Analysis of Ecstasy (MDMA)-induced transcriptional responses in the rat cortex

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ABSTRACT **3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a popular drug of abuse. MDMA is pharmacologically classified as an entactogen because of its affinities to classical hallucinogens and stimulants. Oral ingestion of a single dose of the drug is associated with euphoria, elevated self-confidence, and heightened sensory awareness in humans. Evidence for neurotoxicity in the human serotonin (5-HT) system has been provided. In rats, a single injection of MDMA induces hyperthermia and formation of reactive oxygen species. These effects may cause MDMA-associated, long-term 5-HT depletion, with the cortex being quite sensitive to the biochemical effects of MDMA. It has been suggested that these MDMA effects may be associated with molecular changes in this brain region. To test these ideas, we have made use of the cDNA array analysis, which can provide a more global view of the molecular changes secondary to MDMA injections. Our results show that the genes regulated by MDMA encode proteins that belong to signaling pathways, transcription regulators, or xenobiotic metabolism. Our observations indicate that cortical cells respond to the acute administration of MDMA by modulating transcription of several genes that might lead to long-term changes in the brain.—Thiriet, N., Ladenheim, B., McCoy, M. T., Cadet, J. L. Analysis of Ecstasy (MDMA) -induced transcriptional responses in the rat cortex. *FASEB J.* 16, 1887–1894 (2002)**

Key Words: MDMA · cortex · gene expression · cDNA arrays · SYBR green PCR

THE WIDELY USED recreational drug, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy), is a ring-substituted phenyl-isopropylamine that is related to both amphetamines and hallucinogens (1). In rats, MDMA induces hyperthermia and hyperkinesias (2) and increases locomotor activity (3). These acute effects of MDMA are consistent with massive serotonin (5-HT) release (4). Using in vivo microdialysis, a single injection of MDMA was shown to increase extracellular 5-HT concentration in the cortex and striatum via reversal of cellular transporters (5). 5-HT released in this fashion can then stimulate its various receptors, several of which (including the 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C} serotonergic receptors) have been shown to be involved in the effects of the drug (6). Stimulation of cyclic AMP and Ca^{2+} signaling cascades regulated by these receptors can then result in increased transcription of immediate early genes (IEGs) (7, 8). MDMA's administration has indeed been reported to regulate the transcription of *c-fos* and *egr-1* (NGFI-A) genes in several rat brain structures (9, 10). These genes are known to play key roles in the conversion of short-term neuronal stimulation into long-lasting changes in cell function (11, 12).

In animal models, administration of MDMA at doses (10–20 mg/kg) within the range used by humans (13) have been reported to produce damages to forebrain 5-HT-containing axons in rat (14). In the rat, acute 5-HT release is followed by a >80% reversible depletion of 5-HT in the cortex 3–6 h after MDMA administration (15, 16). Levels of 5-HT and its metabolite 5-HIAA are reported to be reduced 4 days after single MDMA injections (16). This depletion persists for at least 1 wk and is related to neurotoxic effects of the drug on 5-HT terminals (15). The activity of tryptophan hydroxylase (TPH), the rate-limiting enzyme for the biosynthesis of 5-HT, is also reduced very early after MDMA administration, an effect that lasts at least 2 wk (17). More recently, MDMA has also been shown to cause apoptotic cell death in two different studies using cell cultures (18, 19).

The mechanisms by which MDMA causes its deleterious effects have yet to be completely clarified. Nevertheless, they are thought to involve the formation of MDMA metabolic products such as N-methyl-α-methyldopamine (MeDA), orthoquinones, and quinonethioethers (20–22). MDMA is converted into MeDA by demethylenation via a reaction that is catalyzed by cytochrome P-450 isozyme (22). MeDA is unstable and is metabolized in the presence of NADPH into a quinone that forms an adduct with glutathione (22). These reactions involved superoxide and hydroxyl radicals (22, 23). The reactive oxygen species (ROS) are thought to be involved in MDMA-induced neurotoxicity (24–26). These ideas are supported by demonstrations that hydroxyl radical scavengers can attenuate MDMA-induced neurotoxicity in rats (27–29) and by reports that CuZn-superoxide dismutase (CuZn-SOD) transgenic mice are protected against MDMA-induced toxic effects (30, 31). The report that repeated injec-

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tions of MDMA can cause changes in the activities of CuZn-SOD, catalase, and glutathione peroxidase and can induce lipid peroxidation provides further support for ROS involvement in MDMA neurotoxicity (32). Taken together, the biochemical and neurotoxic effects of MDMA suggest that this drug might interact with neuronal systems in complex ways.

To investigate the varied molecular effects of MDMA in the cortex, a structure known to be particularly sensitive to MDMA-induced 5-HT depletion, we used the cDNA array technique to quantify transcript levels of many genes simultaneously (33). We reasoned that this approach might provide a more general portrait of the molecular occurrences stimulated by MDMA. Here we report that MDMA does indeed cause changes in transcripts of several genes that might account for the long-term effects of the drug.

MATERIALS AND METHODS

Animals and drug treatment

Sprague-Dawley rats (Charles River, Raleigh, NC), weighing 250–300 g, received a single injection of MDMA (20 mg/kg) or saline via intraperitoneal route. This dose was chosen because it has been used extensively in previous pharmacological and neurotoxicological studies (10, 34). All animal use procedures were according to the NIH guide for the Care and Use of Laboratory Animals and were approved by the local Care Committee. The rats were killed at various times (0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h, 1 day, 3 days, and 7 days) after drug treatment. After removal of the brain, the frontal cortex was isolated and processed for mRNA isolation.

Probing and hybridization of cDNA arrays

A complete list of the 1176 arrayed genes and their functional classifications is available at the following web page: http://www.clontech.com/atlas/genelists/7860–1 RaTox12.pdf. The families of genes present on the Atlas array membrane include transcription factors, elements of several metabolic pathways (amino acids, lipids, energy, nucleotides), cell cycle regulators, adhesion molecules, cytosolic enzymes, receptors, interleukins, and DNA binding elements, to cite a few.

Details for the sample preparation and microarray processing are available from BD Biosciences Clontech Laboratories (Palo Alto, CA). Total RNA was isolated from the frontal cortex of saline-treated or MDMA-treated rats using the Atlas Pure RNA isolation kit (BD Biosciences Clontech Laboratories). For each condition, two RNA samples consisting of a pool of three animals were analyzed. Each RNA sample was hybridized to one cDNA array membrane. After confirmation of the integrity of total RNA on agarose gel, poly(A)$^+$ RNA isolation and radiolabeled cDNA probe synthesis were carried out using Atlas Pure Total RNA Labeling system (BD Biosciences Clontech Laboratories). A pooled set of primers complementary to the genes represented on the array was used for reverse transcription probe synthesis, which was radiolabeled with ^{32}P-dATP (Amersham Pharmacia Biotech, Piscataway, NJ) and purified by passage over Nucleospin columns (BD Biosciences Clontech Laboratories). The Atlas Rat Toxicology 1.2k Array nylon membrane (BD Biosciences Clontech Laboratories) was prehybridized for 30 min at 68°C in ExpressHyb hybridization solution, then hybridized with radiolabeled ^{32}P-cDNA probes overnight at 68°C. After a high-stringency wash, the membranes were exposed to a PhosphorImaging screen for 5 days at room temperature and scanned using a Storm 840 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) at 100 μm resolution.

Analysis of cDNA arrays and hierarchical clustering

Autoradiographic intensity was analyzed using Array Vision software for Windows NT (Imaging Research Inc., St Catherines, ON). The software places an array template onto the images, aligns the array template to actual target locations (coordinates) in the arrays, and reports quantitative data. This measure represents the hybridization signal. For further analysis, the data were imported into excel (Microsoft, Redmond, WA). The background was set as the array coordinate with lowest expression. This value was subtracted from all coordinate and the data were then exported as tab-limited text file into GeneSpring (Silicon Genetics, Redwood City, CA). Data were normalized to median value of all coordinates on the respective array. Genes were removed from the analysis if the ratio between duplicate saline was >2. Only genes with ratios (expression in MDMA-treated animals/expression in saline-treated animals) >2-fold or <0.5-fold in both independent replicates were considered changed. These stringent criteria were chosen to reduce the chance of false-positive inclusion in this analysis. Similar criteria have been used by us (35) and others (36). A hierarchical cluster analysis with standard correlation (the minimum distance of 0.001 and a separate ratio of 0.5) of the averaged expression of the two experiments was done using GeneSpring software to group the genes according to the similarities of changes in their ratios over time. To make sense of the large data sets, color coding was used; the primary coding scheme in Genespring is to map relative value of the ratio over saline to colors.

Reverse transcription and real-time polymerase chain reaction (real-time quantitative RT-PCR)

We confirmed our microarray results of five representative genes, i.e., the transcription factors NGFI-A and -B; the enzymes, glutathione peroxidase (Gpx-1), and heme oxygenase (Hmox2); as well as the 5-HT receptor 3 (5HTR 3), using the real-time RT-PCR technique (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany). Total RNA was extracted from cortices of six to eight animals per time point and reverse transcription was performed with oligodT primers using Advantage RT for PCR Kit (BD Biosciences Clontech Laboratories, Palo Alto, CA). PCR experiments were then performed using light cycler technology and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals).

HPLC-purified and gene-specific primers (**Table 1**) corresponding to PCR targets on the Atlas Rat Toxicology 1.2 Array were obtained from the Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD). PCR reactions were performed as follows. DNA Master SYBR Green I mix (containing *Taq* DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye) was incubated with primers and cDNA template. The amplification program consisted of one cycle of 95°C with 15 min hold ("hot start"), followed by 50 cycles of denaturation (95°C, 15 s), annealing (specific annealing temperature, 20 s), and extension (72°C, 15 s). Fluorescence data collection was performed at the end of each extension phase. Amplification was followed by melting curve analysis using the program run for one cycle 95°C (0 s), 65°C (1 0 s), and 95°C (0 s). A negative control without cDNA template was run with every assay to assess the overall specificity and to

TABLE 1. *Sequences of the primers used for the quantitative real-time PCR amplification*

Gene name	UPSTREAM Primer	DOWNSTREAM Primer
NGFI-A	TGC AGG CAC AGC CTT GCA GTA CCC	GGG ACT GGT AGG TGT TAT TAA GAG CC
NGFI-B	CCG GTG ACG TGC AGC AAT TTT ATG AC	GGC TAG AAT GTT GTC TAT CCA GTC ACC
GPX1	CTC GGT TTC CCG TGC AAT CAG TTC	TGA GCG CAG TGG GAT CGT CAC TG
HMOX2	GGA TCG GAT TCA CTA CGT AGG ACA G	GCA TTC ATC CTG GCG CGG TAG AAC
5-HTR3	CAT ACC ATC CAG GAC ATC AAC ATT TCC	GAG GCT GAC TGC GTA GAA TAA AGG C
Clathrin	AAG TAT CCG TAA GTG GAG	GGG GTT AAA GTC ACA CAG

verify that no primer-dimer was generated. The relative standard curve was established with serial dilution of a cDNA solution with an unknown concentration that corresponds to a mix of five samples randomly picked. Template concentrations using the relative standard curve were given arbitrary values. The mean concentration of clathrin light chain (CLAT) was used to control for input RNA because it is considered a stable housekeeping gene. The mean CLAT concentration was determined once for each cDNA sample and used to normalize all other genes tested from the same cDNA sample. The relative change in gene expression was recorded as the ratio of normalized data over saline. We used one-way ANOVA, followed by Fisher's protected least significant difference (PLSD) test for testing differences between the different times and the saline-treated animals. All analyses were done using the program Statview 4.02 (SAS Institute, Cary, NC). The null hypothesis was rejected at $P < 0.05$.

RESULTS

cDNA arrays were used to identify gene expression profiles in the rat cortex after an acute injection of MDMA (20 mg/kg). We chose the criteria of 2.0-fold changes in gene expression in both replicate experiments in order to select genes whose expression was altered by MDMA. This approach identified a total of 28 genes, with 19 being up-regulated and 9 being down-regulated by the drug. These changes are presented in **Table 2**. The values correspond to the means of the ratio over saline obtained at the different times. The genes regulated by MDMA can be subdivided into nine functional families. These include Bcl2 family proteins, cell surface antigens, cytokines, cytoskeleton/matrix protein, G-proteins, intracellular kinase and phosphatase network members, metabolism, transcription regulators, and receptors (Table 2). Table 2 also provides GenBank accession numbers for all the genes.

The genes differentially expressed after an acute administration of MDMA can be further subdivided according to their expression profile. For example, some genes were affected early after the injection and returned to control within few hours, whereas the expression of others remained changed for longer periods after the MDMA injection. A hierarchical cluster analysis with standard correlation of the averaged expression of the two experiments was generated using the computer program GeneSpring (**Fig. 1**). The cluster algorithm in GeneSpring arranges the genes according to their expression profiles in such a way that genes with similar patterns of expression across all the time points are clustered adjacent to each other. A dendrogram constructed during the clustering algorithm is shown on the left of the cluster and describes the relationship between the genes. Because genes with similar patterns of expression in response to MDMA are grouped together by common branches of the dendrogram (37), we were able to further divide the large cluster into five subclusters corresponding to five different MDMA-induced molecular signatures. It is thought that genes falling within the same clusters might participate in similar cellular metabolic processes (33, 37).

Cluster A (Fig. 1) includes six genes that showed MDMA-induced decreases in their expression 4 h and 1 day after drug administration. This cluster contains two genes (MIP1α and MIP3) that encode cytokines. These proteins are reported to be involved in neurodegenerative processes such as Alzheimer's disease and multiple sclerosis (38).

Cluster B (Fig. 1) includes nine genes with increased expression 1–2 h after MDMA administration. This cluster contains genes that encode several proteins involved in metabolic pathways, such as heme oxygenase (Hmox2), which participates in metabolic processes and in cellular stress responses. The ribosomal protein (Rps29) and polyubiquitin (PUb) are involved in protein synthesis and breakdown. Two proteins, lactate dehydrogenase (LDH-B) and cellular glutathione peroxidase 1 (Gpx-1), which are involved in cellular detoxification, are induced. Because this cluster includes several genes that participate in metabolic pathways (Gpx-1, Hmox2, LDH-B) and protein turnover (PUb, Rps29), they might participate in adaptive responses to MDMA-induced oxidative stress (24–26).

Cluster C contains six genes whose maximal expression was 30 min postdrug, with subsequent slow reversal to saline levels at ~ 4 h after MDMA injection (Fig. 1). The transcription factors NGFI-A and B fall within this cluster. These changes might be secondary to early activation of 5-HT receptors. The cluster includes genes that code for proteins involved in stress responses, namely, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Gapdh protein has recently been shown to be involved in various aspects of cellular function, including activation of transcription and tubulin assembly (39).

Cluster D consists of three genes whose expression reached their highest level at 8 h (Fig. 1). CAK β, a focal adhesion kinase, interacts with proteins in the

TABLE 2. *Classification of up and down-regulated genes in the rat cortex after MDMA injection*

Genbank accession	Description	Ratio									Up/down
		0.5 h	1 h	2 h	4 h	8 h	16 h	1 day	3 days	7 days	
	Bcl-2 family proteins										
AF027954	BCL2-related ovarian killer protein (Bok)	0.89	0.99	1.22	0.61	0.95	0.61	0.49	0.41	0.50	↓
	Cell surface antigens										
X55288	CD28 T-cell surface antigen (CD28)	0.97	0.68	0.71	0.41	0.72	0.80	0.54	0.59	0.54	↓
AF017437	Integrin-associated protein form 4 (IAP)	0.73	0.42	0.58	0.52	1.39	0.31	0.82	0.73	0.81	↓
	Cytokines										
U22414	Macrophage inflammatory protein 1 alpha (MIP1α)	0.55	1.15	0.73	0.78	1.25	0.87	0.44	0.88	0.89	↓
U90447	Macrophage inflammatory protein 3 (MIP3)	1.23	1.12	1.05	0.56	0.54	0.95	0.41	0.77	1.67	↓
	Cytoskeleton/matrix proteins										
X05834	Fibronectin (Fib)	0.94	0.60	1.10	1.45	2.31	0.90	1.12	1.54	1.08	↑
U61261	Laminin alpha 3 subunit (Lamα3)	1.02	0.83	0.79	1.08	1.54	2.00	0.90	1.09	2.07	↑
U33553	Neuroglycan C (NGlyC)	0.71	0.57	0.59	0.46	0.69	1.16	0.62	0.61	0.81	↓
V01227	Tubulin alpha 1 (Tubα1)	1.85	2.18	2.16	1.24	0.99	1.03	1.19	1.29	1.22	↑
	G-proteins										
L35921	GTP binding protein gamma 9 subunit (Gγ9)	1.63	2.18	2.11	0.91	0.72	1.22	0.85	1.51	1.39	↑
M83676	ras-related protein (RAB12)	1.54	2.99	3.76	1.37	1.25	1.18	1.37	0.98	1.06	↑
	Intracellular kinase and phosphatase network members										
D45854	Cell adhesion kinase β (CAK β)	1.80	1.20	1.83	1.06	1.50	0.75	1.07	0.84	0.48	↓
X52952	MEK kinase c-mos (Mos)	0.49	0.24	0.42	0.54	0.73	1.39	0.31	0.79	1.38	↓
M65159	Protein tyrosine phosphatase, striatum enriched (PTP)	2.15	1.89	2.08	1.22	0.82	0.99	1.47	1.43	1.48	↑
M99567	Phospholipase C beta 3 (PLCβ3)	1.29	1.61	1.99	1.02	1.13	0.90	1.04	1.31	2.02	↑
L01702	Receptor protein-tyrosine phosphatase α (RPTP-α)	1.70	2.42	2.22	1.13	1.01	0.77	1.45	0.86	0.98	↑
	Metabolism										
X59051	40S ribosomal protein S29 (Rps29)	1.58	2.00	2.48	1.00	1.01	1.14	0.84	0.97	0.88	↑
X12367	Cellular glutathione peroxidase I (Gpx1)	2.07	2.98	3.66	0.94	1.77	1.00	1.43	0.96	1.02	↑
J05405	Heme oxygenase 2 (Hmox2)	2.08	2.53	2.76	1.06	1.20	1.20	1.21	1.14	1.54	↑
M63983	Hypoxanthine-guanine phosphoribosyltransferase (Hprt)	2.84	2.43	1.87	0.91	1.21	0.85	0.88	1.02	1.05	↑
M17701	Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	3.22	2.76	2.18	0.86	1.03	0.89	0.79	1.00	0.92	↑
U07181	Lactate dehydrogenase B (LDH-B)	1.46	2.52	2.83	1.00	0.82	0.93	1.17	0.92	0.80	↑
D16554	Polyubiquitin (PUb)	2.17	2.21	1.92	0.80	0.90	1.12	1.00	1.16	1.21	↑
	Receptors										
U59672	5-Hydroxytryptamine receptor 3 receptor (5-HTR3)	0.73	0.60	1.10	1.15	0.75	1.40	1.05	1.55	2.74	↑
U92289	Prostaglandin D2 receptor (PGDR2)	1.06	1.02	1.27	1.81	2.26	1.07	0.25	0.80	0.85	↑
	Transcription										
L03556	Homeobox protein 1.3 (HOX1.3)	0.76	0.86	0.70	0.46	0.80	0.44	0.29	0.83	0.78	↓
M18416	Nerve growth factor-induced protein I-A (NGFI-A, egr-1)	2.59	1.99	2.51	1.02	0.67	0.86	0.89	1.15	0.80	↑
U17254	Nerve growth factor-induced protein I-B (NGFI-B)	3.36	2.08	2.00	0.98	1.30	0.96	0.86	1.11	1.05	↑

extracellular matrix such as fibronectin, which is found in this subcluster (40). Cluster E (Fig. 1) consists of three genes that showed a triphasic expression profile, with small increases at 8–16 h and 7 days and decreases at 1 day after MDMA. Laminin, an element of the extracellular matrix, belongs to this cluster. The kinase Mos is a regulator of the MAP kinases involved in the regulation of several cellular processes (41). PGDR2, the receptor for prostaglandin D2, belongs in this cluster. The prostaglandin D2 is involved in sleep regulation (42), a function known to involve 5-HT (43) and to be disturbed by MDMA (44).

To confirm the data obtained using the cDNA array approach, we selected five of the genes for quantitative measurements using light cycler technology. **Figure 2** shows the expression profile for those five genes. They include NGFI-A and -B, Gpx-1, Hmox2, and the 5-HT receptor (5-HTR 3). After normalization to the CLAT mRNA level in each sample, data were expressed as ratios compared with saline-treated animals. The quantitative PCR results show qualitative profiles similar to the data obtained in the array experiments (compare values in Table 2 to those in Fig. 2). The immediate early genes NGFI-A and B showed increases as early as 30 min; these values remained elevated for 2 h, then returned to control levels ~4 h (Fig. 2). The gene encoding Gpx-1 is regu-

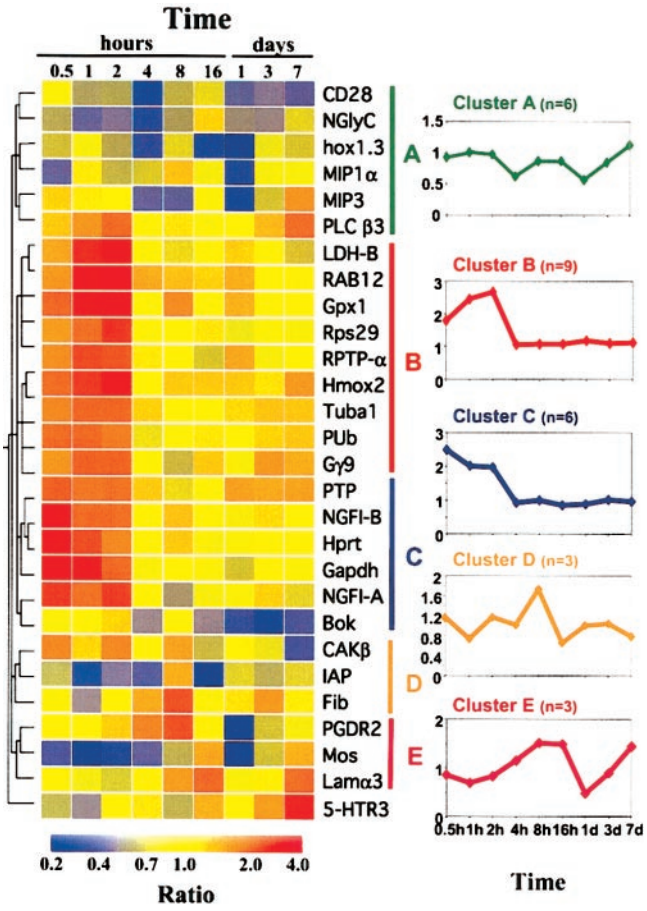

Figure 1. Cluster analysis of ratio profiles for 29 genes in the rat cortex between 30 min and 7 days after MDMA administration. Genes were selected according to the criteria described in Materials and Methods. GeneSpring grouped them into 5 clusters based on similar profiles of changes during the course of the study. Each row represents a given gene and each column a given time. A color scale is provided to represent relative changes. Red corresponds to increased ratios and blue represents decreased ratios in comparison to control values. Graphs indicate the mean profiles of changes for the genes that comprise each cluster.

lated in a fashion similar to the IEGs (Fig. 2). On the other hand, the increase in Hmox2 was more delayed, occurring between 2 and 4 h postdrug. 5-HTR 3 transcripts show a biphasic pattern with a small but significant up-regulation at 4 h, followed by larger increases 3 and 7 days post-MDMA administration.

DISCUSSION

Our experiment used a comprehensive molecular approach to identify changes in gene expression that occur in the rat cortex in response to MDMA administration. Because previous approaches have allowed analyses of, at most, a few genes at a time, it was often difficult to develop comprehensive hypotheses of drug effects. In the case of MDMA, only a few genes had actually been examined (9, 10). Our present observations extend the results of other investigators who have shown that MDMA can cause transcriptional changes in the brain (9, 10). We will limit our discussion to genes involved in transcriptional control, xenobiotic metabolism, and possible adaptive changes related to long-term effects of the drug.

Receptors

We found significant increases in the expression of 5-HTR 3 receptors. However, other 5-HT receptors (5-HTR 1F, 2A, 2B, 2C, 5A, 5B, 4, 6) found on the cDNA arrays did not meet our stringent criteria for change. For example, 5-HTR 2A, 5-HTR 5A, 5-HT 5B, and 5-HTR 6 showed peak effects of 0.53, 1.85, 0.51, and 1.40, respectively. Our results are consistent with a previous report that MDMA had no significant effect on the expression of 5-HTR 2A and 2C binding in animals killed 30 days after drug treatment (45), whereas a transient down-regulation of 5-HTR 2 binding has been described 6 h after MDMA injections to rats (46). Although MDMA treatments have been reported to induce changes in 5-HTR 1A in several brain regions, including the cortex (47, 48), the absence of this receptor on the cDNA arrays prevents us from confirming these results.

Changes in 5-HTR 3 expression are of interest because of the long-term effects of MDMA on the 5-HT system (15). The 5-HTR 3, a ligand-gated ion channel, is structurally and functionally distinct from the other six classes

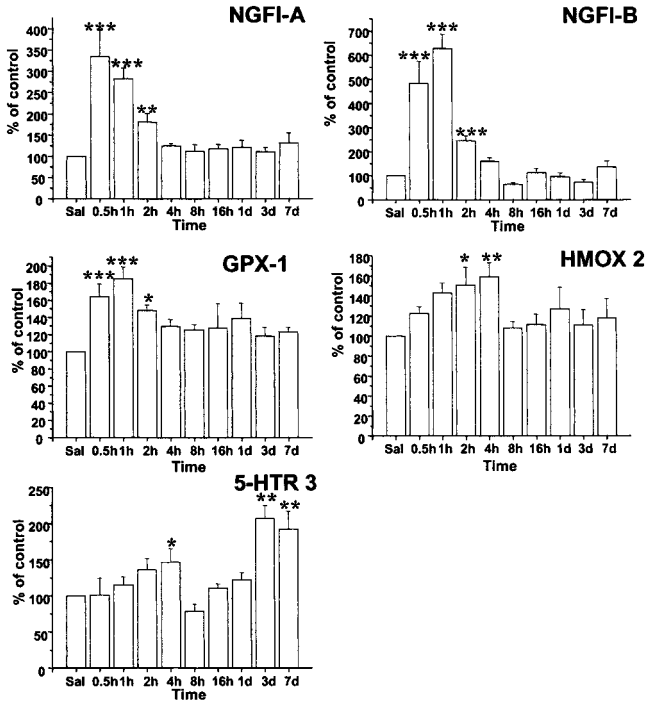

Figure 2. Quantitative PCR confirmation of MDMA-induced changes in 5 transcripts. The mRNA levels were measured as fluorescent intensities using quantitative real-time PCR and normalized to light-chain clathrin mRNA levels. Values represent means ± SE (percentage of saline-treated animals). Statistical analysis was done by ANOVA, followed by Fisher's PLSD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *Compared to saline control group.

of 5-HT receptors, which are all coupled to G-proteins and mediators of synaptic responses (49). Ligand-gated ion channels are responsible for rapid chemical transmission of nerve impulses at synapses where binding of transmitters results in the rapid opening of Na^+-selective pores (50). Presynaptic 5-HTR 3 receptors are thought to be involved in modulation of the release of 5-HT or of other neurotransmitters including dopamine (51). A modulator role for 5-HTR 3 receptors has been proposed for GABA release, but this needs further clarification (51). In the hippocampus and neocortex, these receptors are located postsynaptically in the perikarya and dendrites of GABAergic interneurons (52). They have been shown to induce fast excitatory responses subsequent to 5-HT application to cultures of hippocampal cells (53). Similar findings have been obtained by stimulating the amygdala (54). In our experiments, 5-HTR 3 transcripts were somewhat up-regulated at the 4 h point (+50%), followed by a rapid reversal to control levels (see Fig. 2). These values stayed within normal ranges until they increased substantially (+100%) at 3–7 days, a time when MDMA-mediated cortical 5-HT depletion is well documented (15). This delayed increase in expression might serve to maintain normal 5-HT neurotransmission in the 5-HT-depleted cortex. In a similar fashion, the early up-regulation of 5-HTR 3 transcripts could be an adaptation to the 5-HT depletion (80%) that follows the rapid 5-HT release caused by MDMA administration (15). The observations of increased 5-HTR 3 transcripts might be due to compensatory responses to a transient down-regulation of 5-HTR 3 protein secondary to overstimulation after the early massive release of 5-HT after MDMA administration. This last idea is supported by evidence that other receptor channels, such as AMPA receptors (55) and Na^+ channels (56), can be endocytosed and eliminated by ubiquitinization and proteosomic degradation (57). The observed MDMA-induced early (30 min-1 h postdrug) up-regulation of ubiquitin provides partial support for this suggestion.

Signaling pathways and transcription control

Similar to the action of other amphetamine analogs, MDMA interacts with monoamine uptake transporters and stimulates the release of those amines (5). MDMA inhibits both monoamine oxidase A and B, which are responsible for monoamine degradation (58). These actions result in increased extracellular concentrations of 5-HT and the subsequent stimulation of their receptors (6). Stimulation of these receptors is probably at least partially responsible for the MDMA-induced behavioral effects, such as hyperactivity [for review, see (14)]. Moreover, stimulation of 5-HT receptors can cause increases in the intracellular concentration of several second messengers (cyclic AMP, Ca^{2+}, inositol 1,4,5-triphosphate (IP_3), diacylglycerol) and regulates the activity of various kinases (6). Thus, stimulation of these signaling cascades may participate in MDMA-induced early changes observed in NGF1-A (known as *egr-1, Tis8, Krox24*) and NGFI-B mRNAs (see Table 2 and Fig. 2). These observations confirm those of others who have reported MDMA-induced increases in NGFI-A expression in several brain regions (9). The NGFI-A gene has been shown to be rapidly and transiently activated in response to various neuronal stimuli, including other psychostimulants such as amphetamine and cocaine (59). The transcription factors encoded by NGFI genes stimulate discrete programs of late response gene expression and play key roles in the conversion of short-term neuronal stimulation into specific long-lasting changes in cell function (12). Indeed, our array experiments did identify some genes that showed somewhat delayed MDMA-induced increases. These genes encode several proteins that belong to intracellular transduction cascades and link membrane receptors to the nuclear machinery (60); they include G-protein subunits (G-γ9 and RAB12), kinases, and phosphatases (CAK β, Mos, RPTPα, and PTP) (see Table 2). So it is not farfetched to suggest they could be NGFI-A and B target genes that might participate in MDMA long-term neuroplastic effects. This suggestion is supported by reports that changes in kinase/phosphatase activity are associated with both short-term and long-lasting alterations in neuronal function (61).

Cytoskeletal and matrix proteins

It is of interest to relate the preceding discussion to the MDMA-induced changes in cytoskeletal and matrix proteins. For example, tubulin α1 was increased between 1 and 2 h after drug administration. Tubulin mRNA is enriched in brain regions that contain neurons undergoing neurite extension (62). Thus, MDMA might trigger early reorganization of cortical neuronal networks, with secondary long-term behavioral effects such as the behavioral sensitization that is observed even after a single injection of amphetamines (63). Increased expression of the extracellular matrix proteins fibronectin and laminin α3, which interact with integrin cell surface receptors (64), suggests that these proteins participate in the brain plastic adaptation to the biochemical and 5-HT-depleting effects of MDMA since they are known to be involved in synaptic plasticity and regeneration (64). These ideas need to be tested further.

Metabolic pathways

The dose of MDMA used in our study is known to cause hyperthermia and ROS formation (2, 25). Under these conditions, cells usually adapt their transcriptional responses by either stimulating antioxidant systems, removing damaged macromolecules, or down-regulating nonessential genes (65). These types of metabolic stresses are known to induce the expression of various transcripts, including heme oxygenase, glucose-related proteins, and ubiquitin (66). The present study did indeed identify changes in several genes that encode proteins involved in metabolism and stress responses. Gpx-1 transcript levels were increased at 30 min to 2 h after the MDMA injection. We observed increased transcription of heme oxygenase 2. As mentioned

above, MDMA is metabolized via pathways that can induce the formation of superoxides and peroxides via redox cycling (20–22). Thus, changes in Gpx-1 involved in detoxifying hydroperoxides (67) and heme oxygenase 2, which is inducible by hydrogen peroxide (68), might constitute elements of the adaptive antioxidant responses to MDMA-mediated oxidative stress.

CONCLUSION

Our observations suggest that a single dose of the drug can induce multiple transcriptional changes in the rat neocortex. Our results confirm and extend observations that the expression of several transcription factors can be altered by MDMA administration. These findings show that a single injection of this drug might be responsible for durable neuronal alterations in the rodent brain. Our demonstration that MDMA affects the transcripts of proteins involved in cell-cell and cell–matrix interactions suggests that this drug can indeed cause long-term neuroplastic changes. Moreover, our investigation has identified changes in several genes that encode proteins known to be involved in cellular detoxification processes, thus supporting the view that free radicals mechanisms are activated by MDMA.

Because the cDNA approach has allowed for a simultaneous analysis of MDMA diverse effects on the brain, this approach will help investigators to develop more instructive hypotheses on the mode of actions of the drug. These studies should help draw a more extensive canvass of the molecular and cellular interactions that form the substrates of MDMA multifaceted effects. FJ

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Received for publication May 29, 2002.
Accepted for publication August 6, 2002.