

Active and passive MDMA ('ecstasy') intake induces differential transcriptional changes in the mouse brain

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Running title: MDMA-induced transcriptional changes in mice

Keywords: Addiction, MDMA, ecstasy, transcriptomics, mouse brain, gene expression

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ABSTRACT

3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) is a recreational drug widely used by adolescents and young adults. Although its rewarding effects are well established, there is controversy on its addictive potential. We aimed to compare the consequences of active and passive MDMA administration on gene expression in the mouse brain since all previous studies were based on passive MDMA administration. We used a yoked-control operant intravenous self-administration paradigm combined with microarray technology. Transcriptomic profiles of ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus were analyzed in 27 mice divided in contingent MDMA, yoked MDMA and yoked saline groups, and the observed changes were validated by qRT-PCR. The comparison of contingent MDMA and yoked MDMA versus yoked saline mice allowed identification of differential expression in several genes, most of them with immunological and inflammatory functions, which pinpoints to direct effects of the drug on the brain transcriptome. In the comparison of contingent MDMA versus yoked MDMA administration, hippocampus and the dorsal raphe nucleus showed statistically significant changes. The altered expression of several genes involved in neuroadaptive changes, which may be related to learning self-administration behaviour, could be validated in dorsal raphe nucleus. In conclusion, our study shows a strong effect of MDMA administration on the expression of immunological and inflammatory genes in all the four brain regions studied. In addition, experiments on MDMA self-administration suggest that the dorsal raphe nucleus may be involved in active MDMA seeking behaviour, and show specific alterations on gene expression that support the addictive potential of this drug.

INTRODUCTION

3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) is a recreational drug used around the world by young adults. MDMA induces euphoria and well being in humans (Parrott, 2001), and its rewarding/reinforcing effects have been well established in animal models (Cole & Sumnall, 2003). Although the addictive potential of this substance is still a matter of debate, there is evidence showing that a proportion of MDMA users meet the *Diagnostic and Statistical Manual of Mental Disorders* (DSM) criteria for dependence (Cottler *et al.*, 2001; Leung & Cottler, 2008; Stone *et al.*, 2006). MDMA acutely increases brain levels of dopamine (DA), serotonin (5-HT) and noradrenalin in monkeys, rats and mice by potently inhibiting neurotransmitter reuptake mechanisms (Green *et al.*, 2003). Repeated administration of MDMA in humans produces long-term psychiatric disorders, including anxiety and mood alterations, as well as cognitive deficits (Zakzanis *et al.*, 2007), which may be associated with persistent neuroadaptations dependent on changes in gene expression.

Single or repeated administration of MDMA in animals induce changes in gene expression similar to what has been observed following treatment with other psychostimulants such as cocaine, amphetamine or methamphetamine (Hemby, 2006, Yuferov *et al.*, 2005, Zhang *et al.*, 2005). Acute administration has been reported to dose-dependently increase the expression of several immediate early genes, such as *c-fos* and *Egr1* in different brain structures (Stephenson *et al.*, 1999; Shirayama *et al.*, 2000). Similarly, the *Rnd3* gene involved in actin cytoskeleton modulation and cell adhesion was up-regulated in the striatum of mice after acute MDMA administration (Marie-Claire *et al.*, 2007). Repeated treatment with MDMA increased *DeltaFosB* expression in mice (Olausson *et al.*, 2006) and induced pronounced alterations in gene

expression of glutamate transporters as well as AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl D-aspartate) and metabotropic glutamate receptor subunits in different brain regions in rats (Kindlundh-Hogberg *et al.*, 2008). In addition, changes in pro-dynorphin (*Pdyn*) and pro-enkephalin (*Penk*) gene expression have been observed in several brain areas of rats treated either acutely or repeatedly with MDMA (Adams *et al.*, 2005; Di Benedetto *et al.*, 2006). Using microarray technology, alterations in the expression of numerous genes involved in the modulation of signalling pathways, transcription regulators or xenobiotic metabolism have been demonstrated in the frontal cortex of rats following a single MDMA administration (Thiriet *et al.*, 2002). Although these data provide evidence for the effects of non-contingent administration of MDMA on gene expression in the brain, there are no studies available using models of MDMA operant self-administration, which are more relevant to the human pattern of drug consumption. In this sense, the use of a yoked-control operant intravenous self-administration paradigm coupled with microarray studies have shown different profiles of gene transcript alterations in the nucleus accumbens shell and core comparing contingent versus non-contingent heroin and cocaine administration (Jacobs *et al.*, 2004; Jacobs *et al.*, 2005), which suggests that the learning component associated with active drug-taking is a critical factor affecting changes in gene transcription.

This study was designed to identify changes in gene expression in different brain structures (ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus) in mice receiving repeated contingent or yoked administration of MDMA in order to better understand the consequences of MDMA consumption and seeking behaviour. These structures are known to mediate different aspects of drug reward and instrumental conditioning (Belin *et al.*, 2009; Ikemoto, 2010), participate in the neurochemical and

behavioural effects of MDMA (Cole & Sumnall, 2003) and show gene expression changes following acute (Stephenson *et al.*, 1999; Thiriet *et al.*, 2002) and repeated (Kindlundh-Hogberg *et al.*, 2008; Olausson *et al.*, 2006) non-contingent MDMA administration.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice weighing 20–24 g at the beginning of the experiments were initially housed five per cage in a room with controlled temperature (21 ± 1 °C) and humidity ($65 \pm 10\%$), with a reversed light/dark cycle (lights off from 08:00 to 20:00 hours), and with *ad libitum* food and water. The experiments took place during the dark phase. Behavioural tests and animal care were conducted in accordance with the standard ethical guidelines (National Institutes of Health, 1995; European Communities Directive 86/609 EEC) and approved by the local ethical committee (CEEA-PRBB).

Drugs

MDMA hydrochloride was obtained from Lipomed, A.G. (Arlesheim, Switzerland) and dissolved in sterile 0.9% physiological saline solution.

Surgery and self-administration procedure

Mice were anesthetized with an intraperitoneal (i.p.) injection of a ketamine/xylazine mixture (5:1; 0.10 ml/10 g) and then implanted with an indwelling intravenous (i.v.) silastic catheter in the right jugular vein, as previously described (Orejarena *et al.*, 2009). The animals were pre-treated with ketoprofen 5 mg/kg subcutaneously (s.c.) for post-surgery analgesia. After surgery, the mice were housed individually for the remainder of the experiments. In order to avoid clots and infection, the animals were flushed through the catheter with 0.02 ml of a solution containing heparin (30 UI/ml), cefazoline (50 mg/ml) and sodium chloride (0.09%) for 5 days.

The patency of the catheters was evaluated once a week by the injection of 0.1 ml of thiopental (5 mg/ml). If prominent signs of anaesthesia were not apparent within 3 s of the infusion, the mouse and its corresponding data were removed from the experiment. Three days after surgery, the animals were randomly assigned to either contingent or yoked groups. Contingent mice were trained to self-administer MDMA (0.125 mg/kg/infusion delivered in a volume of 23.5 μ l over 2 s) in single daily 3-h sessions. Acquisition of drug self-administration was performed using a fixed ratio 1 (FR1) schedule of reinforcement such that one nose poke in the active hole resulted in one MDMA infusion, while nose poking in the inactive hole had no programmed consequences. As previously reported (Orejarena *et al.*, 2009), mice had to achieve all of the following conditions to be included in the analysis: (i) less than 20% deviation from the mean of the total number of infusions earned in three consecutive sessions (80% stability), (ii) at least 65% responses at the active hole, and (iii) a minimum of five infusions earned per session. Each contingent mouse was connected to two yoked mice; one receiving an identical dose of MDMA (yoked MDMA) and the other a saline solution (yoked saline). When a contingent mouse had a failed catheter or did not meet the acquisition criteria, the corresponding yoked mice were discarded from the study. A light stimulus, located above the active hole, was paired with the delivery of the drug or saline according to the response of the contingent mouse. To avoid interference by acute transcriptional changes, animals were sacrificed by cervical dislocation eight hours after the last exposure to the self-administration boxes. The brains were quickly removed, and the following brain areas were dissected according to Franklin and Paxinos (Franklin & Paxinos, 1997): ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus. Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice, and stored at -80 °C for later quantification of gene expression.

RNA isolation and microarray hybridization

Twenty-seven mice (9 animals per group of contingent MDMA, yoked MDMA and yoked saline) and four brain areas (ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus) were used in the expression microarray study. Three pools consisting of three mice per pool were used for each experimental group. The pools were organized to homogenize the average number of nose pokes in the different pools. The pooled individuals were the same for all brain regions. The frontal cortex and dorsal raphe nuclei tissue samples belonging to the same pool were pooled before RNA extraction to optimize the yielding of the isolation, given the limited tissue size. In contrast, for the hippocampus and ventral striatum, which are larger brain structures, RNA was isolated separately from each animal and then pooled for the array hybridization. Samples from all tissues were homogenized using the TissueRuptor system (Qiagen. Düsseldorf, Germany) and total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen. Düsseldorf, Germany) according to the manufacturer's protocol. RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies. Wilmington, DE, USA) and integrity was evaluated using the Bioanalyzer 2100 platform (Agilent Technologies. Santa Clara, CA, USA). RNA samples were stored at -80°C until analyzed. For the microarray experiment, we used the GeneChip® Mouse Expression Set 430 array (Affymetrix. Santa Clara, CA, USA), which contains probes that cover over 39,000 transcripts and variants from over 34,000 genes. A total of 36 chips were used: three pools of three individuals per condition (contingent, MDMA, yoked MDMA and yoked saline) and four brain areas. Two µg of RNA from each pool were used to hybridize arrays at the Genomics Unit of Hospital Clínic-IDIBAPS (Barcelona, Spain). Chips were scanned

using a GenePix4000B scanner (Molecular Devices, Inc. Sunnyvale, CA, USA) and raw data were obtained using the GenePix Pro 4.0 (Molecular Devices. Sunnyvale, CA, USA) and GCOS softwares (Affymetrix. Santa Clara, CA, USA).

Quantitative RT-PCR

To confirm the most relevant results obtained in the microarray study, total RNA from the four brain regions of contingent MDMA, yoked MDMA and yoked saline mice was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems. Foster city, CA, USA). For this experiment, we used the same pools that were previously subjected to hybridization with the arrays. The Mouse Endogenous Control Array (Applied Biosystems. Foster city, CA, USA) was used to select endogenous controls. Real Time-PCR experiments were performed for 15 genes using the LightCycler 480 II system and the Universal Probe Library (Roche Applied Science, Penzberg, Germany). Gene assays were designed using the Universal ProbeLibrary Assay Design Center software (Roche Applied Science, www.roche-applied-science.com). Sequence of the primers and probes used are available upon request. Beta-actin (*Actb*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were used to normalize the relative amounts of mRNA.

Statistical and bioinformatic analyses

The self-administration behavioural data were analyzed using a three-way repeated measures ANOVA with group as a between subject factor and hole and day as within subjects factors followed by post-hoc tests for individual comparisons when appropriate. Statistical significance was set at $p < 0.05$.

For the microarray data, we used the Bioconductor software for R environment and the *affy* library (www.bioconductor.org) (Gentleman *et al.*, 2004). The quality assessment of the chips was performed using the *affyPLM* library. Background correction, normalization and summarization were performed using the background method, Robust Multichip Average (RMA) (Irizarry *et al.*, 2003) and the median-polish method, respectively. For gene filtering we discarded those probes that did not correspond to known genes and considered a threshold of $\log_2(60)$ for signal filtering and an interquartile range (IQR)>25% for variability filtering. The IQR method discards genes showing low expression variance among arrays without considering the comparisons performed, and is described to increase statistical power (Hackstadt & Hess, 2009). The Linear Modeling for Microarray Analysis (LIMMA) package (Smyth, 2004) was used for class comparison, by which we compared the expression patterns of the pairs contingent MDMA-yoked MDMA, contingent MDMA-yoked saline, and yoked MDMA-yoked saline. Correction for multiple testing was achieved by adjusting the p-value with a False Discovery Rate (FDR) of 5%. Functional group over-representation analysis of genes with significant differential expression was performed using the DAVID Annotation Tool (david.abcc.ncifcrf.gov) (Dennis *et al.*, 2003) and was supported by literature searches.

Gene expression networks were constructed using the Ingenuity Pathway Analysis 8.8 software (Ingenuity Systems. Redwood city, CA, USA). To assess the direct effect of the drug, genes differentially expressed in both the contingent MDMA-yoked saline and the yoked MDMA-yoked saline comparisons (after 5% FDR) were considered in ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus. To evaluate drug reinforced learning, gene networks were performed considering genes differentially expressed in the comparisons of contingent MDMA-yoked MDMA and

contingent MDMA-yoked saline in hippocampus and in dorsal raphe nucleus (after 15% FDR). This software estimates a score, calculated with the right-tailed Fisher's Exact Test, based on the probability of finding the observed number of differentially expressed genes in a given network by chance.

The identification of over-represented transcription factor binding sites in the different sets of differentially expressed genes was performed using Single Site Analysis with the oPOSSUM 2.0 software (www.cisreg.ca/cgi-bin/oPOSSUM/opussum) (Ho Sui *et al.*, 2005) using default parameters and sorting the results by Z-score.

In the quantitative RT-PCR experiments, gene expression changes for each comparison were evaluated using a U-Mann-Whitney non-parametric test, and statistical significance was set at $p < 0.05$.

RESULTS

MDMA self-administration

The average number of active and inactive nose pokes carried out by contingent mice trained to self-administer MDMA (0.125 mg/kg per infusion) as well as for yoked MDMA and yoked saline mice is shown in Figure 1. Seventy percent of the contingent mice met all the acquisition criteria within a short time period (8 ± 0.76 days), and showed a mean cumulative intake of 19.7 ± 1.62 mg/kg of MDMA during the entire training period. Saline- or MDMA-yoked animals did not discriminate between holes on any of the training sessions. Eleven training sessions were performed until all contingent mice reliably acquired MDMA self-administration behaviour. Three-way repeated measures ANOVA comparing responses in the active and inactive holes for all groups during the entire testing period revealed a significant main effect of group [$F(2,24) = 80.600$, $p < 0.001$], a significant main effect of hole [$F(1,24) = 77.770$, $p < 0.001$], a significant group x hole interaction [$F(2,24) = 77.498$, $p < 0.001$], and a significant group x day interaction [$F(20,240) = 1.969$, $p < 0.01$]. Subsequent Bonferroni post-hoc analysis revealed significant differences between contingent mice versus both yoked groups ($p < 0.001$). Discrimination between holes was significant only in the contingent MDMA group from day 1 through day 11 (Table S1).

MDMA-induced transcriptional changes

To assess possible transcriptional changes caused by active or passive MDMA administration, gene expression profiles in the four brain areas (ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus) from contingent MDMA, yoked MDMA and yoked saline mice were compared between the three possible pairs of experimental

situations using microarray technology. This study design allowed to identify genes modulated by the direct pharmacological effect of MDMA on the brain (those differentially expressed in both the contingent MDMA-yoked saline and yoked MDMA-yoked saline comparisons) as well as genes involved in the cognitive processes related to active MDMA self-administration (those differentially expressed in the contingent MDMA-yoked MDMA comparison and in the contingent MDMA-yoked saline comparison).

Active and passive MDMA administration versus saline: direct effect of the drug

When gene expression levels were compared between yoked MDMA and yoked saline mice, significant differences (5% FDR) were observed in the four brain structures studied, ranging from 503 genes in hippocampus to 1340 genes in ventral striatum. Some of these particular genes were also identified in the comparison of contingent MDMA versus yoked saline, ranging from 16 in dorsal raphe nucleus to 192 in hippocampus (Supplementary Fig. 1a, Tables S2-S5). Genes identified in the two comparisons were analyzed for functional group over-representation using the DAVID database (except from raphe, due to the low number of common positive genes in the two comparisons), and similar clusters were obtained in all brain regions, the most significant ones being those involved in immune or inflammatory response, as well as in response to wounding or to stress (Fig. 2).

The construction of interaction networks for the common positive genes in the two comparisons revealed gene networks of inflammatory and immune response as the best scored in all four brain regions (Supplementary Fig. 2). Interestingly, all the identified networks included the *Lcn2* gene, which showed a dramatic differential expression in all regions, as well as *Tgtp* (frontal cortex and hippocampus;

Supplementary Fig. 2b and 2d, respectively). The analysis of over-representation of transcription factor binding sites could not identify a common transcription factor for all brain regions (data not shown).

Based on their function, we chose 10 genes among those that were differentially expressed in the two comparisons, with a Log Fold Change over 1.5 or below -1.5 (difference in expression over 2.8-fold) for further validation: seven genes related to immunological functions (*Lcn2*, that was differentially expressed in all four brain regions, and *Ctla2a*, *Gbp2*, *Igtp*, *Iigp1*, *Iigp2* and *Tgtp*, that were identified in hippocampus and frontal cortex) and three genes involved in neurological processes (*Sgk1* and *Sgk3* in dorsal raphe nucleus and *Slc17a7* in ventral striatum) (Table 1). QRT-PCR experiments validated the results of the microarray analysis, confirming the overexpression of these genes caused by active and passive MDMA intake in these particular brain structures, with the exception of differences in *Sgk3* and *Iigp2* that were not confirmed in the dorsal raphe nucleus and in hippocampus, respectively (Table 1).

Contingent MDMA self administration versus yoked MDMA: drug reinforced learning

The contingent MDMA versus yoked MDMA comparison displayed significant (5% FDR) gene expression differences only in hippocampus (n = 945) and dorsal raphe nucleus (n = 1) (Supplementary Fig. 1a). Among them, 537 genes were also identified when we compared hippocampus of contingent MDMA and yoked saline mice and, thus, were assumed to be more consistent (Table S6). Functional clustering performed with the DAVID database targeted some interesting neurological functions, such as synapse, synaptosome, neurotransmitter secretion, regulation of neurotransmitter levels, nervous system development, neuron projection, axonogenesis, axon guidance, dendrite, neurite morphogenesis, neuron morphogenesis, neuron differentiation and

learning and memory (Table S7). However, none of these 537 genes displayed a Log Fold Change over 1 or below -1 (differential expression greater than 2-fold) (Supplementary Fig. 1b, Table S6), low values that discouraged further validation by qRT-PCR. For this reason, we changed the significance threshold to a less restrictive FDR value (from 5% to 15%) and the number of genes showing differential expression in both comparisons increased from 537 to 706: 61 in the dorsal raphe nucleus (Table S8), 645 in hippocampus and none in the frontal cortex nor in the ventral striatum. Among them, only four genes (*Camk2a*, *Kalrn*, *Ddn* and *Egr3*; Table 2) displayed a Log Fold Change over 1.5 or below -1.5 in the contingent MDMA versus yoked MDMA comparison. Interestingly, these genes are involved in neuroadaptation and synaptic plasticity. All four genes were overexpressed in the dorsal raphe nucleus of contingent mice and the differential expression in this brain structure was validated by qRT-PCR (Table 2).

Gene network construction on all the genes showing differential expression at 15% FDR revealed that the best scored network in hippocampus (score = 47; Fig. 3a) involves cell-to-cell signalling and interaction/nervous system development and function, including some interesting genes, such as *Syp*, *Stxbp1*, *Cplx2*, *Vamp2*, *Ngln2*, *Nrxn1* and *Nrxn2*, involved in synaptic vesicle fusion, synapsis formation and neurotransmitter release. Interestingly, the best scored network in dorsal raphe nucleus considering 15% FDR (score = 26; Fig. 3b) also includes genes involved in cell-to-cell signalling, interaction/nervous system development and function and behavior, such as *Camk2a* and *Kalrn* (both with a differential expression validated by qRT-PCR).

Over-representation of transcription factor binding sites analysis in hippocampus and dorsal raphe nucleus showed no common regulatory mechanisms. The best scored prediction in hippocampus pointed at the transcription factor Mzf1_1-4 (Z-score

= 68.75) and at Pdx1 in dorsal raphe nucleus (Z-score = 16.64), both predicting binding sites of these transcription factors in more than 90% of the differentially expressed genes. The four genes whose differential expression was validated by qRT-PCR (*Camk2a*, *Kalrn*, *Ddn* and *Egr3*) have predicted binding sites for Pdx1.

DISCUSSION

The aim of the present study was to identify alterations in brain gene expression due to the pharmacological effect of MDMA administration, as well as neuroadaptive changes underlying the learning process associated with operant MDMA self-administration. For that purpose, we have validated a new operant paradigm consisting in master mice that are trained to acquire a stable operant behaviour to self-administer a reinforcing dose of MDMA (Orejarena *et al.*, 2009). Each master mouse is connected to an MDMA yoked animal that passively receives an identical dose of MDMA and to another yoked mouse that receives saline infusions. This yoked-control operant intravenous self-administration paradigm was combined with microarray technology. The results of this experimental design suggested that (i) MDMA modulates the expression of genes involved in inflammatory and immune response in different brain areas; and (ii) the dorsal raphe nucleus may participate in the neuroadaptive changes leading to active MDMA seeking behaviour.

Changes in gene expression relevant to the direct effects of the drug were evaluated by comparing the contingent MDMA and the yoked MDMA mice to the yoked saline mice. In this case, most of the hits corresponded to genes involved in immunological or inflammatory response. Among them, we identified a strong overexpression of *Lcn2* in all the brain regions, which was also present in all the best scored gene networks identified. The *Lcn2* gene encodes lipocalin2 that mediates astrocytosis under inflammatory conditions and is induced after chronic or thermal stress in brain reward regions (Krishnan *et al.*, 2007, Lee *et al.*, 2009, Roudkenar *et al.*, 2009). We also validated the overexpression of other genes (*Ctla2a*, *Gbp2*, *Igtp*, *Iigp1*, *Iigp2* and *Tgtp*) both in hippocampus and in frontal cortex. All of them, except for *Ctla2a*, are genes coding for GTPases that are induced by type II interferon (INF- γ)

(Carlow *et al.*, 1998; Miyairi *et al.*, 2007; Vestal *et al.*, 1998; Yamada *et al.*, 2009; Zerrahn *et al.*, 2002; Zhang *et al.*, 2003), and are involved in some cellular processes mediating interferon control of immune and inflammatory responses. The *Ctla2a* gene encodes the cytotoxic T lymphocyte antigen-2 alpha protein which is a cysteine proteinase inhibitor protein initially discovered in mouse activated T-cells and mast cells (Denizot *et al.*, 1989). A recent study described a dendritic and axonal localization for this protein in the mouse brain (Luziga *et al.*, 2008) and a possible role in neuronal function. Our results showing that repeated MDMA administration produces generalized changes in the expression of genes related to inflammatory and immunological responses are in accordance with previous evidence demonstrating that exposure to MDMA disrupts the immune system (Connor, 2004), which may contribute to its neurotoxic effects (Torres *et al.*, 2010). Our results differ from previous data obtained after acute MDMA exposure in murine models, where mainly serotonin receptors, transcription factors, cytoskeletal, cell adhesion and metabolic genes were differentially expressed in cortical areas or in the striatum (Thiriet *et al.*, 2002; Marie-Claire *et al.*, 2007).

Another direct consequence of the exposure to MDMA was the upregulation of genes involved in neuroadaptations and synaptic plasticity, including the *Sgk1*, *Sgk3* and *Slc17a7* genes. *Sgk1* and *Sgk3*, which encode the serum/glucocorticoid regulated kinase 1 and 3, respectively, were identified in the dorsal raphe nucleus, composed mainly by serotonergic neurons. Both have been described to be involved in memory consolidation in hippocampus (Von Herten & Giese, 2005) and regulate glutamatergic neurotransmission (Boehmer *et al.*, 2003a, Boehmer *et al.*, 2003b, Boehmer *et al.*, 2006, Boehmer *et al.*, 2005, Strutz-Seebohm *et al.*, 2005a, Strutz-Seebohm *et al.*, 2005b). In addition, *Sgk1* increases neurite formation and dendrite growth in spinal cord and

hippocampal neurons (David *et al.*, 2005, Yang *et al.*, 2006). Although these functions have been described in hippocampus and are related to the glutamatergic neurotransmission, they may also occur in other cell types, such as serotonergic neurons. Interestingly, *Slc17a7*, encoding the vesicular glutamate transporter 1 (Vglut1), was upregulated in ventral striatum. Vglut1 is involved in synaptic plasticity and plays an important role in excitatory transmission (Fremeau *et al.*, 2004). In this regard, our findings are consistent with a recent study showing gene expression changes in several glutamine transporters and receptors after repeated MDMA administration (Kindlundh-Hogberg *et al.*, 2008). Although the ventral striatum is mainly formed by GABAergic neurons, it contains glutamatergic afferences, and overexpression of Vglut1 may be localized in glutamatergic axons after axonal transport of the corresponding mRNA molecules followed by translation *in situ* as previously described (Donnelly *et al.*, 2010; Wei, 2011). In order to evaluate gene expression changes related to the learning component of the operant task to obtain MDMA infusions, we compared brain expression profiles of contingent MDMA versus yoked MDMA mice. The highest number of statistically significant changes in gene expression at 5% FDR was observed in the hippocampus, with a single hit in the dorsal raphe nucleus. Although in our experimental conditions these changes were too subtle to be validated by qRT-PCR, our results support the crucial role of the hippocampus in the control of memory and cognitive functions. These results are also in agreement with previous studies showing that cocaine-induced conditioned place preference (CPP) depends on molecular changes that occur in the hippocampus of trained rats (Krasnova *et al.*, 2008, Tzschentke, 1998). When we used a less restrictive 15% FDR threshold, several additional genes showed differential expression in hippocampus and in dorsal raphe nucleus and we identified common gene networks in the two structures. Analysis of over-representation of

transcription binding sites could not identify a common mechanism regulating transcription in active MDMA self-administration in both brain regions. The dorsal raphe nucleus was the only brain region in the comparison of contingent MDMA versus yoked MDMA groups with differentially expressed genes in the microarray experiments that could be further validated by qRT-PCR, including *Camk2a*, *Kalrn*, *Ddn* and *Egr3*. Interestingly, recent evidence suggests that this structure is involved in encoding reward-related aspects of motivated behaviour (Bromberg-Martin *et al.*, 2010, Nakamura *et al.*, 2008), and although none of the validated genes were statistically significant under 5% FDR in the initial microarray study, all four indeed passed a less restricting FDR threshold of 11% (Table 2). The *Camk2a* gene encodes the Ca²⁺/calmodulin-dependent protein kinase II alpha (CaMKIIalpha), which mediates activity-dependent synaptic plasticity. A recent study demonstrated its essential role in dendritic spine enlargement, long-term potentiation and learning (Yamagata *et al.*, 2009). Dendritic spine morphogenesis is also induced by kalirin-7, an isoform encoded by the *Kalrn* gene (Penzes & Jones, 2008). In addition, another study links these two proteins with the same signalling pathway that controls functional and structural spine plasticity (Xie *et al.*, 2007). In this regard, the NMDA glutamate receptor activation in pyramidal neurons causes CaMKII-dependent phosphorylation of kalirin-7 leading to a rapid enlargement of existing spines. Kalirin is also involved in neurite outgrowth through the nerve growth factor (NGF) signalling pathway (Chakrabarti *et al.*, 2005). In addition, we identified these two genes in the best scored network in dorsal raphe nucleus, in which the ras-dependent protein kinase ERK is a central node (Fig. 3b). This is in agreement with a previous study supporting the role of the ERK pathway in the development of addiction-like properties of MDMA (Salzmann *et al.*, 2003). On the other hand, the *Ddn* gene encodes a dendritically localized mRNA that is translated to

the protein dendrin, potentially involved in neuroplasticity events and modulation of post-synaptic cytoskeleton (Kremerskothen *et al.*, 2006). In addition, *Egr3* is a member of the *Egr* gene family, a group of synaptic activity-inducible immediate early genes involved in neuroplasticity related to memory and learning (Guzowski, 2002, Li *et al.*, 2005, Li *et al.*, 2007). Interestingly, the best characterized gene of the family is *Egr1*, and its expression is increased by MDMA in rat prefrontal cortex, striatum and hippocampal dentate gyrus (Shirayama *et al.*, 2000). Our results may suggest a role for dorsal raphe nucleus in the motivational and learning processes needed to actively self-administer MDMA since it is the only brain region, besides hippocampus, showing several statistically significant changes in the comparison of contingent MDMA versus yoked MDMA mice. In addition, the best scored gene network identified common functions within this brain region and hippocampus, with genes involved in cell-to-cell signaling and nervous system development functions. And finally, validated genes that are upregulated in the dorsal raphe nucleus are involved in neuroplasticity and neuron remodelling.

In conclusion, using the yoked-control operant intravenous self-administration paradigm, which is the most relevant animal model to study the addictive potential of drugs of abuse in humans, we showed that repeated exposure to MDMA induces the expression of genes related to inflammatory and immunological responses in several brain structures including the ventral striatum, frontal cortex, dorsal raphe nucleus and hippocampus. In addition, the gene expression changes identified in the dorsal raphe nucleus following MDMA self-administration suggest that this brain region may be involved in motivated learning associated with active MDMA seeking behaviour. However, due to sample size limitation, as we evaluated 9 mice per condition in pools

of three animals of contingent MDMA, yoked-MDMA and yoked-saline, further studies should be performed in order to confirm these results.

ACKNOWLEDGEMENTS

This work was supported by the Spanish “Ministerio de Ciencia e Innovación” (SAF2007-64062), “Instituto de Salud Carlos III” (RD06/001/001 and PI070709), the Catalan Government (SGR2009-00131 and SGR2009-00971), the ICREA Foundation (ICREA Academia-2008), “Plan Nacional Sobre Drogas” (PNSD#2009/022 and PNSD#2009/026) and the DG Research of the European Commission (GENADDICT, LSHM-CT-2004-05166; and PHECOMP, LSHM-CT-2007-037669). Partial support from FEDER funds is also acknowledged. MR is a recipient of a Miguel de Servet contract from “Instituto de Salud Carlos III”. These institutions had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

FINANCIAL DISCLOSURES

None of the authors reported any biomedical financial interests or potential conflicts of interest.

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FIGURE LEGENDS

Figure 1: Operant yoked-control responding for intravenous infusions of MDMA (0.125 mg/kg/infusion). (a) The contingent group received an infusion of MDMA with every active nose-poke ($n = 9$), (b) the yoked MDMA group received an MDMA infusion everytime the contingent mouse made an active nose-poke ($n = 9$), (c) the yoked saline group received a saline infusion everytime the contingent mouse made an active nose-poke ($n = 9$). The data represent means + SEM active and inactive nose-pokes in 3 h sessions during the acquisition period. The asterisks denote significant differences between active and inactive nose-pokes for each training day. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA).

Figure 2: Top ten of the most significant overrepresented biological categories that showed differential expression after exposure to MDMA (contingent MDMA and yoked MDMA mice versus yoked saline). The number of positive genes included in each category is indicated on the right side of each bar. Biological categories correspond to the following Gene Ontology (GO) terms: GO:0002376 (immune system process), GO:0006955 (immune response), GO:0006952 (defense response), GO:0006954 (inflammatory response), GO:0009611 (response to wounding), GO:0009605 (response to external stimulus), GO:0006950 (response to stress), GO:0005576 (extracellular region), GO:0005886 (plasma membrane) and GO:0048518 (positive regulation of biological process).

Figure 3: Gene network graphical representation of interaction between differentially expressed genes after MDMA reinforced learning (contingent MDMA versus yoked MDMA and yoked saline mice). The best scored gene network in (a) hippocampus and in (b) dorsal raphe nucleus includes genes involved both in cell-to-cell signaling and interaction as well as in nervous system development functions. Genes differentially expressed in the two comparisons in the same direction are represented as nodes depicted in red (up-regulated) or green (down-regulated) and biological relationship between two nodes is represented with a solid or dashed line (indicating direct or indirect interaction, respectively). Each node is displayed with different shapes that indicate the functional class of the gene product shown on the right. Modulatory effects on expression are indicated by arrows.

Supplementary Figure 1: Differentially expressed genes in four brain regions in mice that self-administer MDMA (contingent MDMA), mice that receive the drug passively (yoked MDMA) and mice receiving a saline solution (yoked saline), identified through transcriptomic microarray analysis. (a) Venn Diagrams of the microarray data showing statistically significant genes (FDR < 5%) differentially expressed between contingent MDMA-yoked MDMA (con-non con), contingent MDMA-yoked saline (con-sal) and yoked MDMA-yoked saline (non con-sal). (b) Volcanoplots of the contingent MDMA-yoked MDMA comparison showing the significance (logOdds) and the Log Fold Change. Significance threshold (FDR < 5%) is represented by a horizontal line.

Supplementary Figure 2: Gene network graphical representation of interaction between differentially expressed genes after MDMA exposure (contingent MDMA and yoked MDMA versus yoked saline mice). (a) Infection mechanism and infection disease gene network affected in ventral striatum; (b) Inflammatory response and immunological disease gene network affected in frontal cortex; (c) Molecular transport, cell death and cell cycle gene network affected in dorsal raphe nucleus; (d) Antimicrobial response and inflammatory response gene network affected in hippocampus. Genes differentially expressed in both comparisons in the same direction are represented as nodes depicted in red (up-regulated) or green (down-regulated) and biological relationship between two nodes is represented with a solid or dashed line (indicating direct or indirect interaction respectively). Each node is displayed in different shapes corresponding to the functional class of the gene product showed in the legend. Modulatory effects on expression are indicated by arrows.