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METHAMPHETAMINE PRECONDITIONING CAUSES DIFFERENTIAL CHANGES IN STRIATAL TRANSCRIPTIONAL RESPONSES TO LARGE DOSES OF THE DRUG

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□ Methamphetamine (METH) is a toxic drug of abuse, which can cause significant decreases in the levels of monoamines in various brain regions. However, animals treated with progressively increasing doses of METH over several weeks are protected against the toxic effects of the drug. In the present study, we tested the possibility that this pattern of METH injections might be associated with transcriptional changes in the rat striatum, an area of the brain which is known to be very sensitive to METH toxicity and which is protected by METH preconditioning. We found that the presence and absence of preconditioning followed by injection of large doses of METH caused differential expression in different sets of striatal genes. Quantitative PCR confirmed METH-induced changes in some genes of interest. These include small heat shock 27 kD proteins 1 and 2 (HspB1 and HspB2), brain derived neurotrophic factor (BDNF), and heme oxygenase-1 (Hmox-1). Our observations are consistent with previous studies which have reported that ischemic or pharmacological preconditioning can cause reprogramming of gene expression after lethal ischemic insults. These studies add to the growing literature on the effects of preconditioning on the brain transcriptome.

Keywords: methamphetamine, preconditioning, striatum, BDNF, heat shock proteins

INTRODUCTION

Methamphetamine (METH) is an illicit drug which has become an international public health problem. Specifically, METH abuse is associated with many negative consequences which include altered behavioral and cognitive functions (Murray 1998; Scott *et al.* 2007; Darke *et al.* 2008).

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Withdrawal from METH causes anhedonia and intense craving for the drug (Zweben *et al.* 2004; Sekine *et al.* 2006; Darke *et al.* 2008). The negative neuropsychiatric consequences of METH abuse are thought to be due to drug-induced neurodegenerative effects in METH addicts (Scott *et al.* 2007). Patterns of METH abuse are multiple but usually involve the intake of small doses of the drug followed by gradual increases to larger doses of the psychostimulant (Kramer *et al.* 1967). Neuropsychological tests have revealed that METH addicts who abuse these large doses suffer from cognitive deficits (Simon *et al.* 2002; Sekine *et al.* 2003) and structural abnormalities in their brains (Chang *et al.* 2007; Sekine *et al.* 2008). METH-dependent patients indeed suffer from decreases in dopamine (DA) (Volkow *et al.* 2001) and of serotonin (5-HT) transporters (Sekine *et al.* 2006).

Many of these neuropathological changes have been replicated in animal models (Krasnova and Cadet 2009). Specifically, METH can cause decreases in DA, 5-HT, and DA transporters (DAT) in various brain regions (Cadet *et al.* 1994; Deng *et al.* 1999; Ladenheim *et al.* 2000; Thomas and Kuhn 2005; Cadet *et al.* 2007). These experiments focused on the use of moderate to large doses of METH injected during single-day binges (Cadet *et al.* 2003). However, several groups have now experimented with administration of increasing METH doses over several days prior to challenging the animals with toxic doses of the drug and have found that these patterns of drug administration can provide protection against METH toxicity (Johnson-Davis *et al.* 2003; Danaceau *et al.* 2007; Graham *et al.* 2008; Cadet *et al.* 2009a). Cadet and colleagues (2009a) have recently suggested that this pattern of drug administration is comparable with other models of brain preconditioning (Calabrese 2008; Obrenovitch 2008) and might involve similar molecular mechanisms of protection (Cadet and Krasnova 2009). For example, it has been reported that brain preconditioning by various manipulations is associated with differential gene expression in the presence of ischemic injuries (Dirnagl *et al.* 2003; Stenzel-Poore *et al.* 2003; Dhodda *et al.* 2004; Koerner *et al.* 2007; Stenzel-Poore *et al.* 2007). We thus conducted the present study to test if the absence and presence of METH preconditioning might be also associated with METH-induced differential gene expression in the striatum, a brain region which is known to be affected by METH (Krasnova and Cadet 2009).

MATERIALS AND METHODS

Animals.

Male Sprague-Dawley rats (Charles Rivers Laboratories, Raleigh, NC), weighing 330-370 g in the beginning of the experiment were used in the present study. Animals were housed in a humidity- and temperature-con-

trolled room and were given free access to food and water. All animal procedures were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Care Committee.

Drug Treatment and Tissue Collection.

Following habituation, rats were injected intraperitoneally with either (±)-METH-hydrochloride (NIDA, Baltimore, MD) or an equivalent volume of 0.9% saline for a period of three weeks as described elsewhere (Graham *et al.* 2008; Cadet *et al.* 2009a). The saline- or METH-pretreated animals received either saline or METH (5 mg/kg x 8 at 1 h intervals) challenges 72 hours after the preconditioning period. This dose of METH is known to cause significant decreases in the levels of monoamines in the rat striatum (Krasnova and Cadet 2009). The four groups of animals were: saline/saline (SS), saline/METH (SM), METH preconditioning/saline (MS), and METH preconditioning/METH (MM). The animals were euthanized 24 h after the injection of the last dose of METH. Their brains were quickly removed, brain regions were dissected on ice, snap frozen on dry ice, and stored at -80°C until used in microarray analyses or quantitative PCR experiments as described below.

RNA Extraction and Microarray Hybridization.

Total RNA was isolated using Qiagen RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and showed no degradation. Microarray hybridization was carried out using Illumina's RatRef-12 Expression BeadChips arrays (22, 227 probes) (Illumina Inc., San Diego, CA). In brief, a 600 ng aliquot of total RNA from each striatal sample was amplified using Ambion's Illumina RNA Amplification kit (Ambion, Austin, TX). Single-stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP (Roche Diagnostics Corporation, Indianapolis, IN). 750 ng of each cRNA sample were hybridized to Illumina arrays at 55 ºC overnight according to the Illumina Whole-Genome Gene Expression Protocol for BeadStation (Illumina Inc.). Hybridized biotinylated cRNA was detected with cyanine3-streptavidine (GE Healthcare, Piscataway, NJ) and quantified using Illumina's BeadStation 500GX Genetic Analysis Systems scanner.

Microarray Data Analysis.

The raw data for the analyses of the four groups of animals are available upon request. The Illumina BeadStudio software was used to measure fluorescent hybridization signals. Data were extracted by BeadStudio (Illumina Inc.) and then analyzed using GeneSpring software v. 7.3.1

(Silicon Genetics, Redwood City, CA). Raw data were imported into GeneSpring and normalized using global normalization. The normalized data were used to identify changes in gene expression in four group comparisons: MS vs SS, SM vs SS, MM vs SS, and MM vs MS. A gene was identified as significantly changed if it showed increased or decreased expression according to an arbitrary cut-off of 1.7-fold changes at $p < 0.025$.

Real-time PCR.

Total RNA extracted from the rat striatum was also used to confirm the expression of genes of interest by real-time RT-PCR as previously described (Krasnova *et al.* 2007; Krasnova *et al.* 2008). In brief, unpooled total RNA obtained from 5-7 rats per group was reverse-transcribed with oligo dT primers and Advantage RT for PCR kit (Clontech, Mountain View, CA). PCR experiments were performed using light cycler technology and LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to manufacturer's protocol. Sequences for gene-specific primers corresponding to PCR targets were obtained using LightCycler Probe Design software (Roche). The primers were synthesized and HPLC-purified at the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD). Quantitative PCR values were normalized using 18S rRNA and quantified. The results are reported as relative changes which were calculated as the ratios of normalized gene expression data of each group compared to the SS group.

Statistical Analysis.

Statistical analysis was performed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference post-hoc comparison (StatView 4.02, SAS Institute, Cary, NC). Values are shown as means ± SEM. The null hypothesis was rejected at $p < 0.05$.

RESULTS

Identification of genes regulated by METH preconditioning and by METH challenges in the rat striatum.

As reported elsewhere, METH preconditioning caused protection against METH-induced depletion in striatal DA and 5-HT levels (Cadet *et al.* 2009a). In order to assess transcriptional effects of toxic doses of METH in the rat striatum, we used Illumina RatRef-12 Expression BeadChips arrays that contain 22, 523 probes. The results of 3 comparisons between the four groups of rats: MS vs SS, SM vs SS, and MM vs MS are presented in the Venn diagram (Fig. 1). To be identified as changed, the genes had to show 1.7-fold difference with control expression at $p < 0.025$. A total of 230 genes were differentially impacted in the three

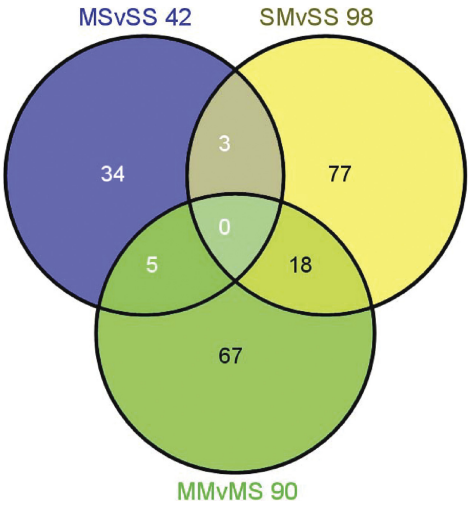

FIGURE 1. METH preconditioning induces differential striatal transcriptional responses to large doses METH. The Venn diagram shows the overlap of genes identified by the three sets of comparisons. The animals were injected and euthanized as described in the text. RNA was extracted from rat striatal tissues. The microarray experiments were performed as described in the method section. Genes were identified as significantly changed if they show greater than ±1.7-fold changes at $p < 0.025$.

comparisons, the distribution and overlap of these genes are shown in Fig. 1. Partial lists of these genes are given in tables 1-4.

Effects of METH preconditioning on striatal gene expression.

Table 1 shows that chronic administration of low non-toxic doses of METH caused significant changes in the expression of 42 genes, with 30 being up-regulated and 12 down-regulated (MS vs SS). Of these genes, there were 34 that were found only in the MS vs SS comparison while 3 were found co-localized within the SM vs SS and 5 genes within the MM vs MS comparison. The most up-regulated gene was the predicted gene, Cd97, which showed 27-fold increases. (We are using only abbreviations in the text since the full name of the genes can be found in the tables that provide the list of METH-regulated genes). METH preconditioning caused 18-fold increases in the expression of Gabrr2 which is a receptor in GABA-mediated inhibitory synapses in the brain (Schmidt 2008). Another gene of interest is Fgf3, a member of the Fgf family of trophic factors (Itoh and Ornitz, 2008), which shows about 12-fold increases after repeated injections of non-toxic doses of METH.

Effects of METH challenges on striatal gene expression in the absence of METH preconditioning.

Table 2 shows partial lists of genes affected by binge METH challenge in the striatum in the absence of METH preconditioning (SM vs SS).

TABLE 1. Effects of METH preconditioning alone on striatal gene expression.

Fold Changes MS/SS	Gene Symbol	Common	Description
18.32	Adam18	tMDCIII	a disintegrin and metallopeptidase domain 18
18.18	Gabrr2	Gabrr2	gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2
13.91	Sftpb	Sp-b	surfactant associated protein B
11.89	Fgf3	Int2	fibroblast growth factor 3
8.73	Grpca	Grpca	glutamine/glutamic acid-rich protein A
1.89	Fmo2	Fmo2	flavin containing monooxygenase 2
1.75	E2f1	E2f1	E2F transcription factor 1
–1.71	Lox		Rattus norvegicus lysyl oxidase (Lox), mRNA.
–2.92	Foxi2	Fkhl5; Foxf1	forkhead box I2
–16.23	Pcdhac1	Pcdhac1; rCNRvc1	protocadherin alpha subfamily C, 1

Data were obtained from the MS vs SS comparison. Predicted genes are not listed. The genes are listed in descending order according to METH-induced fold changes in gene expression.

TABLE 2. METH-induced increases in striatal gene expression in the absence of METH preconditioning.

Fold Changes SM/SS	Gene Symbol	Common	Description
44.60	Hcst	Hcst	hematopoietic cell signal transducer
21.13	Il1a	IL-1 alpha	interleukin 1 alpha
15.80	Lfng	Lfng	lunatic fringe gene homolog (Drosophila)
14.83	H19	H19	Rattus norvegicus H19 fetal liver mRNA (H19), misc RNA.
12.53	Bcl2l10	Bcl2l10	Bcl2-like 10
11.22	Mb	Mb	myoglobin
10.88	Timp1	Timp; TIMP-1	tissue inhibitor of metallopeptidase 1
8.19	Hspb1	Hsp25; Hsp27	heat shock 27kDa protein 1
4.83	Cd44	CD44A; METAA; RHAMM	CD44 antigen
4.45	Lcn2	Lcn2	lipocalin 2
4.41	S100a3	S100a3	S100 calcium binding protein A3
3.05	Fmo2	Fmo2	flavin containing monooxygenase 2
2.82	Gfap	Gfap	Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA.
2.74	Pdpn	E11; Gp38; OTS-8; RTI40; T1-alpha	podoplanin
2.67	Emp3		epithelial membrane protein 3
2.64	Serping1		serine (or cysteine) peptidase inhibitor, clade G, member 1
2.53	Parp3	Adprtl3	poly (ADP-ribose) polymerase family, member 3
2.50	Cd14	Cd14	CD14 antigen
2.34	Chi3l1	Chi3l1	chitinase 3-like 1
2.32	Tyrobp	Karap	Tyro protein tyrosine kinase binding protein

Continued

TABLE 2. *Continued.*

Fold Changes SM/SS	Gene Symbol	Common	Description
2.28	Gpd1	GPDH; Gpd3	glycerol-3-phosphate dehydrogenase 1 (soluble)
2.25	Tmbim1		transmembrane BAX inhibitor motif containing 1
2.20	Nes	Nes	nestin
2.17	Cox6a2	COX6B; COX6AH	cytochrome c oxidase, subunit VIa, polypeptide 2
2.16	Prelp	Prelp	proline arginine-rich end leucine-rich repeat protein
2.13	Plp2	A4-LSB	proteolipid protein 2
2.12	C1qb	C1qb	complement component 1, q subcomponent, beta polypeptide
2.02	Ptpn6	Ptph6; Shp-1	protein tyrosine phosphatase, non-receptor type 6
1.96	Sv2c	Sv2c	synaptic vesicle glycoprotein 2c
1.95	Prkcdbp	Srbc; DIG-2	protein kinase C, delta binding protein
1.95	Vamp5	Vamp5	vesicle-associated membrane protein 5
1.95	S100a4	CAPL; MTS1	S100 calcium-binding protein A4
1.93	Fxyd5	RIC	FXYD domain-containing ion transport regulator 5
1.92	Ddit4l	Ddit4l	DNA-damage-inducible transcript 4-like (Ddit4l), mRNA.
1.92	Col5a1	Col5a1	procollagen, type V, alpha 1
1.90	Fgfrl1	Fgfr5	fibroblast growth factor receptor-like 1
1.90	Arf6	Arf6	ADP-ribosylation factor 6
1.89	Hla-dma	RT1-DMa; RT1.DMa	major histocompatibility complex, class II, DM alpha
1.89	Pycard	Asc	PYD and CARD domain containing
1.85	Cdo1	Cdo1	cysteine dioxygenase 1, cytosolic
1.84	Clic1	Clic1	chloride intracellular channel 1
1.82	Ccl21b		chemokine (C-C motif) ligand 21b (serine)
1.81	Stat3		signal transducer and activator of transcription 3
1.80	Hmox1	Ho1; Heox; Hmox; Ho-1; HEOXG; hsp32	heme oxygenase (decycling) 1
1.79	Chek2	Chk2; Rad53	CHK2 checkpoint homolog (S. pombe)
1.79	Casp4	Casp11	caspase 4, apoptosis-related cysteine peptidase
1.76	Eif4ebp1	PHAS-I	eukaryotic translation initiation factor 4E binding protein 1
1.74	Tgfb1	Tgfb1	transforming growth factor, beta 1
1.73	Lgals3	gal-3	lectin, galactose binding, soluble 3
1.71	ZnT3	Slc30a3	solute carrier family 30 (zinc transporter), member 3
1.70	Emp1	TMP; CL-20; EMP-1; ENP1MR	epithelial membrane protein 1

The data were generated from the SM vs SS comparison. Predicted genes are not included. The genes are listed in descending order according to fold changes in gene expression.

METH challenge caused significant changes in a total of 98 genes. Of these, 79 were up-regulated (Table 2) and 19 were down-regulated (not shown). The most significantly changed gene was Hcst which showed about a 45-fold increase. Other up-regulated genes of interest include Bcl2-like 10, Hsp27/HspB1, GFAP, Hmox-1, and caspase 4. GFAP expres-

sion has been shown to be induced by toxic doses of METH (Deng *et al.* 1999; Krasnova *et al.* 2010). The members of the Bcl2 family of mitochondrial proteins are also influenced by toxic doses of the drug (Jayanthi *et al.* 2001) and are involved in METH-induced neuronal apoptosis (Cadet *et al.* 1997). The expression of Hsp27/HspB1 (Jayanthi *et al.* 2009) and of Hmox-1 (Cadet *et al.* 2009b; Jayanthi *et al.* 2009) is also changed by toxic doses of METH. Some of these genes are similar to those reported by another group (Thomas et al., 2004).

Effects of METH challenges on striatal gene expression in the presence of METH preconditioning.

Table 3 shows a partial list of genes whose expression was affected by the injections of large doses of METH in the presence of METH preconditioning (MM vs MS). Ninety genes were affected in that comparison. Of these, 32 were up-regulated and 58 were down-regulated by the large dose METH challenge. Sixty seven of these genes were changed only after METH challenge in the striata of rats preconditioned with METH while 18 genes were also contained in the MM vs MS comparison (Fig. 1). In addition, 5 genes showed changes in expression in the MS vs SS comparison. The most up-regulated gene was H19. Other up-regulated genes of interest include Timp1, Nes, GFAP, and Vgf. HspB1 which was up-regulated in the absence of METH preconditioning is also up-regulated in the MM vs MS comparison, but to a lesser extent. In contrast, S100a3 and GFAP were up-regulated to similar extent in the absence or presence of METH preconditioning.

Differential METH-induced striatal gene expression in the absence and presence of METH preconditioning.

In order to dissect the effects of METH preconditioning further, we compared the levels of gene expression between the MM and the SM groups (MM vs SM). We found that 77 genes were affected, with 36 being up-regulated and 41 down-regulated. Table 4 shows a partial list of these genes. The most up-regulated gene was Igsf7. Other up-regulated genes of interest include Lif and Egfl6. Down-regulated genes of interest include BDNF and Nurr1.

Quantitative PCR for genes of interest

We examined METH-induced changes in the expression of Hsp27/HspB1 which was up-regulated to differential degrees in both the SM and MM groups in the microarray experiments. METH injections caused about 22- and 7-fold increases in the levels of Hsp27/HspB1 mRNA in the SM and MM groups, respectively (Fig. 2A). The changes observed in the MM were significantly less pronounced than those observed in the SM group. We also measured the expression of HspB2,

TABLE 3. METH-induced changes in striatal gene expression in the presence of METH preconditioning.

Fold Changes (MM/MS)	Gene Symbol	Common	Description
13.43	H19		Rattus norvegicus H19 fetal liver mRNA (H19), misc RNA.
11.12	Gys2	GLYSN	glycogen synthase 2
5.67	Timp1	Timp; TIMP-1	tissue inhibitor of metallopeptidase 1
4.47	Hspb1	Hsp25; Hsp27	heat shock 27kDa protein 1
3.50	Cd44	CD44A; METAA	CD44 antigen
3.30	S100a3	S100a3	S100 calcium binding protein A3
2.95	Cxcl10	IP-10; Scyb10	chemokine (C-X-C motif) ligand 10
2.20	Nes	Nes	nestin
2.19	Gfap	Gfap	Rattus norvegicus glial fibrillary acidic protein (Gfap), mRNA.
2.18	Pdpn	E11; Gp38; OTS-8	podoplanin
1.94	Chi3l1	Chi3l1	chitinase 3-like 1
1.83	Tmbim1		transmembrane BAX inhibitor motif containing 1
1.82	Tyrobp	Karap	Tyro protein tyrosine kinase binding protein
1.81	Vgf	Vgf	VGF nerve growth factor inducible
1.81	Serping1		serine (or cysteine) peptidase inhibitor, clade G, member 1
1.76	Prelp	Prelp	proline arginine-rich end leucine-rich repeat protein
-1.72	Slc4a1		solute carrier family 4, member 1
-1.75	Gucy1b2	SGC; Gucy1b2a; Gucy1b2b	guanylate cyclase 1, soluble, beta 2
-1.75	Grem1	drm; Cktsf1b1	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)
-1.79	Ces2	rCES2; CES RL4	carboxylesterase 2 (intestine, liver)
-1.81	Hcn1	Hcn1	hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
-1.86	Sstr1		Rattus norvegicus somatostatin receptor 1 (Sstr1), mRNA.
-1.87	Ahr		Rattus norvegicus aryl hydrocarbon receptor (Ahr), mRNA.
-1.94	Rnasel	Rnasel	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
-1.95	Pnck	Camk1b	pregnancy upregulated non-ubiquitously expressed CaM kinase
-2.13	Slit1		Rattus norvegicus slit homolog 1 (Drosophila) (Slit1), mRNA.
-3.37	Rtn4r		Rattus norvegicus reticulon 4 receptor (Rtn4r), mRNA.
-4.18	St8sia6	Siat8f	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6
-14.21	Arl10	Arl10	ADP-ribosylation factor-like 10
-15.94	Nfatc2ip		nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein
-20.45	Dnase1l3		deoxyribonuclease I-like 3
-27.30	Cldn3	Cldn3	claudin 3
-30.18	Ntn2l	Ntn3	netrin 2-like (chicken)
-42.53	Pla2g1b	Pla2g1b	phospholipase A2, group IB
-44.28	Col23a1	Col23a1	procollagen, type XXIII, alpha 1

The data were generated from the MM vs MS comparison. Predicted genes are not included. The genes are listed in descending order according to fold changes in gene expression.

TABLE 4. Differential METH-induced striatal gene expression in the absence and presence of METH preconditioning.

Fold Changes (MM/SM)	Gene Symbol	Common	Description
23.64	Igsf7	Igsf7; Mair-II	immunoglobulin superfamily, member 7
15.17	Lif	Lif	leukemia inhibitory factor
10.55	Nanog	Nanog	Nanog homeobox
7.40	Fut11		fucosyltransferase 11
7.38	Kcnq2	Kcnq2	potassium voltage-gated channel, subfamily Q, member 2
1.88	Chrna7	BTX	cholinergic receptor, nicotinic, alpha polypeptide 7
1.74	Egfl6	Egfl6	EGF-like-domain, multiple 6
-1.72	Lxn		latexin
-1.75	Bdnf		brain derived neurotrophic factor
-1.76	Hgfac	Hgfac	hepatocyte growth factor activator
-1.79	Abcb1b	Mdr1; Pgy1; Abcb1	ATP-binding cassette, sub-family B (MDR/TAP), member 1B
-1.86	Hcn1	Hcn1	hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
-2.02	Nr4a2	Nurr1	nuclear receptor subfamily 4, group A, member 2
-2.07	Hs3st2	Hs3st2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
-2.61	Klk8	bsp1	kallikrein 8 (neuropsin/ovasin)
-2.65	Nov	Nov	nephroblastoma overexpressed gene
-2.77	Cnga1	HCN; Cncg	cyclic nucleotide gated channel alpha 1
-11.10	Npm2	Npm2	nucleophosmin/nucleoplasmin 2
-20.75	Neurod1	Neurod1	neurogenic differentiation 1

Data were obtained from the MM vs SM comparison. Predicted genes are not listed. The genes are listed in descending order according to the METH-induced fold changes.

another member of the HspB family of small heat shock proteins (sHSPs) (Hu *et al.* 2008) which has been shown to exert protection against ischemia-induced damage (Morrison *et al.* 2004). The METH injections caused significant increases in HspB2 mRNA levels in the absence but not in the presence of METH preconditioning (Fig. 2B).

The PCR experiments also confirmed that the expression of Hmox-1 was up-regulated in the SM but not in the MM group (Fig. 3A). In addition, we also measured the expression of Hmox-2, another member of the Hmox family even though it was not identified as being regulated by METH in the microarray data. As shown in Fig. 3B, there were small decreases in the MM group which were significantly different from the SM group. Because Hmox-1 expression is regulated by NRF2 protein translocation from the cytosol to the nucleus (Surh *et al.* 2009), we tested the idea that multiple injections of METH might cause increases in Nrf2 mRNA. We found that the METH challenge did cause increases in Nrf2 expression in the absence (SM) but not in the presence of METH pre-

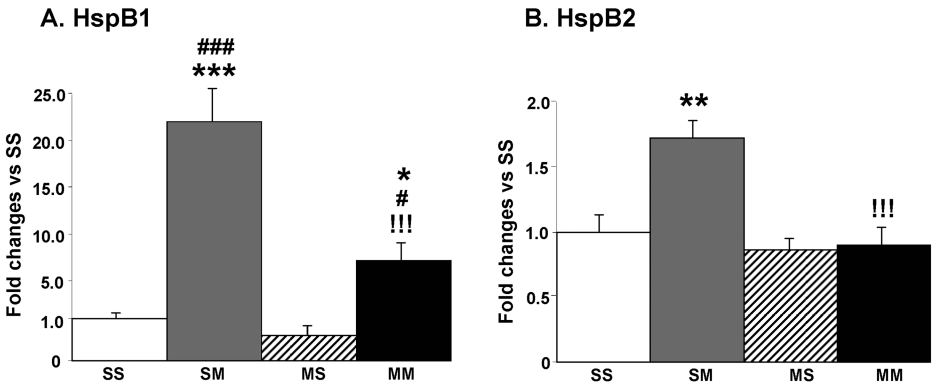

FIGURE 2. Quantitative PCR validates the effects of large doses of METH on striatal HspB1 and HspB2 mRNA levels. Data were obtained using RNA isolated from 5-6 animals per group and measured individually. The mRNA levels were normalized to 18S rRNA levels. The values are shown as means ± SEM in comparison to the SS group. METH caused substantial increases in (A) HspB1 in both the SM and MM groups and (B) HspB2 only in the SM group. Keys to statistics: *, **, *** $p <$ 0.05, 0.01, 0.001, respectively, in comparison to the SS group; #, ### $p < 0.05$, 0.001, respectively, in comparison to the MS group; !!!, $p < 0.001$, in comparison to the SM group.

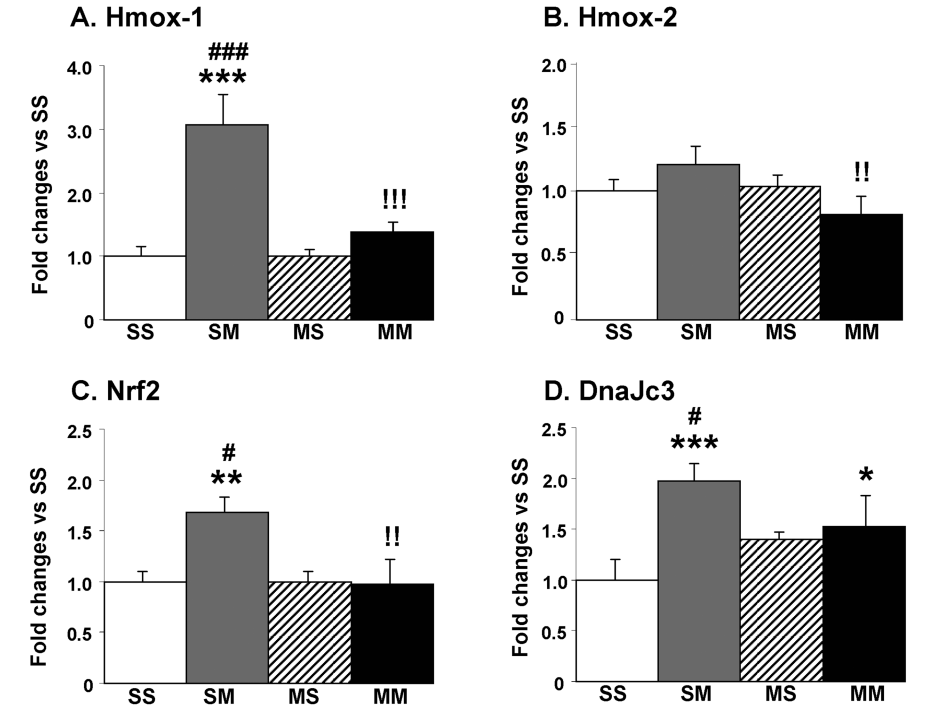

FIGURE 3. Effects of large doses of METH on striatal Hmox-1, Hmox-2, Nrf2, and DnaJc3 mRNA levels. Data were obtained using RNA isolated from 5-6 animals per group and measured individually. The mRNA levels were normalized to 18S rRNA levels. The values are means ± SEM in comparison to the SS group. METH caused substantial increases in (A) Hmox-1 in the SM group but not in (B) Hmox-2. There were also METH-induced increases in the expression of (C) Nrf2 in the SM group and of (D) DnaJc3 in both the SM and MM groups. Keys to statistics: *, **, *** $p < 0.05$, 0.01, 0.001, respectively, in comparison to the SS group; #, ### $p < 0.05$, 0.001, respectively, in comparison to the MS group; !!, !!!, $p < 0.001$, respectively, in comparison to the SM group.

conditioning (MM) (Fig. 3C). Because Hmox-1 is up-regulated by endoplasmic reticulum (ER) stress (Gozzelino *et al.* 2010), we also tested the possibility that DnaJc3 (p58IPK) which is involved in protecting cells against ER stress (Rutkowski *et al.* 2007) might be induced by METH. Indeed, the large METH doses caused significant increases in DnaJc3 expression in the absence but not in the presence of METH preconditioning (Fig. 3D).

Because the microarray experiments identified BDNF as being down-regulated in the MM in comparison to the SM group and because BDNF has been shown to provide protection against transneuronal degeneration of DA neurons (Canudas *et al.* 2005), we sought to confirm these data by quantitative PCR. Figure 4A shows that there were significant decreases in BDNF expression in the presence of METH preconditioning (MM group). We also measured the expression of GDNF that has been shown to protect against METH toxicity (Cass *et al.* 2006). There were some decreases in GDNF mRNA levels in MM group that did not reach statistical significance (Fig. 4B).

DISCUSSION

The main findings in these experiments are that acute injections of large doses of METH caused differential gene expression in the striata of rats depending on whether the animals have been pre-exposed repeatedly to smaller non-toxic doses of the drug or not. The present observations allowed for the creation of differentially expressed genes after exposure to large doses of METH. These results are important because there are very few reports on the effects of chronic METH treatment on large scale gene expression in the rat striatum. We hope that the results of our study

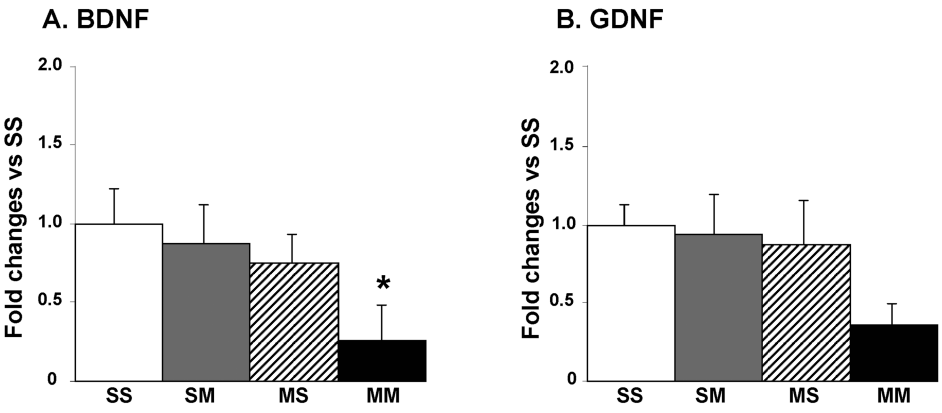

FIGURE 4. Effects of large doses of METH on striatal BDNF and GDNF mRNA levels. Tissues were processed and mRNA levels measured as described in the text. METH caused significant decreases in (A) BDNF in MM group. (B) GDNF expression was not significantly affected in any of the groups. Keys to statistics: * $p < 0.05$, in comparison to the SS group.

will provide a comprehensive database for future investigations of METH preconditioning, METH toxicity, and drug-induced neuroplastic changes in the rat striatum.

In this study, we found that the vast majority of genes regulated by the single-day binge METH injections are different from those regulated by acute METH injections (Cadet *et al.* 2001; Jayanthi *et al.* 2009). These differences might be due to the fact that data reported in the previous studies were obtained from animals euthanized within the first 4 hours after the METH injections (Cadet *et al.* 2001; Jayanthi *et al.* 2009). It is important to point out that the genes identified in the striatum are also different from the genes identified in the midbrain after METH preconditioning, indicating that the preconditioning process might be regionally specific in terms of METH-induced gene expression (Cadet *et al.* 2009b). These regional differences might be secondary to the fact that the data of the former study came from the rat midbrain which is the site of origin for dopaminergic neurons whereas the present data were obtained from intrinsic striatal non-dopaminergic neurons. Nevertheless, our data support the idea that METH preconditioning is associated with significant alterations of the striatal transcriptional responses to large doses of METH. In what follows, we discuss the role of some interesting genes whose METH-induced changes in expression were confirmed by quantitative PCR.

Mammalian HSPs, which include Hsp27/HspB1, are molecular chaperones that participate in the proper folding of proteins and help to maintain their native conformations during stressful events (Arya *et al.* 2007). Hsp27/HspB1 is a novel regulator of intracellular redox state (Arrigo 2007). HSPs also participate in the transfer of improperly folded proteins to the proteasome for degradation. HSPs are induced by heat shock, hypoxic and ischemic events, and oxidative stress (Arrigo 2007; Arya *et al.* 2007). Recent studies have documented a role for these proteins in neurodegenerative processes and have demonstrated that HSPs are important in cellular protection against aggregation-prone proteins and in animal models of neurodegeneration (Muchowski and Wacker 2005). Thus, our demonstration of METH-induced expression of the chaperones, Hsp27/HspB1 (Franklin *et al.* 2005) and HspB2 (Hu *et al.* 2008), suggests that striatal cells are able to mount adaptive defensive HSP-modulated networks against the toxic effects of the drug. Hsp27/HspB1 appears to exert its protective effects, in part, by inhibiting caspase-dependent apoptotic pathways (Garrido *et al.* 1999; Voss *et al.* 2007).

We also found that the METH challenge caused 3-fold increases in Hmox-1 expression in the absence of METH preconditioning and that these increases were attenuated in the striata of the METH preconditioned rats. Because Hmox-1 is an enzyme that can be induced by oxida-

tive stress (Calabrese *et al.* 2004; Li *et al.* 2007) and because METH toxicity is mediated, in part, via oxidative stress (Krasnova and Cadet 2009), the present observations suggest that the METH challenge might have caused more oxidative stress in the striata of animals pretreated with saline. However, since saline-pretreated animals do show METH toxicity, these METH-induced increases in Hmox-1 might not be sufficient to protect post-synaptic cells from drug-related damage. It is important to point that overexpression of Hmox-1 can protect against METH toxicity in vitro (Huang *et al.* 2009). Nevertheless, it appears that a substantial increase in the level of the enzyme might be necessary before protection against METH toxicity can be observed in vivo. This remains to be demonstrated.

The expression of BDNF which is involved in the regulation of cell survival (Canudas *et al.* 2005) and in synaptic plasticity (Kuipers and Bramham 2006), was significantly decreased by the METH challenge only in the presence of METH preconditioning. These results were unexpected since we had observed increases in BDNF expression in the midbrain where the DA neurons are located (Cadet *et al.* 2009b). The present observations in the rat striatum suggest that the increases in BDNF in midbrain dopaminergic neurons might cause increases in the release of BDNF in the striatum and compensatory down-regulation of its expression in intrinsic striatal cells via epigenetic changes in the regulation of BDNF through promoter methylation (Dennis and Levitt 2005). These epigenetic changes might also involve decreased recruitment of acetylated histones on BDNF promoters since histone deacetylase (HDAC) inhibitors have been reported to cause increases in BDNF transcription (Wu *et al.* 2008). In any case, these dichotomous results in the terminal regions and in the cell body area emphasize the need to determine regional effects of toxic agents on the brain.

This discussion also applies to the effects of METH on striatal Hspb2 and Hmox1 mRNA levels which showed increases in response to the challenge with high doses of METH in the absence but not in the presence of METH preconditioning. The situation was reversed in the midbrain of these animals because METH preconditioning enhanced the METH challenge-induced increases in Hspb2 and Hmox-1 mRNA levels in the rat midbrain (Cadet *et al.*, 2009b). These results support our thesis that the brain cannot be thought of as a homogeneous structure when assessing the molecular effects of preconditioning and/or toxic compounds.

In summary, we found that the challenge with large doses of METH is associated with differential transcriptional responses in the rat striatum in the absence and presence of preconditioning with repeated injections of small nontoxic doses of the drug. These results suggest that intrinsic striatal cells exposed to low METH doses develop a certain degree of tolerance to the effects of the drug on the expression of genes that are triggering the nefarious METH effects. Future studies are underway to test

the possibility that METH preconditioning can also protect against METH-induced cell death in the striatum.

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