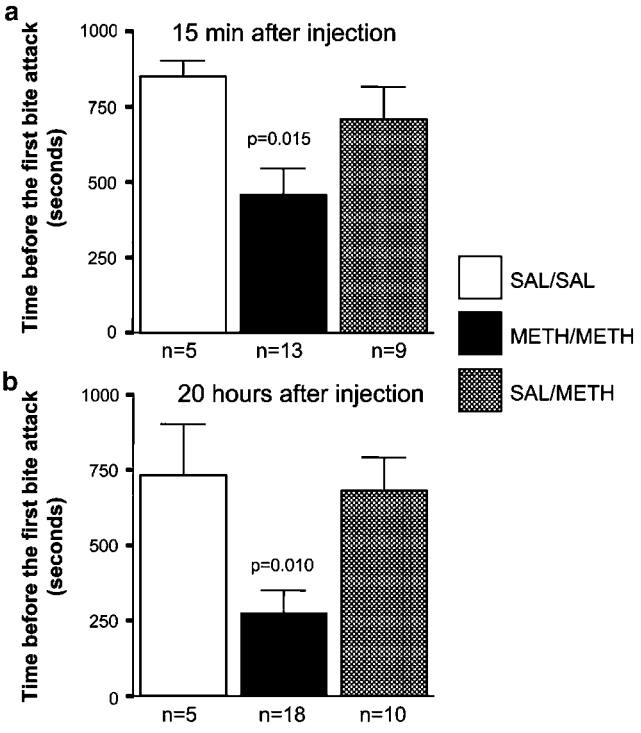

Figure 1 Chronic treatment with METH increases aggressiveness in mice measured 15 min or 20 h after the last injection. Latency time before the first bite attack on the intruder was recorded in resident–intruder paradigm. Latency time was measured 15 min (a) or 20 h (b) after injection of METH (METH/METH and SAL/METH groups) or saline (SAL/SAL group). Note that the time before the bite attack was significantly shorter in mice chronically treated with METH both at the 15-min and 20-h time points. ANOVA revealed significant differences among the groups (ANOVA, $F=4.000$, $df=2, 24$, $p=0.032$ and $F=6.26$, $df=2, 32$, $p=0.005$ at 15-min and 20-h time points, respectively). *Post hoc* analysis revealed significant difference between the METH/METH and SAL/SAL groups ($p=0.015$ and 0.01 at 15-min and 20-h time points, respectively).

behaviors (see Figure 1). Qualitative observations revealed stereotypic behaviors in mice chronically treated with METH, but not in mice that received a single injection of METH. The most common stereotypic behaviors were running in circles, constant grooming, or poking heads into the bedding. These behaviors typically began several minutes after the METH challenge and continued for several hours. No apparent stereotypy was evident 20 h after METH challenge. Stereotypy apparently did not preclude measurements of locomotor activity in our experiments, since, as illustrated in Figure 2a, despite stereotypy, mice treated with METH chronically showed greater hyperlocomotion after METH challenge than did saline-treated mice challenged with same dose of METH.

Antibody Microarray Analysis

Mice chronically injected with METH (METH/METH mice) were compared to mice chronically injected with saline (SAL/SAL mice). Tissues from the striatum and frontal cortex were collected 4 h after the last injection. The 4-h time point lies within the interval of time when mice chronically treated with METH display increased aggressiveness. This time point was selected because 4 h after injection only mice chronically treated with METH exhibited hyperlocomotion, while mice that received a single injection of METH did not. Thus, METH-induced behavioral changes at this time point were likely to be due to neuroadaptative changes rather than due to the acute effects of METH. The average difference in protein abundance between METH/METH and SAL/SAL mice measured using AB microarrays from five independent experiments was less than 9% for all but two proteins, which indicated 13 and 15% differences, respectively (data not shown). For the purpose of further evaluation, we define proteins as possibly affected if they replicated greater than 5% increases or decreases in 4 or 5 out of 5 independent experiments comparing METH/METH mice with SAL/SAL mice (Table 1). By these criteria, there were seven downregulated and one upregulated proteins after chronic METH treatment, including Erk2 and 14-3-3e (Table 1); these results suggested possible alterations in MAP kinase-related pathways in mice chronically treated with METH.

Analysis of MAP Kinase-Related Pathways Using Kinetworks Protein Kinase Screen

Striatum. Three other groups of mice were injected using either METH/METH ($n=11$), SAL/METH ($n=9$), or SAL/SAL ($n=9$) treatment regimens. Tissues were collected 4 h after the last injection. Protein samples from two or four animals were pooled for each analysis to reduce individual variability and to reduce the cost of the analysis. Four pools for the METH/METH and SAL/SAL groups and three pools for the SAL/METH group were prepared. According to Kinexus recommendations, fold changes in immunoreactivity greater than 1.25 in a single experiment may be considered as evidence of altered abundance of a protein. In this study, we chose to apply more stringent criteria for changes. Changes were considered to be robust if the fold changes from repeated independent experiments (four experiments for the METH/METH mice and three experiments for the SAL/MET mice) were greater than 1.5. Kinetworks analysis confirmed lower abundance of Erk2 (both total and phosphorylated Erk2) in the striatum of mice chronically treated with METH (Table 2). Importantly, two different antibodies were used to measure Erk2 in the Kinetworks analysis, and both revealed similar magnitudes of changes (Table 2). Erk2 reduction revealed by Kinetworks screen (Table 2) was greater than reduction revealed using antibody microarrays (Table 1), indicating, possibly, greater sensitivity of Kinetworks screen compared to antibody microarrays. It is important to note, however, that different specificities of antibodies and different groups of animals were used in AB microarrays and Kinetworks screen. Measurements of other MEK1/2/Erk1,2 pathway-related protein kinases revealed decreases in Erk1 and several upstream protein kinases, including Raf1(72), MOS, MEK1, as well as in several downstream protein kinases, including RSK2, GSK3α, and GSK3β (Table 2). The level of the upstream regulator of the Erk1/2 pathway, protein kinase A (PKA), was decreased, whereas levels of the PKCα and PKCζ subunits of protein kinase C (PKC) were increased in the striata of METH/METH mice. Decreases in MEK1, Erk2p, GSK3α, and MEK7 in METH/METH mice

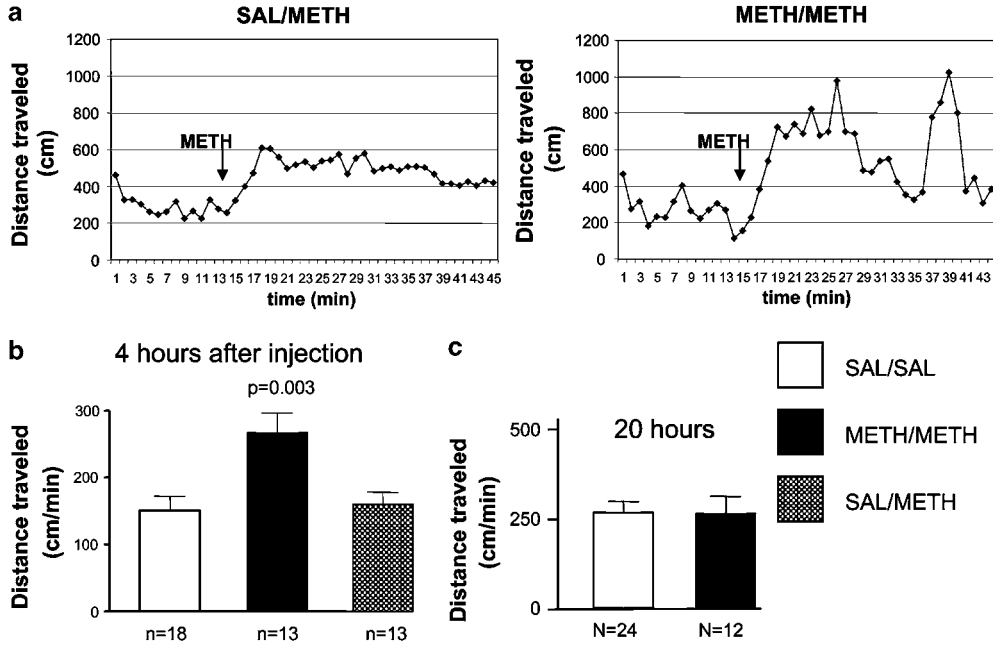

Figure 2 Chronic treatment with METH sensitizes mice to the locomotion-stimulating effect of METH. Effect of METH on locomotor activity in mice. (a) Effect of METH at the 15-min time point after challenge with METH. Mice chronically treated with saline (left panel) or with METH (right panel) were placed in locomotor activity monitored 19 h after the last injection, and the distance traveled was recorded for 15 min. Mice then received an injection of METH (6 mg/kg) and locomotor activity was recorded for another 30 min. Data are the means for 12 mice in each of the groups and are given in 1-min blocks. Arrow indicates the time of injection of METH. (b) Effect of METH challenge 4 h after injection. Separate groups of mice received 6 mg/kg METH (SAL/METH and METH/METH groups) or saline (SAL/SAL group). After 4 h, mice were placed in an activity monitor. Mice were allowed to habituate to the activity monitor for 15 min and locomotor activity was recorded for 15 min. Data are shown as average distance per minute traveled during the 15 min of observation. Locomotor activity measured 4 h after injection was increased in mice that were chronically treated with METH (METH/METH mice), but was not increased in mice that received a single dose of METH (SAL/METH mice) compared with SAL/SAL mice (ANOVA, $F = 7.081$, $df = 2, 41$, $p = 0.002$; *post hoc* tests showed significant ($p = 0.003$) differences between SAL/SAL and METH/METH mice). (c) Locomotor activity measured 20 h after the last injection was not increased in METH/METH mice compared with SAL/SAL mice. Data are mean ± SEM.

Table 1 Antibody Microarray (Proteins Changed in the Striatum of Mice Chronically Treated with METH[a])

	Ratio of protein level in METH/METH mouse to SAL/SAL mouse				
	Pairs of mice				
Protein name	**1**	**2**	**3**	**4**	**5**
Erk 2	0.86	0.90	0.92	1.09	0.94
14-3-3e*	1.03	0.86	0.90	0.91	0.90
p53	0.91	1.04	0.87	0.89	0.91
p21 (Cip1/WAF1)*	0.89	0.92	0.93	1.00	0.90
Integrin b3 (CD61)*	0.95	0.99	0.87	0.94	0.90
NFAT-1**	0.94	0.97	0.90	0.93	0.94
NHE-3*	1.00	0.93	0.93	0.90	0.98
Arginase I	1.10	1.07	1.11	0.96	1.06

*$p < 0.05$, **$p < 0.01$ one-sample *t*-test (two-sided).
Tissue was collected 4 h after the last METH or saline injection.
[a]Change greater than 5% replicated in four out of five METH/METH and SAL/SAL pairs of mice.

were statistically significant ($p < 0.05$). Of the changes observed after chronic treatment with METH, only Erk1, Erk2, and GSKα showed similar changes in the striata of mice treated with a single injection of METH; however, differences did not reach statistical significance (Table 2).

Frontal cortex. The level of GSK3α in the frontal cortex was significantly ($p < 0.05$) decreased after chronic treatment, but not after a single injection of METH (Table 3). Other changes in the frontal cortex did not reach statistical significance and included nominal increases in PKCα, PKCζ, and PKCδ and decrease in PKCμ(120) after both repeated and single injections of METH (Table 3). There were also increases in levels of PKCβ1, PKCε, and PKCγ in the frontal cortex after repeated injections of METH (Table 3). Level of Erk1p was decreased after chronic treatment, but not after a single injection of METH (Table 3).

Total and Phosphorylated MEK1 in the Striatum at 4- and 20-h Time Points after METH Injection

The Kinetworks analysis revealed significant reduction of striatal MEK1 after repeated METH injections, but only modest changes after a single injection (Table 2). As these observations had suggested a role for MEK1 in aggressiveness associated with chronic treatment with METH and because MEK1 plays a central role in the MEK1,2/Erk1,2 pathway, total and phosphorylated MEK1 expression was assessed in the striata from other groups of animals using antibodies different from those used in the Kinetwork analysis. Immunoreactivity was measured 20 h after METH

Table 2 Analysis of MAP Kinase-Related Pathways in Striatum Using Kinexus Kinetworks Protein Kinase Screen

		Abundance[a]			Fold change	
Protein kinase	**Abbreviated name**	**Saline**	**Meth/Meth**	**Saline/Meth**	**Meth/Meth**	**Sal/Meth**
Oncogene Raf 1 (72)	Raf1(72)	2816±626	956±370	2350±920	**−2.9**	−1.2
v-raf homolog B1	RafB	5380±2675	6754±4361	7162±2018	1.3	1.3
v-mos homolog 1	MOS	27965±4734	16534±4890	21528±3574	**−1.7**	−1.3
Cancer Osaka thyroid oncogene	COT	9871±614	7362±1527	7690±1782	−1.3	−1.3
MAP kinase kinase 1 (MKK1)	MEK1	11417±2102	4138±903**	9644±646	**−2.8****	−1.2
Extracellular regulated kinase 1	Erk1	50125±10974	24027±10658	25745±6559	**−2.1**	**−1.9**
Erk1 phosphorylated	Erk1p	8895±338	5174±1979	4171±1798	**−1.7**	**−2.1**
Extracellular regulated kinase 2	Erk2	51759±14139	25371±14125	22960±5520	**−2.0**	**−2.2**
Extracellular regulated kinase 2[b]	Erk2	50449±8164	28232±13392	26334±4414	**−1.8**	**−1.9**
Erk2 phosphorylated	Erk2p	3262±638	2390±71*	1446±478	**−1.4***	**−2.2**
Ribosomal S6 kinase 1	RSK1	5486±1757	6038±2422	7577±2874	1.1	1.4
Ribosomal S6 kinase 2	RSK2	7897	4853	8402	**−1.6**	1.0
Glycogen synthase kinase 3α	GSK3α	6107±855	2591±935*	3063±1096	**−2.4***	**−2.0**
Glycogen synthase kinase 3β	GSK3β	2852±214	1363±398	2480±935	**−2.1**	1.1
Protein kinase A	PKA	2217±1003	1363±376	2298±748	**−1.6**	1.0
Protein kinase C α	PKCα	3172±642	5230±1437	4013±313	**1.6**	1.3
Protein kinase C β1	PKCβ	17951±4700	21828±4607	22721±3652	1.2	1.3
Protein kinase C delta	PKCδ	1664±626	1880±604	1853±749	1.1	1.1
Protein kinase C epsilon	PKCε	16593±7995	16402±7340	17859±4164	1.0	1.1
Protein kinase C gamma	PKCγ	22954±3021	27157±6400	25104±4995	1.2	1.1
Protein kinase C lambda	PKCλ	2781±1501	2330±686	1842±668	−1.2	**−1.5**
Protein kinase C μ(115)	PKCμ	4120±934	3337±1018	4035±1103	−1.2	−1.0
Protein kinase C μ(120)	PKCμ	2211±305	2538±645	3046±1058	1.1	1.3
Protein kinase C zeta	PKCζ	11468±2915	16934±4489	11947±1445	**1.5**	1.0
Cyclin-dependent kinase 5	CDK5	15649±2551	12360±5361	16803±1138	−1.3	1.1
MAP kinase kinase 4	MEK4	22760±4691	24274±8172	22495±4878	1.1	1.0
Hematopoietic progenitor kinase 1	HPK1	5634±3005	3392±779	5600±814	**−1.7**	1.0
MAP kinase kinase 7	MEK7	1648±249	749±270*	1172±109	**−2.2***	−1.4
Stress-activated protein kinase (38)	JNK3 38	4921±837	3651±1324	4946±646	−1.3	1.0
Stress-activated protein kinase (45)	JNK3 46	1602±533	1448±381	1678±524	−1.1	1.0
MAP kinase kinase 6 (MEK6)	MEK6	2256±122	2476±467	3210±868	1.1	1.4
p38 Hog MAP kinase	p38	7193±1771	4953±1033	5909±453	−1.4	−1.2

Data were from Western blotting of tissue from 11 mice in the METH/METH, nine in SAL/METH, and nine in SAL/SAL groups. In each of the treatment group tissue was pooled from two or three mice. Four pools of samples were examined for METH/METH, three for SAL/METH, and four for SAL/SAL groups.
Bold indicate fold changes greater than 1.5.
*$p<0.05$; **$p<0.01$, Dunnet *post hoc* test, two tailed. Tissue was collected 4 h after the last METH or saline injection.
[a]Measured by the area under intensity profile curve. Units are intensity × mm. Data are means ± SEM.
[b]Erk2 was measured using two different antibodies.
ANOVA, MEK1: $F=9.16$, df = 2, 8, $p=0.009$; Erk2p: $F=3.46$, df = 2, 8, $p=0.08$; GSK3a: $F=5.23$, df = 2, 8, $p=0.035$; MEK7: $F=5.05$, df = 2, 8, $p=0.039$.

injection when aggressiveness was increased without hyperlocomotion, and also at 4 h after injection when significant hyperlocomotion was evident in METH/METH but not in SAL/METH mice (Figures 1 and 2).

Consistent with the Kinetworks analysis, levels of total MEK1 measured 4 h after injection were significantly lower in METH/METH mice compared with SAL/SAL mice (Figure 3a and b). MEK1 remained significantly decreased at 20 h after the METH injection in METH/METH mice. At that time, a smaller reduction of MEK1 was found in mice that received a single dose of METH (Figure 3a and b). Analysis of the phosphorylated form of MEK1 in mice chronically treated with METH revealed significant reduction at 4 h after the last METH injection (Figure 3a and b). Significantly greater decreases in phosphorylated MEK1 were observed in the METH/METH group at 20 h after injection with METH (Figure 3a and b). Levels of phosphorylated MEK1 were also decreased 4 h after a single

Table 3 Analysis of MAP Kinase-Related Pathways in Frontal Cortex Using Kinexus Kinetworks Protein Kinase Screen

		Abundance[a]			Fold change	
Protein kinase	**Abbreviated name**	**Saline**	**Meth/Meth**	**Saline/Meth**	**Meth/Meth**	**Sal/Meth**
Oncogene Raf 1 (68)	RAF1 (68)	1660±466	2942±480	1447±271	**1.8**	−1.1
Oncogene Raf 1 (72)	Raf1(72)	2980±576	3317±608	3892±547	1.1	1.3
v-raf homolog B1	RafB	20251±17484	17994±7955	5553±1633	−1.1	**−3.6**
v-mos homolog 1	MOS	19466±1516	24359±3198	27356±6265	1.3	1.4
Cancer Osaka thyroid oncogene	COT	15003±2645	9447±1458	13150±1566	**−1.6**	−1.1
MAP kinase kinase 1 (MKK1)	MEK1	11740±1598	11376±2881	13313±1801	1.0	1.1
Extracellular regulated kinase 1	Erk1	55877±16240	53892±17206	64275±14564	1.0	1.2
Erk1 phosphorylated	Erk1p	9654±2883	5805±1208	10625±451	**−1.7**	1.1
Extracellular regulated kinase 2	Erk2	62406±22770	53388±17764	61295±17793	−1.2	1.0
Extracellular regulated kinase 2[b]	Erk2	74668±1224	58750±9247	65032±7819	−1.3	−1.1
Erk2 phosphorylated	Erk2p	4025±598	3645±947	5018±300	−1.1	1.2
Ribosomal S6 kinase 1	RSK1	8764±2204	9692±1437	10788±2800	1.1	1.2
Ribosomal S6 kinase 2	RSK2	4411	7646	7666	**1.7**	**1.7**
Glycogen synthase kinase 3α	GSK3α	6978±867	4180±807	6719±1019	**−1.7***	1.0
Glycogen synthase kinase 3β	GSK3β	3550±676	2973±519	4810±966	−1.2	1.4
Protein kinase A	PKA	1376±371	1660±455	2006±872	1.2	1.5
Protein kinase C α	PKCα	2822±1181	7042±4553	4751±1080	**2.5**	**1.7**
Protein kinase C β1	PKCβ	28938±12121	43626±16242	38377±4418	**1.5**	1.3
Protein kinase C delta	PKCδ	1525±603	4386±1871	3218±758	**2.9**	2.1
Protein kinase C epsilon	PKCε	28275±13868	51317±29905	36297±10378	**1.8**	1.3
Protein kinase C gamma	PKCγ	37306±13465	57421±24405	47484±5701	**1.5**	1.3
Protein kinase C lambda	PKCλ	3946±3156	5207±1882	4335±587	1.3	1.1
Protein kinase C μ(115)	PKCμ	5126±1459	5508±1078	3171±411	1.1	**−1.6**
Protein kinase C μ(120)	PKCμ	4183±2409	2810±196	1618±231	**−1.5**	**−2.6**
Protein kinase C zeta	PKCζ	11412±5098	30312±15647	19014±3254	**2.7**	**1.7**
Cyclin-dependent kinase 5	CDK5	35499±11191	25648±3658	33115±1563	−1.4	−1.1
MAP kinase kinase 4	MEK4	30551±11537	44062±15046	45028±6881	1.4	**1.5**
Hematopoietic progenitor kinase 1	HPK1	7228±2260	7068±1135	5508±1428	1.0	−1.3
MAP kinase kinase 7	MEK7	1805±276	1449±212	2005±175	−1.2	1.1
Stress-activated protein kinase (38)	JNK3 38	10088±1151	8338±663	9120±984	−1.2	−1.1
Stress-activated protein kinase (45)	JNK3 46	1948±204	2777±270	1772±248	1.4	−1.1
MAP kinase kinase 6	MEK6	3987±1256	5894±1835	5494±829	**1.5**	1.4
p38 Hog MAP kinase	p38	9583±2036	10547±1897	11617±1433	1.1	1.2

Data were from Western blotting of tissue from 11 mice in the METH/METH, nine in SAL/METH, and nine in SAL/SAL groups. In each of the treatment group, tissue was pooled from two or four mice. Three pools of samples were examined for each the METH/METH, SAL/METH, and SAL/SAL groups.
[a]Measured by the area under intensity profile curve. Units are intensity × mm. Data are means±SEM.
[b]Erk2 was measured using two different antibodies.
Bold indicate fold changes greater than 1.5.
*$p<0.05$, Dunnet's *post hoc* test, two tailed. Tissue was collected 4 h after the last METH or saline injection.

injection of METH. In contrast, no significant changes in phosphorylated MEK1 were observed 20 h after a single injection of METH.

DISCUSSION

METH abuse is associated with a high rate of aggressive behaviors, affective instability, and poor impulse control (Carey and Mandel, 1968; Ellinwood, 1971; Hawks *et al*, 1969; Szuster, 1990). These observations suggest the possibility that these patients might suffer from dysfunctions within subcortical striato-prefrontal networks that subsume human emotional, cognitive, and social behaviors (Strakowski *et al*, 2005). In the present study, we found significantly increased aggressiveness in mice chronically treated with METH, observations that were not dependent on gross activation of locomotor activity. Antibody micro-

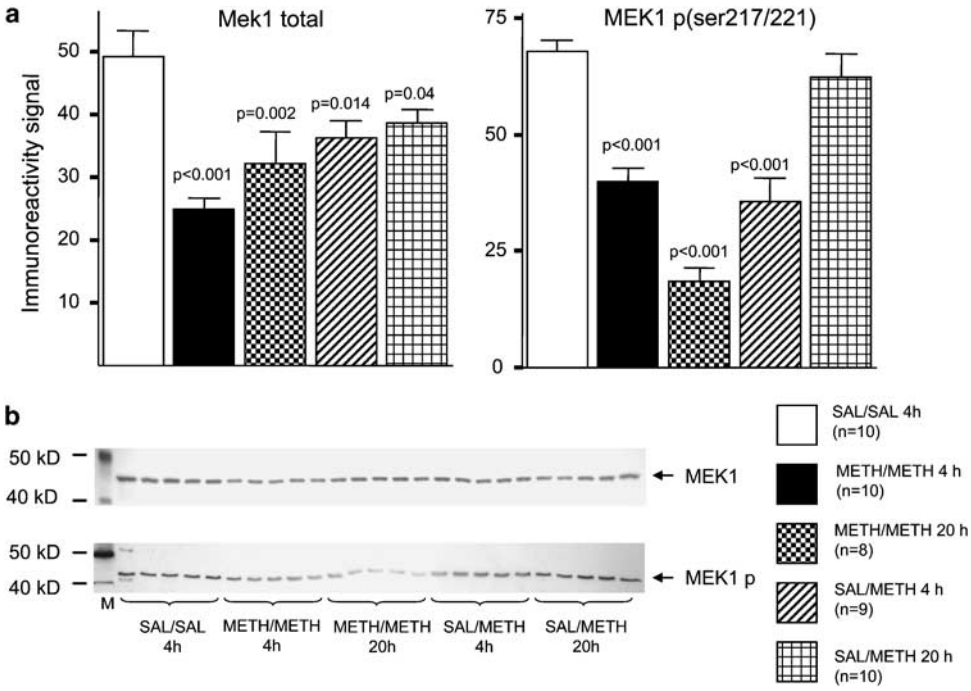

Figure 3 Western blotting analysis of total and phosphorylated forms of MEK1 in the striatum from mice 4 or 20 h after injection. (a) Levels of immunoreactivity. ANOVA revealed significant differences between the treatment groups both for total MEK1 ($F = 7.832$, $df = 4, 42$, $p < 0.001$) and for phosphorylated MEK1 ($F = 26.497$, $df = 4, 42$, $p < 0.001$). *P*-values above the individual bars indicate significant difference from SAL/SAL revealed in the *post hoc* test. (b) Representative Western blots.

array analysis revealed decreases in the levels of Erk2 and 14-3-3e in the striata of the mice chronically treated with METH. As protein kinase Erk2 is thought to be the principal component of the classical MAP kinase pathway and because 14-3-3e is an inhibitor and substrate of PKC (Jones *et al*, 1995), the decreases in these two proteins suggest that repeated METH injections might perturb MAP kinase-related pathways in the striato-prefrontal circuitries. This idea is supported by Western blotting analyses that also revealed changes in multiple components of MAP kinase-related pathways in the striatum and frontal cortex of aggressive mice chronically treated with METH. Thus, MAP kinase-related pathways in the prefronto-striatal circuits might be involved in the manifestations of aggressive behaviors induced by repeated injection of METH.

The use of animal models to get insights into human violence and aggressive behaviors is a challenging task because of the complexity and multidimensional nature of these behaviors (Lederhendler, 2003; Miczek *et al*, 2001). The fact that increased aggressiveness in mice was observed only after long-term treatment with METH suggests that METH-induced aggressiveness in mice might be a valuable model to study aggressiveness associated with METH abuse in humans. This observation is supported by the fact that increased aggressiveness in mice was not dependent on METH-induced hyperlocomotion. These observations suggest that these behavioral changes reflect neuroadaptations in mechanisms involved in the regulation of aggressive behaviors in rodents. Thus, it is not far-fetched to suggest that METH-induced aggressive behaviors, which developed over time, might be secondary to drug-mediated disruptions of inhibitory circuits that might be involved in suppressing aggressive behaviors. One obvious limitation of the current study is that it does not answer fully the question related to how much of the effects seen in the METH/METH group are residual or due to molecular responses to the last METH injection. Future studies using various time intervals after the last injection are needed to clarify this question.

MAP kinases are abundant within neuronal cell bodies and dendrites, where they regulate a variety of functions including neurotransmission and ion channel activities at synapses (Chen *et al*, 2001; Davis *et al*, 2000; Sharma *et al*, 2002). MAP kinase-related pathways, including the classical MEK1,2/Erk1,2 pathway as well as PKC- and PKA-dependent cascades, have been implicated in behavioral abnormalities observed in substance abuse and in affective disorders (Chen *et al*, 1999; Dwivedi *et al*, 2001; Einat *et al*, 2003a, b; Freeman *et al*, 2001a, b; Manji and Chen, 2002; Mizoguchi *et al*, 2004; Valjent *et al*, 2000). These ideas are consistent with our observations that changes in MAP kinase-related proteins in the striatum were more abundant after being repeated than after a single injection of METH. For example, statistically significant decreases in Gsk3α, MEK7, and pErk2 were observed only after repeated injections of METH. In addition, MEK1 was dramatically reduced after chronic METH treatment, but only modestly reduced after a single injection of METH, while phosphorylated MEK1 at 20-h time point was significantly reduced only after repeated METH injections. These changes in phosphorylated MEK1 might be secondary to decreased abundance of the upstream protein kinase Raf1. When taken together, these observations are consistent with the idea that alterations observed after chronic METH treat-

ment are consequent to neuroadaptive mechanisms induced by chronic METH treatment, but not by acute direct affects of METH on neuronal systems.

Our findings that mice that display short attack latency against an intruder after chronic treatment with METH also experience significant changes in MAP kinase-related pathways are consistent with the results of a recent report in which the technique of serial analysis of gene expression (SAGE) was used to measure gene expression in two mice lines genetically selected for short and long attack latency (Feldker *et al*, 2003). This SAGE analysis revealed that mice with short attack latency showed lower expression of genes encoding components of MAP kinase pathways (Feldker *et al*, 2003). The concordance of the results obtained using two different approaches, that is, pharmacological (here) and genetic (Feldker *et al*, 2003), and different techniques (transcripts *vs* protein abundance) strongly supports the idea that MAP kinase-related pathways may be involved in aggressive behaviors. These suggestions are also consistent with the reported involvement of MAP kinase pathways in the regulation of several neurotransmitter systems (Della Rocca *et al*, 1999; Greengard, 2001) that have been implicated in the regulation of aggressivity. These include serotonergic (Birger *et al*, 2003; Davidge *et al*, 2004; Edwards and Kravitz, 1997; Ferris *et al*, 1999; Huber *et al*, 1997; Korte *et al*, 1996; Manuck *et al*, 2002; Miczek *et al*, 2001), dopamine, norepinephrine, GABA, glutamate, acetylcholine, cholecystokinin, substance P, and opioid neurotransmitter systems (Brodkin *et al*, 2002; Chiavegatto *et al*, 2001; Olivier *et al*, 1995; Siegel *et al*, 1999).

Another line of evidence to support the idea that MAP kinase-related pathways may be involved in aggressive behaviors comes from studies of the mood stabilizers, lithium and valporate. In addition to their use to treat affective disorders, lithium and valporate have been used for the management of aggressive behaviors in various pathological conditions, including mental retardation, brain injury, autism, schizophrenia, attention-deficit/hyperactivity disorder, conduct disorder, and pervasive developmental disorder (Bellus *et al*, 1996; Glenn *et al*, 1989; Goetzl *et al*, 1977; Malone *et al*, 1994; McDougle *et al*, 2003; Platt *et al*, 1981; Schiff *et al*, 1982; Shader *et al*, 1974; Sheard, 1975, 1984; Silva *et al*, 1993; Swann, 2003; Weller *et al*, 1999; Wroblewski *et al*, 1997). Lithium has also been reported to block aggressiveness in laboratory animals (Sheard, 1975). The therapeutic effects of lithium and valporate are thought to be related to their regulatory actions on several members of MAP kinase-related pathways (Bhat *et al*, 2004; Chen *et al*, 1999; Hao *et al*, 2004; Manji and Chen, 2002; Manji and Lenox, 1999), similar to those that are affected in mice chronically treated with METH.

In conclusion, the present study identified complex changes in MAP kinase-related pathways in the striatum and frontal cortex of mice that display increased aggressiveness after receiving repeated METH injections. Given the role of these kinase cascades in the regulation of various cortical and striatal neurotransmitter systems that are thought to be involved in the modulation of aggressive behaviors in various animal models, our observations are consistent with the view that these pathways might be involved in the regulation of aggressive behaviors in mice. Further studies are needed to examine how stable over time are behavioral and molecular changes induced by long-term treatment with METH, and to delineate the role of specific protein kinases in specific METH-induced behaviors.

ACKNOWLEDGEMENTS

This research was supported by the Intramural Research Program of the NIDA, NIH, DHHS. We thank Dr CW Schindler for providing activity monitor and helpful suggestions. We also thank B Ladenheim for his expert help in brain dissections.

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