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

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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 mice divided in contingent MDMA, yoked MDMA and yoked saline groups, and several 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, but others being involved in neuroadaptation. 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 neuroadaptative changes and synapse function, which may be related to learning self-administration behaviour, could be validated in these two brain structures. 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 and hippocampus 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 a 'feeling' of 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 contitioning (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 ± 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 µl 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 ºC 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 -80ºC 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 expression changes in genes selected on the basis of their function, 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 only exception was hippocampus in the comparison of contingent versus yoked MDMA mice, where the validations were performed in samples from separate subjects (9 animals instead of three pools of three animals) in order to increase the chances of validation of small gene expression changes. This brain structure is large enough to apply this approach. The Mouse Endogenous Control Array (Applied Biosystems. Foster city, CA, USA) was used to select endogenous controls. Real Time-PCR experiments were performed for 22 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 microrray 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%. To evaluate drug reinforced learning, FDR threshold was initially set at 5%, which allowed identification of differentially expressed genes that showed low fold changes (absolute average fold change = 1.34), in contrast with the figures obtained in genes that were differentially expressed as a consequence of the direct effect of the drug (absolute average fold change = 2.51). Table S1 shows the proportion of differentially expressed genes identified across each range of fold change. Thus, we increased the FDR threshold up to

15% in the comparisons of contingent MDMA-yoked MDMA and contingent MDMA-yoked saline, identifying changes in another brain structure, dorsal raphe nucleus and obtaining a significant increase in the average fold change in this structure (1.66). 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) considering GO biological processes (NFAT) 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). 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 (score = -log (Fisher exact test probability)).

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/opossum) (Ho Sui *et al.*, 2005), using the default parameters of the vertebrate Jaspar Core profile, and sorting the top 20 results by Z-score.

KEGG pathways enrichment analyses as well as Gene Ontology (GO), Cytogenetic band, and microRNA targets enrichment analyses were performed using the WebGESTALT software (bioinfo.vanderbilt.edu/webgestalt).

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 $p < 0.05$.

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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 Fig. 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 S2).

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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 both 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

In this part of the study we focused on genes that were differentially expressed as a consequence of the MDMA effect. We considered only those genes displaying changes in the same direction in the two comparisons: yoked MDMA versus yoked saline and contingent MDMA versus yoked saline (Fig. 2a-I). Significant differences were observed in both comparisons in the four brain structures studied, ranging from 16 in dorsal raphe nucleus to 183 in hippocampus, most of them upregulated in contingent and yoked MDMA mice (Fig. 2a-II; Supplementary Fig. 1a; Tables S3-S6). Those genes identified in the two comparisons were analyzed for functional group over-representation using the DAVID database (except for 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. 2a-III). The pathway "Complement and coagulation cascades" was found to be enriched in the KEGG pathway analysis in frontal cortex, hippocampus and ventral striatum. Also, the pathways "B cell receptor signaling" and "Natural killer cell mediated cytotoxicity" were identified in frontal cortex and hippocampus, respectively.

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For the genes that were differentially expressed in the two comparisons we obtained gene networks of inflammatory and immune response as the best scored in all four brain regions (Supplementary Fig. 2). Interestingly, the NF-kappaB complex was a central node in three of the four identified networks. Remarkably, these gene networks comprise several of the upregulated genes in our microarray experiments, such as *Lcn2* (lipocalin 2), with a dramatic upregulation in all regions, and *Tgtp* (T-cell specific GTPase) (frontal cortex and hippocampus; Supplementary Fig. 2b and 2d, respectively).

As most of the genes were upregulated, we performed an analysis of over-representation of evolutionarily conserved transcription factor binding sites (TFBS) in upregulated genes in every structure, and the results pointed at a possible common regulation mechanism for all brain regions by the REL transcription factor class, specifically NF-kappaB and Rela transcription factors (Table 1a). Since these two transcription factors can form homodimeric or heterodimeric complexes (containing the two proteins or combinations with other NF-kappaB complex proteins), we investigated if the target sequences contained predicted binding sites for both and observed that NF-kappaB binding sites are also predicted in most of Rela targets (79.5%). Remarkably, separate KEGG analysis of these NF-kappaB and Rela targets identified two enriched pathways in common for the two transcription factors: "Jak-STAT signaling" and "Chemokine signaling".

Finally, we performed cytogenetic bands enrichment analysis in all differentially expresed genes to identify clusters of co-regulated genes, and observed two interesting regions in common between hippocampus and frontal cortex: chromosome 11 B1 (ID:10742) and chromosome 17 B1 (ID:9061), including genes encoding interferon gamma induced GTPases and histocompatibility molecules (class I MHC) respectively, that were confirmed only in these two brain regions in the GO enrichment analysis (data

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not shown). Finally, microRNA target enrichment analysis did not show significant results in upregulated genes in all structures (data not shown).

Based on their function, we chose 10 genes among those that were differentially expressed in the two comparisons (contingent MDMA-yoked saline and yoked MDMA-yoked saline), 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 2). 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 2).

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Contingent MDMA self administration versus yoked MDMA: drug reinforced learning

In the second part of the study we focused on genes displaying differential expression as a consequence of active MDMA administation. We considered only those genes with changes in the same direction in the two comparisons that were performed: Contingent MDMA versus yoked MDMA and contingent MDMA versus saline (Fig. 2b-I). Differences in gene expression were observed only in hippocampus (537 genes), with low fold changes (average fold change = 1.34). For a fair comparison, we changed the significance threshold to a less restrictive value (see Materials and Methods) and observed differential expression of a number of additional genes in hippocampus (645, most of them downregulated in contingent mice) and also in dorsal raphe nucleus (61, most of them upregulated in contingent mice) (Fig. 2b-II, Tables S7 and S8). No

differences were observed in frontal cortex or ventral striatum. In hippocampus, functional group over-representation performed using the DAVID database targeted some interesting neurological functions, such as synaptic transmission, regulation of synaptic plasticity, axononogenesis, or learning and memory (Fig. 2b-III). In contrast, no specific neurological functions were identified in dorsal raphe nucleus in this step. Interestingly, KEGG pathway enrichment analyses revealed three altered pathways in hippocampus and dorsal raphe nucleus: "Long-term potentiation", "MAPK signaling" and "Wnt signaling", in which most of the genes were also downregulated in the contingent mice in hippocampus (Fig. 3).

Gene network construction on all these genes revealed that the best scored network in hippocampus (score = 47; Fig. 4a) involves cell-to-cell signalling and interaction/nervous system development and function. Remarkably, most of the genes present in this network are differentially expressed in hippocampus of contingent mice. This network includes genes involved in synaptic vesicle fusion, synapsis formation and neurotransmitter release, such as *Cplx2*, *Vamp2*, *Ngln2*, *Nrxn1* and *Nrxn2*, consistenly with the over-represented GO categories identified. Interestingly, the best scored network in dorsal raphe nucleus (score = 26; Fig. 4b) also includes genes involved in cell-to-cell signalling, interaction/nervous system development and function and behavior, such as *Camk2a* and *Kalrn*.

Analysis of over-representation of TFBS in hippocampus and dorsal raphe predicted a possible common regulatory mechanism in upregulated genes, but not in the downregulated ones (Table 1b). Upregulation in dorsal raphe nucleus and hippocampus could be related to the action of the Nkx2-5 transcription factor, which is the best scored prediction in both structures. The subset of genes predicted to be regulated by Nkx2-5 showed enrichment of long-term potentiation in both hippocampus

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and dorsal raphe nucleus in the KEGG pathways analysis. Downregulation of hippocampal genes could be mediated mainly by the Mzf1_1-4 transcription factor (Table 1b). Cytogenetic bands enrichment analyses did not show any significantly enriched region (data not shown). MicroRNA target enrichment analysis performed in the two brain structures identified, among others, miR-96, with predicted targets in 21 genes downregulated in hippocampus (adjusted p-value = 4.3e-06), all of them showing interesting functions such as cell communication and synapse organization in the GO enrichment analyses.

Based on their function, we selected 8 genes in hippocampus (*Amigo1*, *Bzrap1*, *Gprin1*, *Mapk8ip1*, *Nlgn2*, *Vgf*, *Madd* and *Axin2*) and 4 genes in dorsal raphe nucleus (*Camk2a*, *Kalrn*, *Ddn* and *Egr3*) for further validation. QRT-PCR results confirmed a downregulation of the *Bzrap1*, *Nlgn2* and *Axin2* genes in the contingent mice in hippocampus, and an upregulation of the *Camk2a*, *Kalrn*, *Ddn* and *Egr3* genes in the contingent mice in dorsal raphe nucleus (Table 3). *Camk2a* is present in the "Long-term potentiation pathway" in dorsal raphe nucleus. It is also present in the "Wnt signaling pathway" in this structure, like *Axin2* in hippocampus. *Nlgn2* gene, together with *Camk2a* and *Kalrn* are present in hippocampus and dorsal raphe nucleus gene networks, respectively (Fig. 4) The genes whose upregulated expression was validated by qRT-PCR in dorsal raphe nucleus (*Camk2a*, *Kalrn*, *Ddn* and *Egr3*) have predicted binding sites for Nkx2-5.

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Deleted: We then focused our attention on genes displaying larger differences in gene expression. For that purpose, 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 altered gene expression resulting in 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).

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 neuroadaptative 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 hippocampus and the dorsal raphe nucleus may participate in the neuroadaptative 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.

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). From our analyses we could hypothesize that the expression of many of these genes is regulated by NF-kappaB and Rela, which is also supported by *in silico* networks pointing at the NF-kappaB complex as a central node. This finding is consistent with previous studies showing that MDMA may induce the activation of NF-kappaB (Montiel-Duarte *et al*., 2004; Orio *et al*., 2010; Tiangco *et al*., 2005). However, the expression of neither NF-kappaB nor Rela was altered in our animal model according to microarray data. Our results obtained from repeated MDMA administration differ from previous data obtained after acute MDMA exposure in murine models, where mainly serotonin receptors, several transcription factors, cytoskeletal, cell adhesion and metabolic genes were differentially expressed in cortical areas or in the striatum (Marie-Claire *et al.*, 2007; Thiriet *et al*., 2002).

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 serotoninergic neurons. Both have been described to be involved in memory consolidation in hippocampus (Von Hertzen & Giese, 2005) and regulate glutamatergic

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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 serotoninergic 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). Although not validated, enriched cytogenetic bands and GO analysis also pinpointed class I major histocompatibility complex (MHC) genes in hippocampus and frontal cortex, which, besides from their involvement in immune response, have been related to activity-dependent remodeling and plasticity of connections in the CNS, playing an important role in long-term potentiation (LTP) and long-term depression (LTD) (Huh *et al.*, 2000).

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 was observed in hippocampus, supporting the

crucial role of this brain structure 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). In this regard, several genes differentially expressed in hippocampus are involved in long-term potentiation, as well as in other important pathways for learning and memory processes, which are also altered in hippocampus of rats with cocaine-induced CPP (Krasnova *et al.*, 2008). Surprisingly, several additional genes showed differential expression also in dorsal raphe nucleus in our study, some of them also related to these pathways. Interestingly, recent evidence suggests that dorsal raphe nucleus is involved in encoding reward-related aspects of motivated behaviour (Bromberg-Martin *et al.*, 2010, Nakamura *et al.*, 2008). Also, concerning active MDMA self-administration, we observed more similarities between hippocampus and dorsal raphe nucleus in our study: i) We identified common gene networks involved in cell-to-cell signaling and nervous system development functions in the two structures and ii) analysis of over-representation of TFBS pointed at Nkx2-5 as a common modulator of genes that are upregulated in active MDMA self-administration in both brain regions. However, microarray data did not show altered expression of Nkx2-5. This transcription factor has been described to be involved in neuronal differentiation (Riazi *et al.*, 2005).

Most genes showing differential expression in hippocampus, in contrast with dorsal raphe nucleus, are downregulated in contingent mice. Such downregulation may be related to the effect of transcription factor Mzf1_1-4, which has predicted binding sites in >75% of the genes that are downregulated in this brain structure (Table 1b). However, the expression of this transcription factor is not altered according to microarray data. Also, several downregulated genes in hippocampus are predicted

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targets for the microRNA miR-96, which is expressed in frontal cortex and seems to be involved in modulating gene expression in several neuronal processes such as long-term potentiation and depression (Juhila *et a*l., 2011).

Seven out of 12 genes could be further validated by qRT-PCR, three in hippocampus (*Bzrap1*, *Nlgn2* and *Axin2*) and four in dorsal raphe nucleus (*Camk2a*, *Kalrn*, *Ddn* and *Egr3*). The three genes validated by qRT-PCR in hippocampus are underexpressed in contingent mice and modulate synapse and neurogenesis. The benzodiazapine receptor associated protein 1 (encoded by *Bzrap1)* is an adaptor molecule thought to regulate synaptic transmission by linking vesicular release machinery to voltage gated Ca2+ channels (Wang *et al*., 2000). The *Nlgn2* gene encodes neuroligin 2, which has an important role in organizing the functional properties of synapses, acting preferentially at inhibitory synapses (Gibson *et al*., 2009). Other genes encoding proteins involved in neurotransmission have also been described to be altered by cocaine self-administration in rats (Ahmed *et al*., 2005). Axin2 is a negative regulator of the Wnt signalling pathway, signalling that induces neurogenesis in hippocampal neurons (Jho *et al*., 2002; Lie *et al*., 2005).

The four genes validated by qRT-PCR in dorsal raphe nucleus are overexpressed in contingent mice and are related to synaptic plasticity and neuroadaptations. *Camk2a* encodes the Ca2+/calmodulin-dependent protein kinase II alpha (CaMKIIalpha), which mediates activity-dependent synaptic plasticity and has an essential role in dendritic spine enlargement, LTP 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). 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. 4b). 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).

In our experimental conditions, qRT-PCR validation succeeded in 76% of the cases (considering all the brain structures and comparisons tested for each gene: 47 validated/62 tested). The limited number of replicas in our study may have prevented some validations, specially when differences in gene expression were low in the microarray (see Table S1).

Our results may suggest a role for dorsal raphe nucleus and hippocampus in the motivational and learning processes needed to actively self-administer MDMA since they are the only studied brain regions that show statistically significant changes in the comparison of contingent MDMA versus yoked MDMA mice. In addition, the best scored gene network identified common functions in dorsal raphe nucleus 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, and validated genes downregulated in hippocampus are involved in synapse function and neurogenesis.

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 hippocampus and dorsal raphe nucleus following MDMA self-administration suggest that both brain regions 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 of contingent MDMA, yoked-MDMA and yoked-saline, further studies should be performed to confirm these results.

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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: Gene expression changes caused by the direct effect of MDMA (a) or to MDMA-reinforced learning (b). I) VennDiagrams, with the group of differentially expressed genes considered in each study shadowed in grey. On the left, expression changes in common in the comparisons of contingent MDMA-yoked saline and yoked MDMA-yoked saline. On the right, expression changes that are observed in the comparisons of contingent MDMA-yoked MDMA and contingent MDMA-yoked saline but not in yoked MDMA-yoked saline. CON: contingent. II) Total number of genes that are upregulated or downregulated in the different comparisions performed. III) Selection of over-represented biological categories that include differentially expressed genes. The number of positive genes included in each category is indicated on the right side of each bar. Biological categories correspond to Gene Ontology (GO) terms.

Deleted: 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).

Deleted: 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: Selection of altered KEGG pathways in hippocampus after MDMA-reinforced learning. Heatmap showing the level of expression in the microarray of genes identified in two KEGG pathways. For each experimental group the three replicates are shown.

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Figure 4: 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 (upregulated) or green (downregulated) 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 rigth. Modulatory effects on expression are indicated by arrows. Gene expression changes validated by qRT-PCR are indicated by blue arrows.

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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 (log Odds) 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. The NF-kappaB central node is indicated in each network by an orange arrow. Gene expression changes validated by qRT-PCR are indicated by blue arrows.

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). We selected, from those neurological functions listed above, 18 genes for further validation (*Amigo1*, *Bzrap1*, *Gprin1*, *Mapk8ip1*, *Nlgn2*, *Vgf*, *Madd* and *Axin2*) and qRT-PCR results confirmed a downregulation of three genes in the contingent mice: *Bzrap1*, *Nlgn2* and *Axin2* (Table 2). We then focused our attention on genes displaying larger differences in gene expression. For that purpose, 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 altered gene expression resulting in 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).

Table 1: Over-representation of transcription factor binding sites. a) Predicted targets for transcription factors in common in all four brain regions in upregulated genes due to the direct effect of MDMA. b) Best predicted targets for transcription factors in upregulated and downregulated genes in hippocampus and dorsal raphe nucleus due to MDMA-reinforced learning in contingent mice.

a) MDMA direct effect

	Transcription factor	Target genes	Z-score	p-value
Ventral striatum	NF-kappaB	33	10.9	1.1e-27
	Rela	25	9.9	4.1e-23
Frontal cortex	NF-kappaB	28	11.0	3.8e-28
	Rela	25	11.3	1.3e-29
Dorsal raphe nucleus	NF-kappaB	5	3.1	1.9e-03
	Rela	3	0.3	0.76
Hippocampus	NF-kappaB	32	5.0	5.7e-07
	Rela	30	9.8	1.1e-22

b) MDMA-reinforced learning

	Upregulated				Downregulated			
	Transcription factor	Target genes	Z-score	p-value	Transcription factor	Target genes	Z-score	p-value
Dorsal raphe nucleus	Nkx2-5	31	16.8	2.4e-63	Nhlh1	4	19.5	1.1e-84
Hippocampus	Nkx2-5	57	29.7	7.7e-194	Mzf1_1-4	413	76.5	0

Table 2: Direct pharmacological effect of MDMA: qRT-PCR validation of microarray data of 10 genes

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			Contingent-MDMA vs Yoked-Saline			Yoked-MDMA vs Yoked-Saline		
			Microarray		qRT-PCR	Microarray		qRT-PCR
		Ave Expr [a]	Fold Change	P-value (Adj P-value)	Fold Change	Fold Change	P-value (Adj P-value)	Fold Change
Frontal Cortex								
Lcn2	Lipocalin 2	8.8	**52.8**	5.5 e-8 (3e-4)	**55.6** [b]	**55.7**	4.7e-8 (2.5e-4)	**65.9** [b]
Ctla2a [c]	Cytotoxic T lymphocyte-associated protein 2 alpha	8.8	**3.0**	8.5 e-4 (0.04)	**4.8** [b]	**4.9**	3.7e-5 (8e-3)	**7.3** [b]
Gbp2 [c]	Guanylate binding protein 2	8.7	**8.3**	2.5 e-4 (0.03)	**11.3** [b]	**7.7**	3.3e-4 (0.01)	**12.9** [b]
Igtp	Interferon gamma Induced GTPase	8.4	**4.2**	3.7 e-4 (0.04)	**6.1** [b]	**4.0**	5e-4 (0.02)	**5.3** [b]
Iigp1 [c]	Interferon inducible GTPase 1	7.1	**5.5**	2.1 e-4 (0.03)	**7.9** [b]	**6.4**	1e-4 (0.01)	**9.4** [b]
Iigp2	Interferon inducible GTPase 2	7.4	**3.2**	1.9 e-4 (0.03)	**3.7** [b]	**3.0**	3e-4 (0.02)	**3.0** [b]
Tgtp	T-cell specific GTPase	9.2	**5.8**	1.2 e-4 (0.03)	**7.3** [b]	**6.6**	6.7e-5 (9.2e-3)	**8.1** [b]
Hippocampus								
Lcn2	Lipocalin 2	8.5	**25.0**	1.7e-6 (4e-3)	**33.5** [b]	**36.4**	7e-7 (1.6e-3)	**49.6** [b]
Ctla2a [c]	Cytotoxic T lymphocyte-associated protein 2 alpha	8.2	**3.0**	1.5e-3 (0.01)	**3.7** [b]	**5.2**	9.6e-5 (0.01)	**8.0** [b]
Gbp2 [c]	Guanylate binding protein 2	8.0	**7.5**	4e-3 (0.02)	**12.0** [b]	**6.9**	5e-3 (0.03)	**13.4** [b]
Igtp	Interferon gamma Induced GTPase	8.5	**4.0**	4.8e-3 (0.02)	**7.0** [b]	**4.1**	4.3e-3 (0.03)	**6.8** [b]
Iigp1 [c]	Interferon inducible GTPase 1	7.0	**3.9**	0.01 (0.04)	**5.4** [b]	**4.7**	6e-3 (0.04)	**7.2** [b]
Iigp2	Interferon inducible GTPase 2	7.3	**2.9**	3.7e-3 (0.02)	**NS**	**2.7**	4.9e-3 (0.03)	**NS**
Tgtp	T-cell specific GTPase	9.2	**4.4**	1.6e-3 (0.01)	**5.0** [b]	**4.7**	1.1e-3 (0.02)	**5.5** [b]
Dorsal Raphe Nucleus								
Lcn2	Lipocalin 2	8.7	**26.1**	2.7e-6 (8 e-3)	**37.7** [a]	**52.4**	5e-7 (7 e-4)	**59.3** [a]
Sgk1	Serum/glucocorticoid regulated kinase 1	10.8	**3.1**	1.3e-5 (0.01)	**2.8** [a]	**4.3**	1.6e-6 (1.4 e-3)	**4.1** [a]
Sgk3 [c]	Serum/glucocorticoid regulated kinase 3	8.6	**2.1**	0.01 (0.09)	**NS**	**4.2**	5.3e-4 (9 e -3)	**2.8** [a]
Ventral Striatum								
Lcn2	Lipocalin 2	8.1	**32.8**	1.6e-7 (3.8 e-4)	**37.7** [a]	**41.3**	9.5e-8 (2.2 e-4)	**52.9** [a]
Slc17a7	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter)	8.1	**3.6**	6.9e-4 (0.04)	**2.7** [a]	**3.6**	7.2e-4 (7.4 e-3)	**2.9** [a]

[a] Ave Expr: Average log2-expression for the gene probe over all arrays and channels.
[b] p-value < 0.05; normalized to *Actb*
[c] Genes showing significant differential expression in two independent probe sets. The smallest absolute fold change is shown.
NS, not significant

Table 3: Effect of active MDMA self-administration: qRT-PCR validation of microarray data of three genes in hippocampus and four genes in dorsal raphe nucleus

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Deleted: four genes showing a Log Fold Change of ±1.5

			Contingent-MDMA vs Yoked-MDMA			Contingent-MDMA vs Yoked-Saline		
			Microarray		qRT-PCR	Microarray		qRT-PCR
Gene Symbol	Gene name	Ave Expr [a]	Fold Change	P-value (Adj P value)	Fold Change	Fold Change	P-value (Adj P value)	Fold Change
Hippocampus								
Bzrap1 [c]	Benzodiazepine receptor associated protein 1	10.3	**-1.4**	1e-4 (4e-3)	**-1.6** [b]	**-1.2**	0.01 (0.04)	**-1.3** [b]
Nlgn2	Neuroligin 2	9.9	**-1.3**	6e-4 (6e-3)	**-1.3** [b]	**-1.2**	3e-3 (0.02)	**-1.4** [b]
Axin2	Axin 2	7.4	**-1.3**	2e-3 (9e-3)	**-1.2** [b]	**-1.3**	6e-4 (0.01)	**-1.3** [b]
Dorsal Raphe Nucleus								
Camk2a	Calcium/calmodulin-dependent protein kinase II alpha	7.1	**2.8**	0.01 (0.11)	**2.3** [b]	**1.9**	0.02 (0.12)	**NS**
Kalrn	Kalirin, Rho GEF kinase	7.8	**2.9**	4e-3 (0.10)	**1.5** [b]	**3.2**	2.3e-3 (0.06)	**NS**
Ddn	Dendrin	7.1	**3.5**	8e-3 (0.11)	**5.5** [b]	**3.3**	0.01 (0.09)	**5.3** [b]
Egr3	Early growth response 3	5.4	**4.0**	5e-4 (0.09)	**3.0** [b]	**3.3**	1.5e-3 (0.06)	**2.4** [b]

[a] Ave Expr: Average log2-expression for the gene probe over all arrays and channels.
[b] p<0.05; normalized to *Actb*
[c] Genes showing significant differential expression in two independent probe sets. The smallest absolute fold change is shown.
NS, not significant.

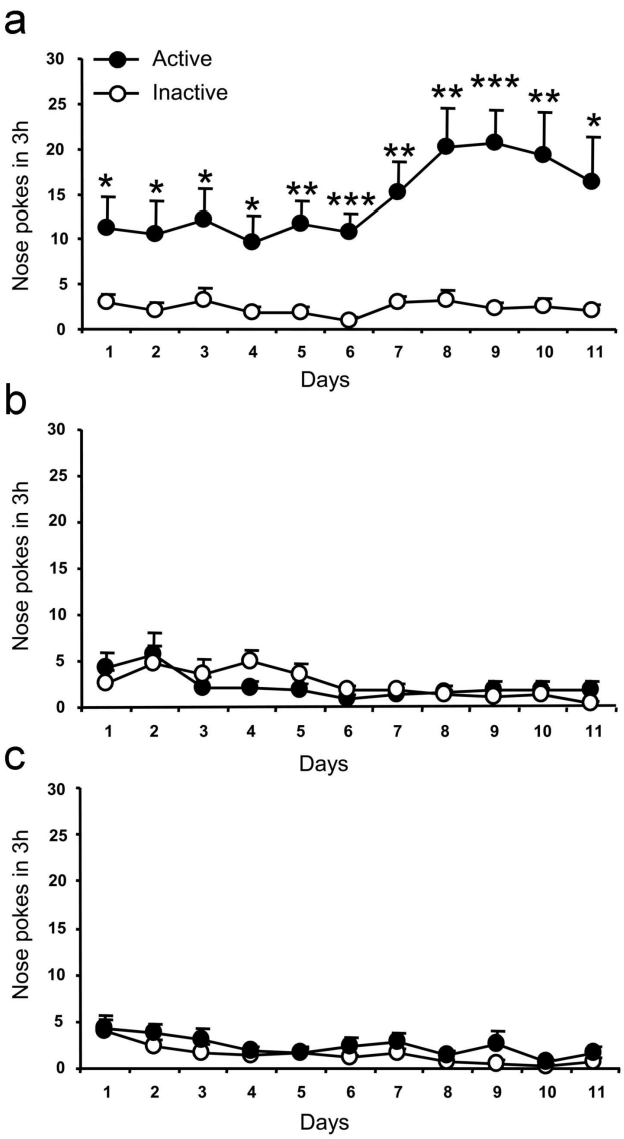

Figure 1: Operant yoked-control responding for intravenous infusions of MDMA (0.125 mg/kg/infusion). (a) The contingent group received an infusion of MDMA with every active nose-poke (n = 9), (b) the yoked MDMA group received an MDMA infusion everytime the contingent mouse made an active nose-poke (n = 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).
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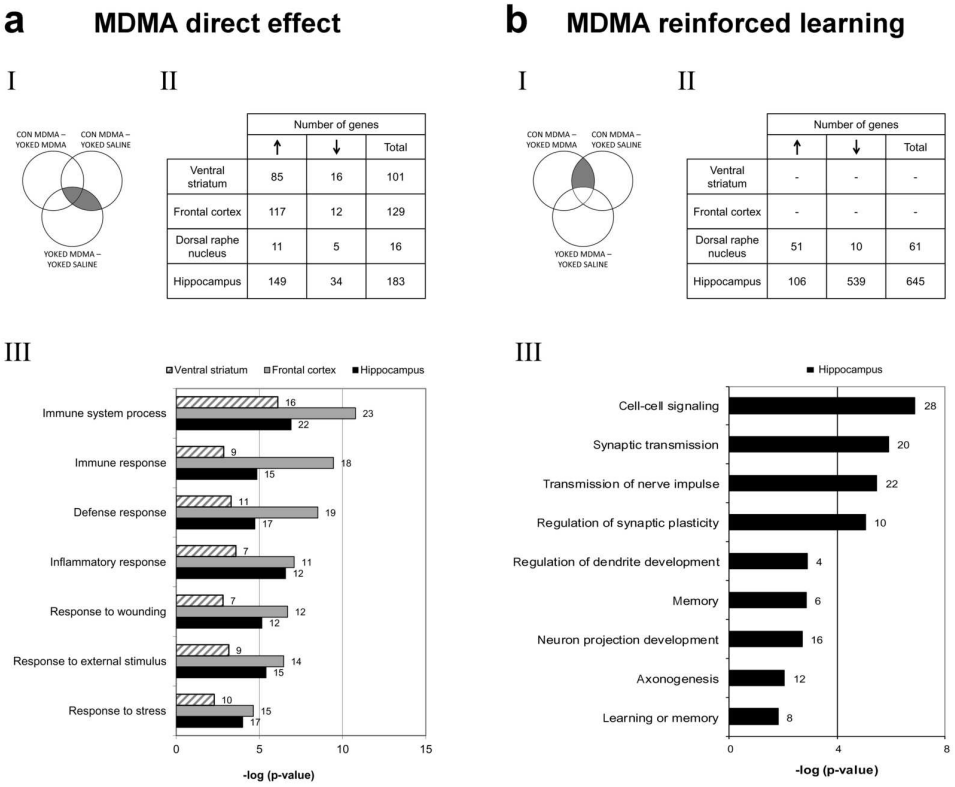

Figure 2: Gene expression changes caused by the direct effect of MDMA (a) or to MDMA-reinforced learning (b). I) VennDiagrams, with the group of differentially expressed genes considered in each study shadowed in grey. On the left, expression changes in common in the comparisons of contingent MDMA-yoked saline and yoked MDMA-yoked saline. On the right, expression changes that are observed in the comparisons of contingent MDMA-yoked MDMA and contingent MDMA-yoked saline but not in yoked MDMA-yoked saline. CON: contingent. II) Total number of genes that are upregulated or downregulated in the different comparisions performed. III) Selection of over-represented biological categories that include differentially expressed genes. The number of positive genes included in each category is indicated on the right side of each bar. Biological categories correspond to Gene Ontology (GO) terms.
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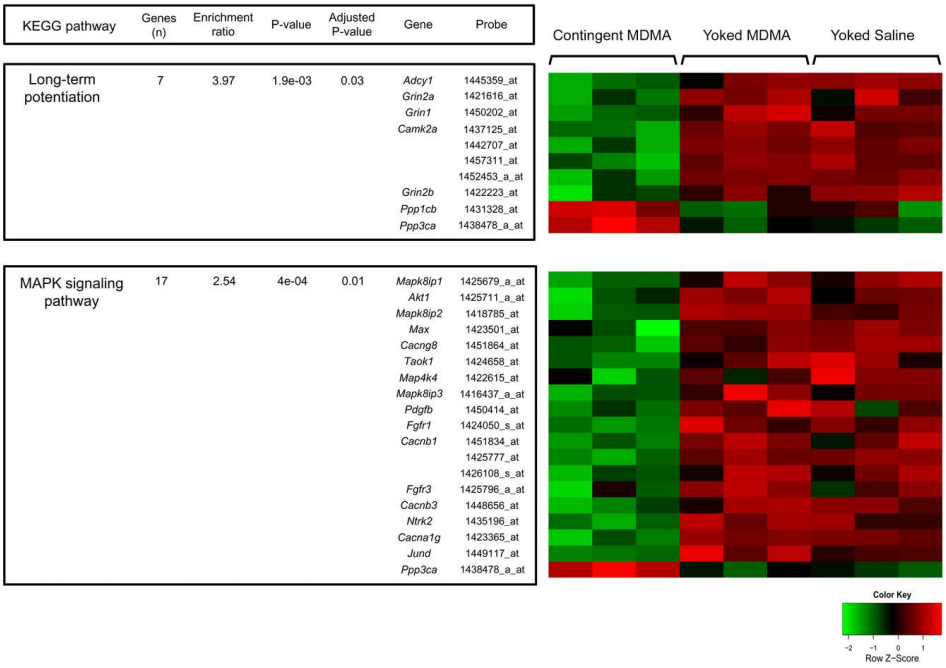

Figure 3: Selection of altered KEGG pathways in hippocampus after MDMA-reinforced learning. Heatmap showing the level of expression in the microarray of genes identified in two KEGG pathways. For each experimental group the three replicates are shown.
119x84mm (300 x 300 DPI)

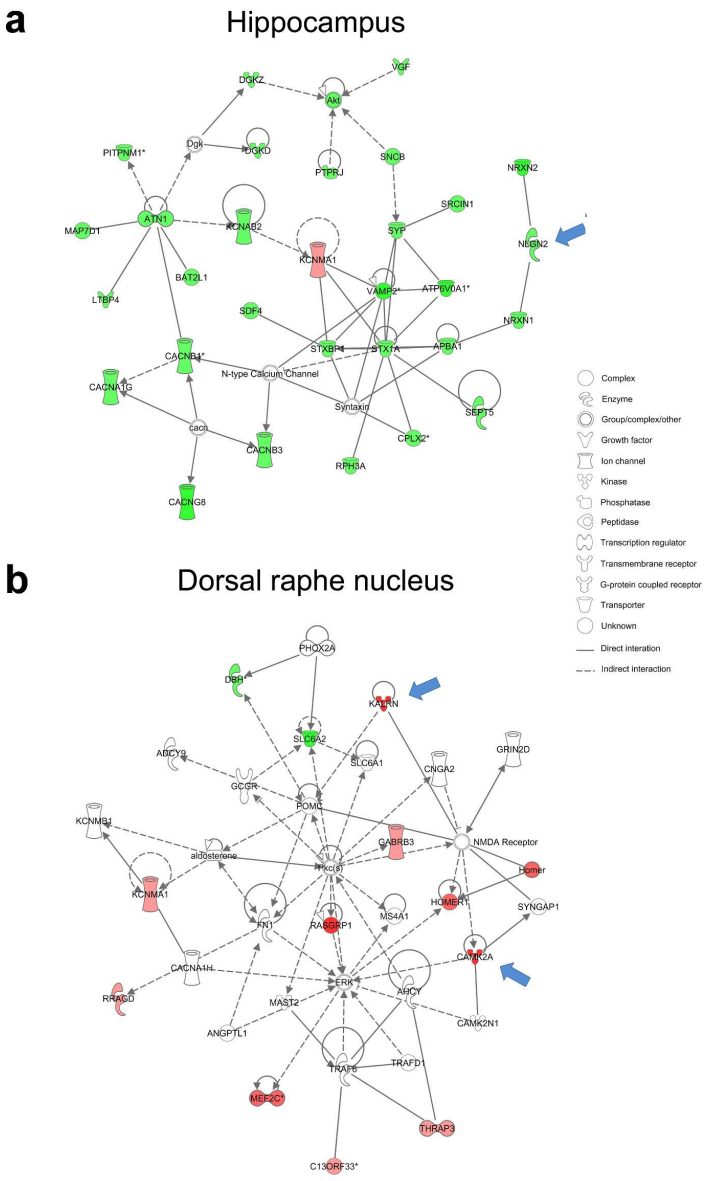

Figure 4: Gene network graphical representation of interaction between differentially expressed genes after MDMA reinforced learning (contingent MDMA versus yoked MDMA and yoked saline mice). The best scored gene network in (a) hippocampus and in (b) dorsal raphe nucleus includes genes involved both 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 (upregulated) or green (downregulated) 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 rigth. Modulatory effects on expression are indicated by arrows. Gene expression changes validated by qRT-PCR are indicated by blue arrows.
260x397mm (300 x 300 DPI)