Chapter 2 | Hippocampal CA1 region shows differential regulation of gene expression in mice displaying extremes in behavioral sensitization to amphetamine: relevance for psychosis susceptibility?

ABSTRACT

Psychosis susceptibility is mediated in part by the dopaminergic neurotransmitter system. In humans there are individual differences in vulnerability for psychosis which are reflected in differential sensitivity for psychostimulants such as amphetamine. We hypothesize that the same genes and pathways underlying behavioral sensitization in mice are also involved in the vulnerability to psychosis. The aim of the current study was to investigate which genes and pathways may contribute to behavioral sensitization in different dopaminergic output areas in the mouse brain. We took advantage of the naturally occurring difference in psychostimulant sensitivity in DBA/2 mice and selected animals displaying extremes in behavioral sensitization to amphetamine. Subsequently, the dopamine output areas prefrontal cortex (PFC), nucleus accumbens (NAc) and the cornu ammonis 1 (CA1) area of the hippocampus were isolated by laser microdissection and subjected to DNA microarray analysis 1 hour after a challenge dose of amphetamine. A large number of genes with differential expression between high and low responders were identified, with no overlap between brain regions. Validation of these gene expression changes with quantitative RT-PCR demonstrated that the most robust and reproducible effects on gene expression were in the CA1 region of the hippocampus. Interestingly, many of the validated genes in CA1 are members of the CRE-family and appeared to be targets of the glucocorticoid receptor (GR) and myocyte enhancer 2 (Mef2) transcription factors. We hypothesize that CRE, Mef2 and GR signaling form a transcription regulating network, which underlies differential amphetamine sensitivity and therefore may play an important role in susceptibility to psychosis.

INTRODUCTION

Psychosis is characterized by a gradual loss of contact with reality, progressing from emotional instability, acoustic and visual disturbances, decreased discriminative ability for real and surreal ideas and memories to more pronounced symptoms like hallucinations, delusions and thought disorders. Psychotic-like symptoms can be induced by psychostimulant drugs like amphetamine (Janowsky and Risch, 1979). Patients with a high susceptibility for psychosis, such as schizophrenia patients, display an increased sensitivity to amphetamine (Strakowski *et al*, 1997), that resembles the behavioral sensitization found in rodents after repeated exposure to amphetamine (Alessi *et al*, 2003; Peleg-Raibstein *et al*, 2008; Peleg-Raibstein *et al*, 2006; Tenn *et al*, 2003). This behavioral sensitization is characterized by a progressive and persisting increase in the behavioral activity and neurochemical responses to psychostimulants, such as stimulation of locomotor activity, stereotypy and dopamine release in the striatum (Featherstone *et al*, 2007; Laruelle and Abi-Dargham, 1999; Morrens *et al*, 2006). Moreover, the number of dopamine (DA) D2 receptors in the high-affinity conformational state is altered in the striatum whereas the total expression of DA D2 receptors is not changed in both sensitized animals and schizophrenia patients (Seeman *et al*, 2007; Seeman *et al*, 2005). Substantial interindividual differences exist in susceptibility to develop psychosis as well as in sensitivity to amphetamine (Alessi *et al*, 2003). It has been hypothesized that individuals that are more sensitive to amphetamine are also more susceptible to become psychotic (Post, 1992; Segal *et al*, 1981). Based on these similarities, the amphetamine-sensitization model can be considered a promising animal model to study several aspects of schizophrenia (Featherstone *et al*, 2007).

Persistent neuroplastic alterations in the reward circuitry, in particular in the mesolimbic dopamine pathway, are associated with the expression of behavioral sensitization (Nestler, 2005a). The mesolimbic dopaminergic pathway originates in the ventral tegmental area (VTA) and projects to the nucleus accumbens (NAc), amygdala, prefrontal cortex (PFC) and other forebrain regions including the cornu ammonis 1 (CA1) subregion of the hippocampus (Floresco *et al*, 2001; Gasbarri *et al*, 1994; Thierry *et al*, 2000). Induction and expression of behavioral sensitization to psychostimulants is a complex process in which various neurotransmitters, in particular dopamine and glutamate, result in downstream molecular adaptations in the VTA-NAc circuitry and other limbic brain regions. In the VTA enhanced glutamatergic neurotransmission results in a sensitized state resembling long-term potentiation (LTP). In the NAc, induction of the transcription factors ΔFosb and CREB appear to be common adaptations in response to chronic exposure to drugs of abuse, contributing to the sensitized state (McClung and Nestler, 2003; McClung *et al*, 2004; Nestler, 2005b; Shaw-Lutchman *et al*, 2003). In addition, the ERK pathway and cAMP-independent activation of Akt-GSK3 may also play a role in long-lasting behavioral sensitization (Beaulieu *et al*, 2007; Emamian *et al*, 2004; Valjent *et al*,

2006). However, still a lot remains unresolved regarding the molecular events that contribute to behavioral sensitization in different brain regions of the mesolimbic dopamine circuitry.

The aim of the current study was to investigate which genes and pathways may contribute to behavioral sensitization in different parts of the mesolimbic circuitry in the mouse brain. We hypothesize that the same genes and pathways underlying behavioral sensitization are also involved in the vulnerability to psychosis. To investigate these molecular pathways we took advantage of the naturally occurring variability in behavioral sensitization to amphetamine in DBA/2 mice, an inbred mouse line (de Jong *et al*, 2007), thus ruling out the influence of genetic differences. We developed a sensitization regimen that allowed us to separate mice in two distinct groups showing very high sensitization and no sensitization to amphetamine, respectively, despite the exact same amphetamine treatment. Large scale gene expression profiles were generated of several dopaminergic output brain regions, including the CA1 region of the hippocampus, the NAc and PFC, in mice selected for extremes in behavioral sensitization to amphetamine, in search of susceptibility genes and pathways underlying the differential behavioral sensitization.

MATERIALS &METHODS

Drugs D-amphetamine ((+)-a-methylphenethylamine sulfate; Unikem A/S, Copenhagen, Denmark) was dissolved in 0.9% sodium chloride. Doses are listed as salt equivalents (mg/kg).

Animals Animal experiments were in accordance with the guidelines issued by the Danish Animal Experimentation Inspectorate. DBA/2 mice (Charles River Laboratories, Salzfeld, Germany) were housed 4 mice per cage in a temperature and humidity controlled environment at a 12 hour light-dark cycle. During the experiment animals had ad libitum access to water and food. Mice were left undisturbed for 14 days prior to initiation of the experiments.

Amphetamine sensitization In experiment 1 mice were divided in four groups based on the treatment received during days 1-5 and on day 20 respectively: group 1 (amph/amph, n=100), group 2 (sal/sal, n=10), group 3 (sal/amph, n=10) and group 4 (amph/sal, n=10). Animals received either d-amphetamine (2.5 mg/kg) or saline for 5 consecutive days (days 1-5). After a 14 day withdrawal period, animals were given a low dose amphetamine challenge (1.25 mg/kg) or saline (day 20) (For a detailed scheme see Figure 1). At the drug challenge (day 20), locomotor behaviour was assessed as described below. Based on the locomotor response to the amphetamine challenge on day 20, the 10% amph/amph animals with the highest locomotor response were designated high responders (HR) (n=10), while the 10% animals with the lowest response were designated low responders (LR) (n=10). The high and low responders were used for subsequent gene expression analysis.

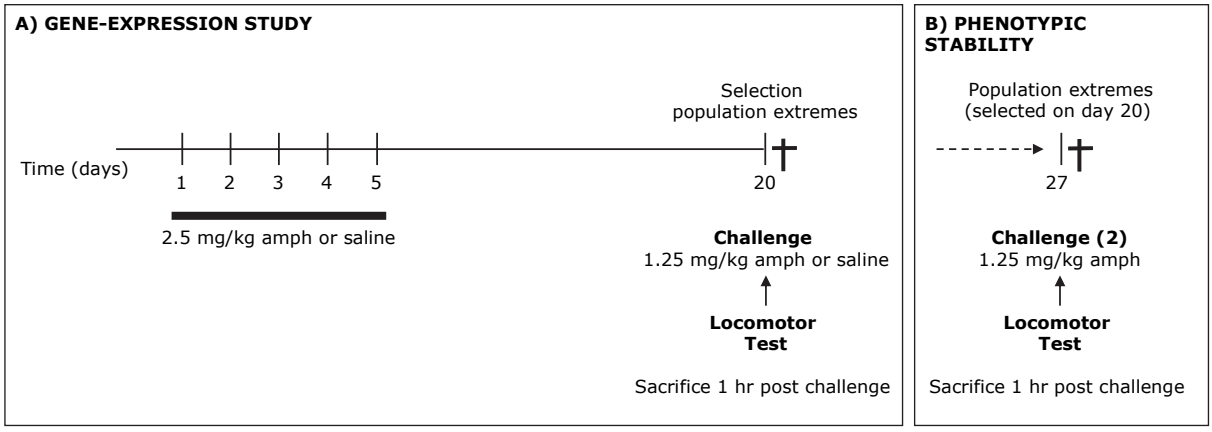

Figure 1A | Animals received either d-amphetamine (2.5 mg/kg) or saline for 5 consecutive days (days 1-5). After a 14-day withdrawal period (day 20) animals were given a low dose amphetamine challenge (1.25 mg/kg) or saline and the 10% population extremes in the AMPH/AMPH group (low and high responders) were selected. In the expression profiling study, mice were sacrificed 1 hour after the challenge on day 20 (experiment 1).B | In the follow-up study (experiment 2), the low and high responders received an additional amphetamine (1.25 mg/kg) challenge on day 27 and were sacrificed 1 hour later. Locomotor tests were performed on the indicated days.

In a follow-up experiment (experiment 2) it was investigated whether the HR and LR phenotype is stable. A new batch of animals was subjected to the same treatment and dosing regimen as in the

first study. The selected 10% HR and LR responders of the amph/amph group (n=10 each) on day 20 were subsequently left undisturbed for an additional 7 days and re-challenged with 1.25 mg/kg on day 27 and locomotor behavior was measured again (Figure 1). The HR and LR responders were used for revalidation of gene expression changes measured in experiment 1.

Locomotor behaviour Animals were placed individually in makrolon locomotor activity cages (20 cm × 35 cm × 18 cm) (Lundbeck). Following a 60 minute habituation period, amphetamine or vehicle was administered and locomotor activity was recorded for an additional 60 min. The locomotor activity cages were equipped with 5 × 8 infrared light sources plus photocells. The light beams crossed the cage 1.8 cm above the bottom of the cage. During the test session, locomotor activity was recorded as crossings of infrared light beams, and total locomotor count represents the accumulated number of crossings over the 60 minute period. The recording of a motility count required interruption of two adjacent light beams, thus avoiding counts induced by stationary movements of the mice. All experiments were conducted during the light phase of the cycle and initiated using a clean cage.

Tissue dissection Selected mice were sacrificed directly after the locomotor activity measurement on day 20 (experiment 1) and on day 27 (follow-up experiment 2). Brains were rapidly dissected and snap-frozen in isopentane (cooled in ethanol placed on pulverised dry ice) and stored at -80C for later use.

Brain amphetamine levels Amphetamine in total brain homogenates was measured in two groups (n=10 each) of mice with locomotor activity counts just below the highest and just above the lowest responders. Amphetamine levels were measured by liquid chromatography/tandem mass spectrometry (LC–MS/MS) to test whether differences in responsiveness could be accounted for by differences in brain drug exposure. Brain tissue was homogenated with four times its weight of Acetonitrile:water (70:30) using a TomtecAutogizer. The supernatant was analyzed like plasma. On line sample preparation and liquid chromatography were performed with turbulent flow chromatography (Cohesive Technologies, UK), using a dual column configuration. MS/MS detection was done with an Applied BiosystemsSciex API 3000 instrument in positive-ion electrospray ionization mode.

Laser microdissection Laser microdissection (LMD) was performed as previously described (Datson *et al*, 2004) on brain tissue from experiment 1. Briefly, coronal brain sections (8 µm) were cut using a cryostat (PALM, Bernried, Germany) at -18ºC. According to the Mouse Brain Atlas (Franklin, 1997)cryosections from CA1 area were collected starting at Bregma -1.58, NAc cryosections between Bregma +1.70 and +1.18 and PFC cryosections (Prelimbic and Infralimbic cortex) between Bregma +2.80 and +2.10. Both hemispheres were used for sectioning. Cryosections were thaw-mounted on

PEN-membrane slides (1440-1000, PALM, Bernried, Germany) which had been pretreated by heating for 4 hours at 180°C and subsequent UV irradiation for 30 min at 254 nm. After sectioning the slices were kept at -80ºC until further use. On the day of LMD, the slides were briefly stained with haematoxylin (10 %) and dehydrated in 70, 95 and 100% ethanol, briefly dipped in xylene and dried at 40ºC. Immediately afterwards, the slides were used for LMD and the laser microdissected tissue fragments were collected in adhesive caps (1440-0250 PALM, Bernried, Germany). A conservative estimate of CA1 was taken to avoid contamination with CA2/CA3. For NAc, an area containing both the core and shell was dissected. For PFC both prelimbic and medial orbital cortical regions were combined (Figure 2). Per mouse a total of 4 sections were dissected and pooled to constitute a sample for subsequent linear amplification and microarray hybridization.

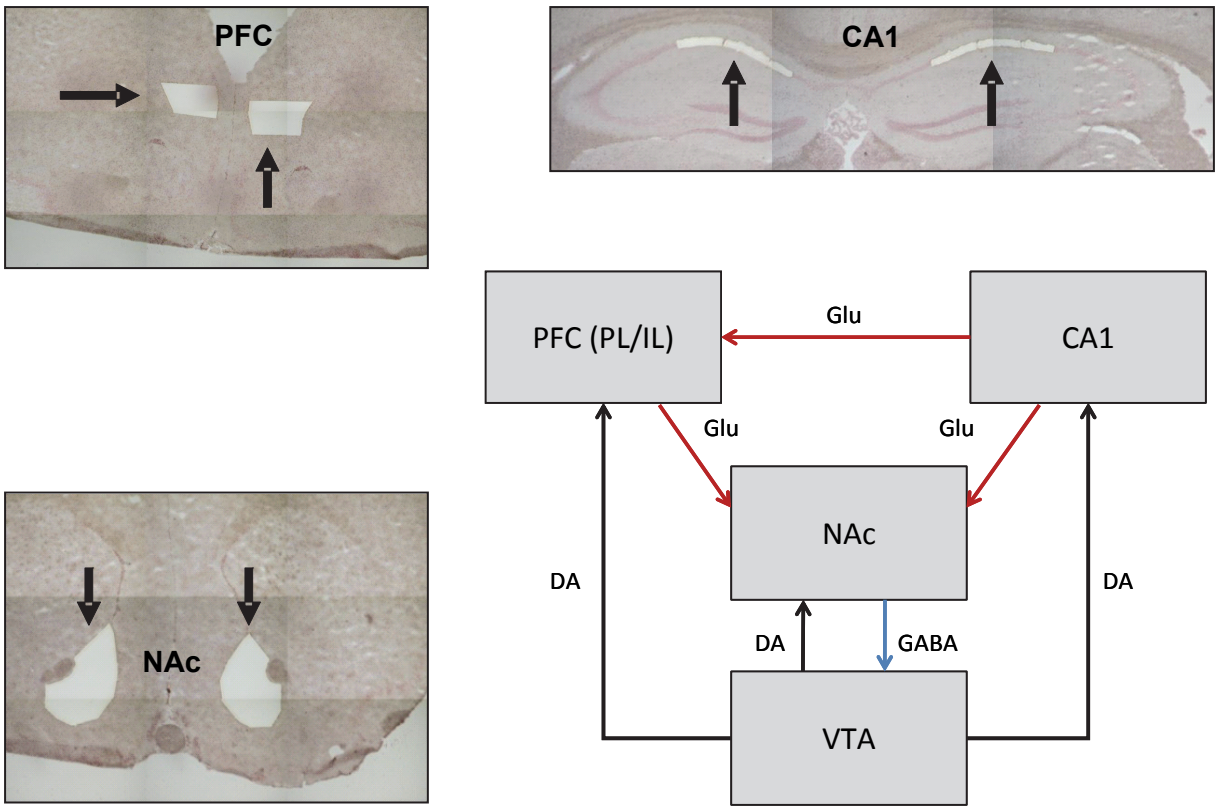

Figure 2 | Scheme showing connection between the selected brain areas including examples of laser microdissection. PFC: prefrontal cortex; IL: infralimbic; PL: prelimbic; (NAc) nucleus accumbens; CA1: cornu ammonis 1 region of the hippocampus; VTA: ventral tegmental area. Red arrows indicate glutamatergic neurons, black arrows dopaminergic neurons and blue arrows GABAergic neurons. Glu: glutamate; DA: dopamine; GABA: gamma-amino-butyric-acid.

RNA isolation, linear amplification and microarray hybridization Immediately after laser microdissection, RNA was isolated using Trizol (15596-026, Invitrogen Life Technologies, Carlsbad) using the manufacturer's protocol. Linear acrylamide was added as a carrier. RNA quality and quantity was checked by analyzing 1 μl of RNA on the Agilent 2100 Bioanalyzer using the RNA 6000 Pico LabChip Kit (5065-4473, Agilent Technologies, Palo Alto, USA). Ten ng of total RNA was used for the first round of linear amplification using the GeneChip One-Cycle Target Labeling and Control reagents (P/N 900493, Affymetrix, Santa Clara, USA). For the second round of amplification 100 ng of input RNA was used, during which the RNA was biotin-labeled using the GeneChip Two-Cycle target Labeling and Control Reagents (P/N 900494, Affymetrix, USA).

GeneChip hybridization Twenty micrograms of biotinylated RNA was subsequently fragmented using DNA Fragmentation Reagents (No. AM8740, Ambion). Thebiotinylated and fragmented RNA was hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix), containing approximately 45,000 probe sets representing 39,000 transcripts and 35,000 different genes. Hybridizations were conducted at the Leiden Genome Technology Center (LGTC, Leiden University, The Netherlands) according to the manufacturer's recommendations (Affymetrix, Santa Clara, USA). A total of 60 microarrays were hybridized, per brain region 10 HR and 10 LR.

Data Analysis Raw images were analyzed and features extracted using Affymetrix Gene Chip Operating Software (GCOS) (Affymetrix, Foster City, CA). For each brain region, the resulting CEL files containing probe level information were then normalized and converted to gene intensity values by the GC-RMA algorithm within BRB Arraytools version 3.7.3 developed by Dr. Richard Simon and the BRB Array development team (Simon *et al*, 2007). To identify differentially expressed genes we applied a two-sample t-test (fold-change>1.2 and p-value cutoff of $p<0.01$) comparison between high to low responders. Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) version 7.5 was used to identify pathways, networks and gene-list matching to published datasets of genes involved in specific transcription regulation systems (MEF, CRE, GR). The gene lists for the specific transcription regulation systems were retrieved from the supplementary material in the relevant publications (Pfenning *et al*, 2007; Wu and Xie, 2006; Zhang *et al*, 2005) and loaded into Ingenuity as comparison datasets.

Real time quantitative PCR (RT-qPCR) Primers for RT-qPCR validation were designed within the target sequence used by Affymetrix for probe design using Primer3 freeware. Primers were checked for specificity using BLAST (NCBI, Bethesda, USA) and for hairpins and self-complementarity using oligo 4.0 (MBI, Cascade, USA). The primer sequences of the validated genes that were measured can

be found in Supplementary Table SI. RT-qPCR measurements were performed on amplified RNA from experiment 1 to replicate the results from the GeneChip analysis. cDNA synthesis was performed using the iScript^{TM} cDNA Synthesis Kit (170-8897, Bio-Rad, Hercules, USA) following the manufacturer's protocol. RT-qPCRwas performed on a Lightcycler 2.0 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) using the Lightcycler FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche). The standard curve method was used to quantify the expression differences (Livak and Schmittgen, 2001). The non-parametric Mann-Whitney Test was used to assess significant differential gene expression between low and high responders.

Brain tissue from follow-up experiment 2 was used to replicate the changes in gene expression between low and high responders found in the CA1 area in an independent experiment. For this purpose, the dorsal hippocampus was dissected from frozen brain and 8 punches containing CA1 tissue were obtained from two 1mm tissue sections. RNA was synthesized to cDNA without further amplification and RT-qPCR and data analysis was performed as previously reported (Christensen *et al*) on a selection of genes that were successfully validated in experiment 1.

RESULTS

DBA/2 mice display large and stable individual differences in sensitization to amphetamine The locomotor responses to the challenge dose of amphetamine (1.25 mg/kg) or saline on day 20 are depicted in Figure 3a. On average, animals that received amphetamine pretreatment on days 1-5 (amph/amph) were more responsive to the acute amphetamine challenge than saline pre-treated mice (sal/amph), signifying the occurrence of sensitization. However, a large inter-individual variability was observed in the amph/amph group. The 10% amph/amph animals with highest locomotor response to amphetamine on day 20 were designated high responders (HR) (n=10), while the 10% animals with the lowest response were designated low responders (LR) (n=10). In an independent follow-up study it was demonstrated that the high and low responder phenotype is stable until at least one week after the first drug challenge (Figure 3b). The slight increase in both groups might signify further incubation of sensitization which is known to occur with prolonged withdrawal periods.

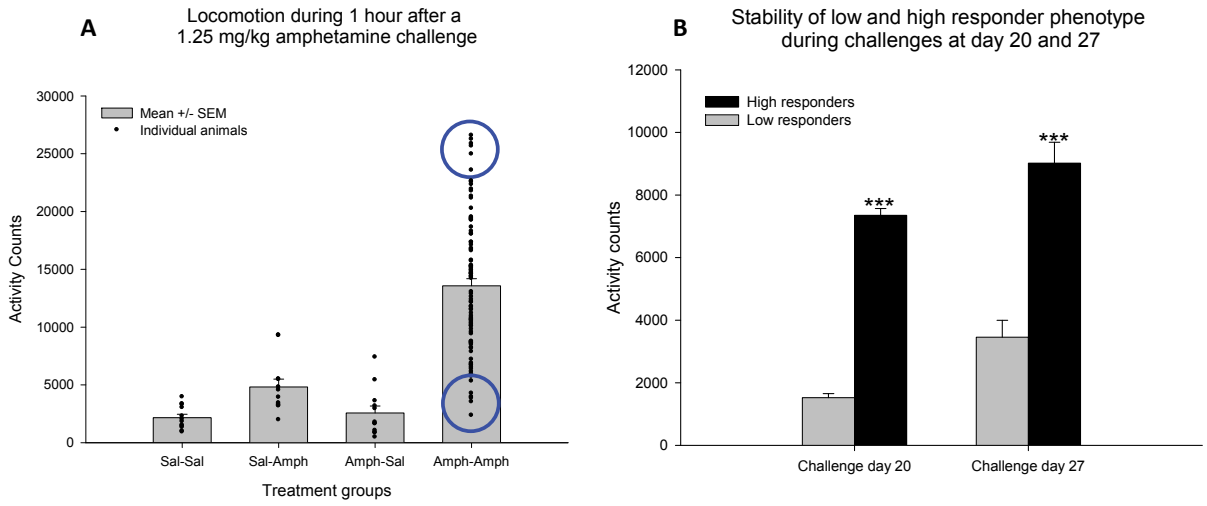

Figure 3A| Locomotor responses to the amphetamine (1.25 mg/kg) or saline challenge on day 20. Data are represented as total activity count over the 60 minute treatment period. SAL/SAL n=10, SAL/AMPH n=10, AMPH/SAL n=10, AMPH/AMPH n=100. Ovals indicated the 10% population extremes (low responders n=10, high responders n=10) in the AMPH/AMPH group selected for gene-expression profiling.B: Locomotor responses of the 10% population extremes in the AMPH/AMPH group (selected on day 20) to the amphetamine (1.25 mg/kg) challenges on days 20 and 27. Data are represented as total activity count over the 60 minute treatment period. Low responders n=10, high responders n=10. *** p<0.001 vs low responders (Mann-Whitney Rank Sum Test).

Amphetamine exposure is not different in high and low responders Amphetamine in total brain homogenates was measured in two groups (n=10 each) of mice with locomotor activity counts just below the highest (21289±377 counts) and just above the lowest responders (4387±406 counts). There was no correlation between exposure and locomotor activity (Supplementary data, Figure SI), indicating that the phenotypic difference in locomotor sensitization could not be attributed to differences in CNS amphetamine exposure.

Identification of differentially expressed genes reveals region-specific molecular signatures To identify potential molecular changes induced by the behavioural sensitization microarray analysis was performed on PFC, NAc and hippocampal CA1 regions collected from 10 HR and 10 LR animals 1 h after a challenge dose of amphetamine on day 20 (Figure 1). This time point was selected in order to examine the early factors behind the long-term changes induced by the challenge stimulus and more importantly, to look under challenged conditions in which the differences between HR and LR are most evident. Differentially regulated genes were identified by statistically comparing GC-RMA mean normalized values of HR to LR. Of the 45,000 probes on the Affymetrix gene chip mouse genome 430 2.0 arrays, we identified 63 (39 up, 24 down), 29 (20 up, 9 down) and 105 (76 up, 29 down) genes that significantly differed in expression between HR and LR in CA1, NAc and PFC respectively by two sample t-test ($p<0.01$, fold-change>1.2) (Figure 4a). These gene lists are referredto as the primary lists (Supplementary material, Table SII). Comparison of the three primary lists revealed no overlapping genes (Figure 4b). Moreover, pairwise correlation analysis of all expression values in the 60 samples showed a clear distinction in region specific expression signatures (Figure 4c). These specific molecular signatures of the analysed brain regions most likely reflect both their specific connectivity and function in a complex circuit as well as their distinct molecular response to amphetamine challenge.

Differential expression between HR and LR was most robust in the hippocampal CA1 region A total of 83 genes were selected for reconfirmation by RT-qPCR from all three brain regions based on overall lowest p-value and highest fold change. In both NAc and PFC the reconfirmation rates were rather low, with a reconfirmation rate of 3 out of 24 genes (12.5%) in the NAc and 5 out of 30 genes (16.7%) in the PFC. In the CA1 the reconfirmation rate was considerably higher, with a success rate of 14 out of 28 genes (50.0%).

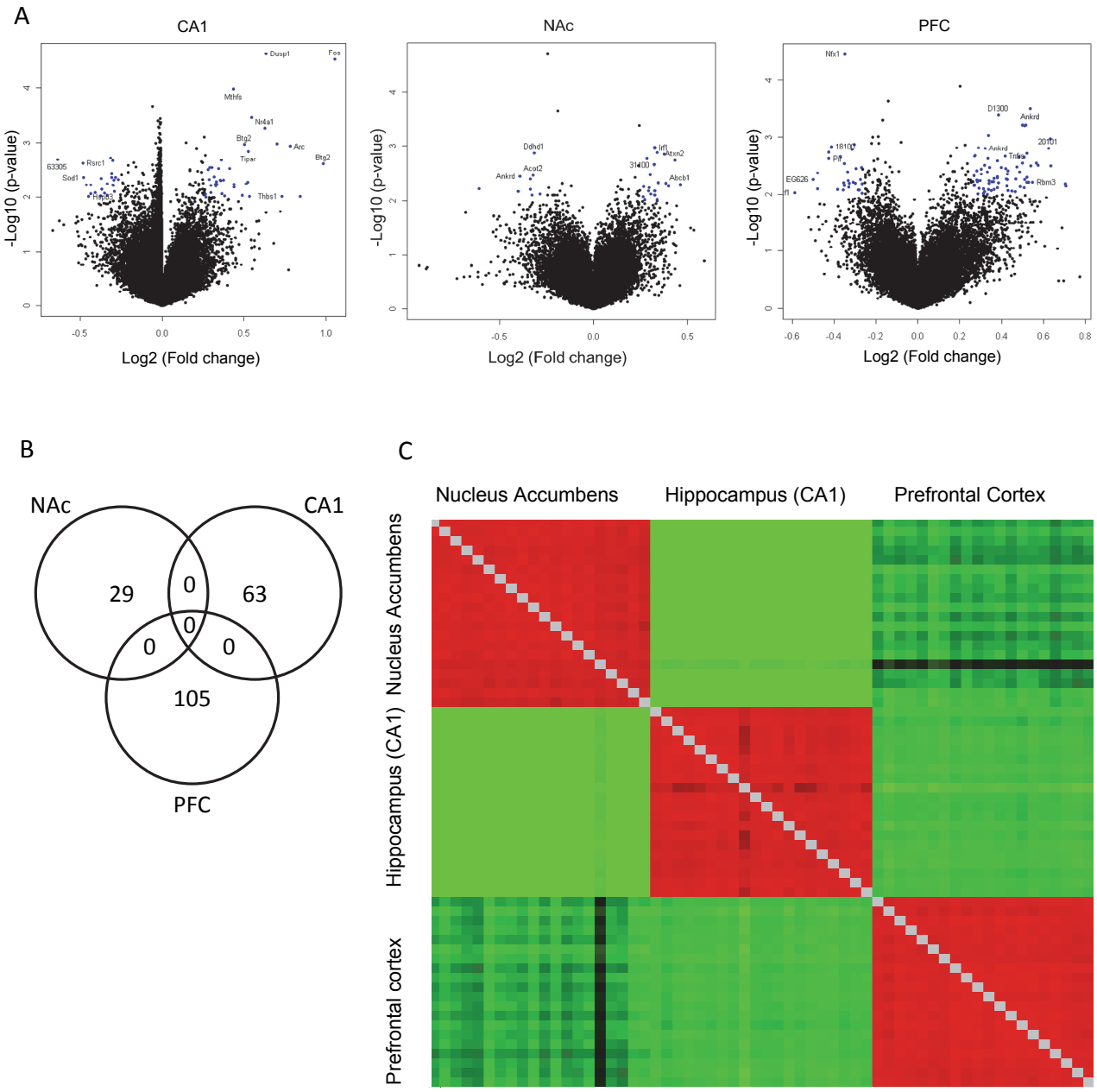

Figure 4A |Volcano plots of -log10 (p-value) vs. log2(Fold change). Labeled are largest fold change and lowest p-value genes. The blue points in each graph indicate the Affymetrix probesets that passed the t-test p<0.01 and FC>1.2 statistical requirements. B: Venn diagram of genes differentially expressed between HR and LR. Genes meeting the F>1.2 fold, p<0.01 criteria have been included. No common genes are identified when comparing CA1, PFC and NAc. C: Correlation matrix of expression levels between all 60 samples in the experiment. Differential expression between tissues is clearly identified. Correlation analysis is not able to differentiate between high and low responder groups.

Gene expression changes in CA1 could be replicated in a novel independent study The expression of several genes that were confirmed to show differential expression in the CA1 area with RT-qPCR in the first experiment were validated in an independent sensitization experiment. Gene expression of six selected genes (Arc, Nr4a1, Dusp1, Fos, Egr2 and Tiparp) was quantified in the CA1 of the phenotypically stable animals that received a second amphetamine challenge (Figure 1). In contrast to the validation described above, the six genes were measured in non-amplified mRNA derived from manually dissected CA1 rather than laser microdissection. Despite these technical differences the results replicated the differential expression between LR and HR that was shown in the first study, although NR4a1 did not reach statistical significance (Figure 5).

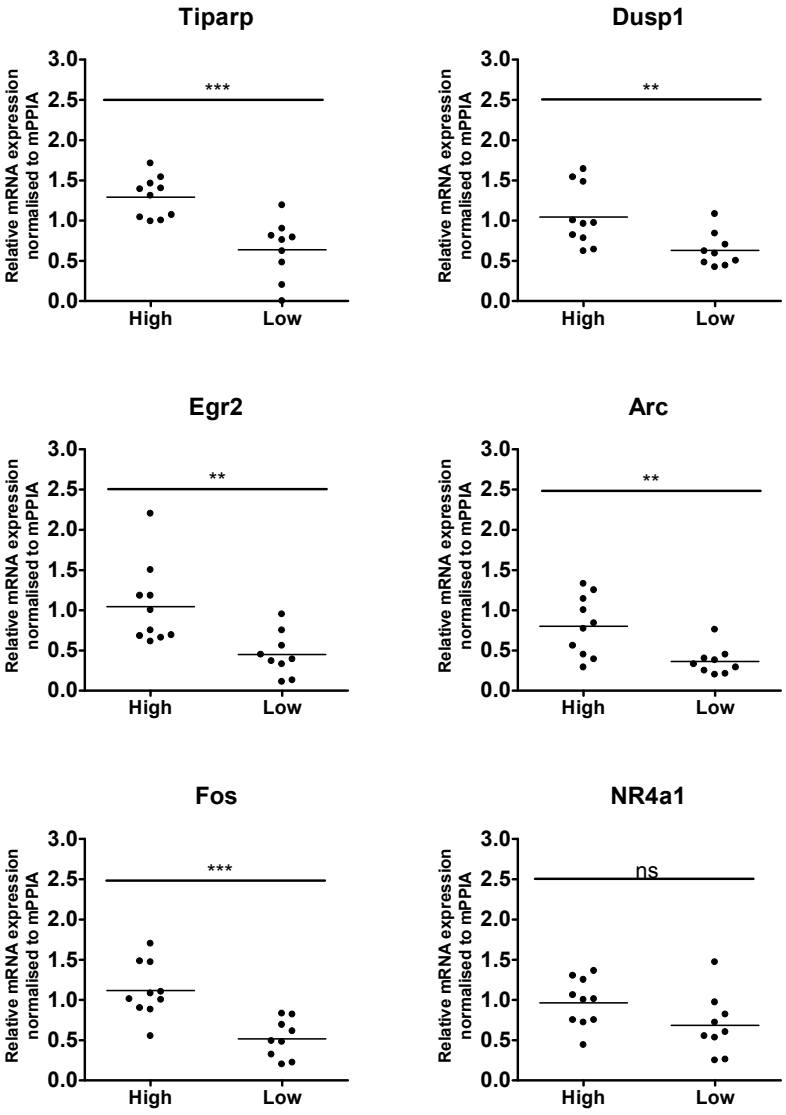

Figure 5 |RT-qPCR validation results of gene expression differences between low and high responders in the CA1 region of the hippocampus in the second animal experiment. ** p<0.01 vs low responders; *** p<0.001 vs low responders (Mann-Whitney Rank Sum Test)

Validated genes overlap with several gene classes, including GR, MEF2, and CRE regulated genes

The genes differentially expressed in CA1 were subjected to Ingenuity Pathway Analysis (IPA). Genes regulated by specific transcription factors or promoter systems as identified by ChIP/ChIP technology were identified from the literature and used to compose gene lists for target genes of transcription factors MEF2, CREB, GR and REST (Supplementary material, Table SIII for details). Each of the gene lists were compared to the 63 genes identified in CA1 and to a list of 2000 randomly selected genes from the entire list of probe sets (~45.000 probes). This comparison indicated a clear overrepresentation of GR, CRE and MEF2 promoter regulated genes among the differentially regulated gene set in CA1 (Figure 6). The comparison was repeated with a large number of randomizations of the R2K set and the differences shown in Figure 6 were found to be stable.

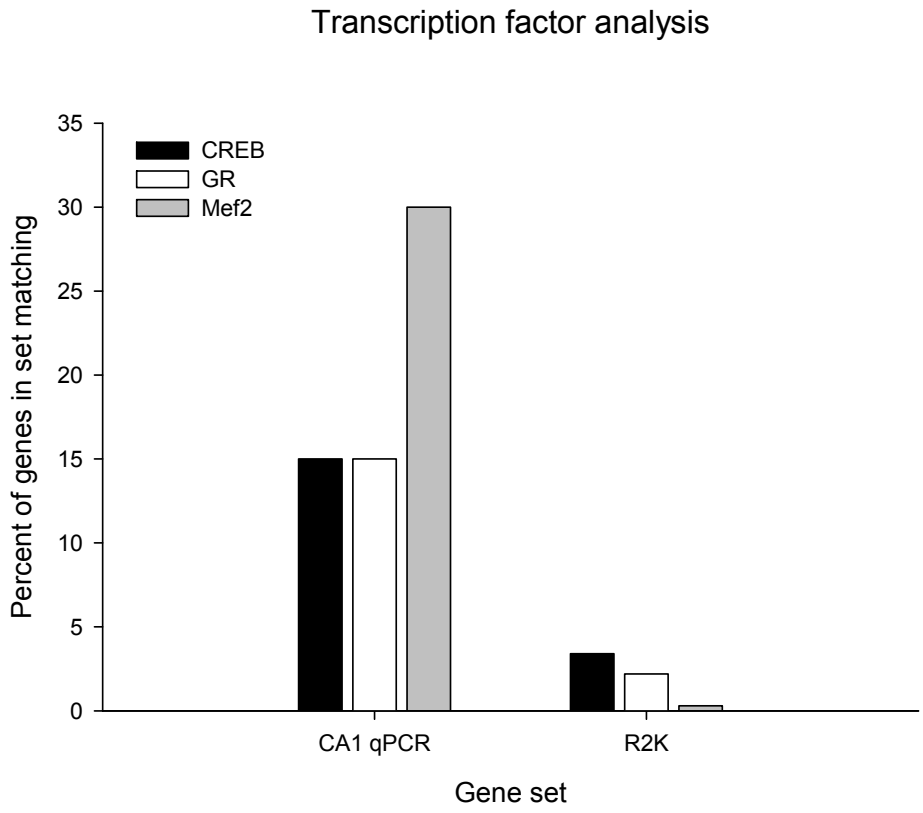

Figure 6 | Comparison of genes regulated in CA1, Nucleus Accumbens (Acc) and Prefrontal Cortex (PFC) to genes involved in specific transcriptional regulation as identified by ChIP/ChIP experiments. For each of the brain areas the comparison is made for the genes identified in expression array and for those confirmed by qPCR. The R2K dataset represents 10 x 2000 random probe sets, indicating background signal and size difference of ChIP/ChIP data sets used. Genes compared are those listed in supplementary Table I, and shown in figure 4. qPCR confirmed genes are those genes from gene expression data set that were confirmed by qPCR with a p-value better than 0.05 in any of the validation experiments (Sup Table III).

DISCUSSION

The aim of this study was to elucidate which genes and pathways underlie the differences in behavioral response to amphetamine in genetically identical mice selected for responsiveness to amphetamine sensitization. The amphetamine sensitization model is suggested to reflect the heightened sensitivity of schizophrenia patients to psychostimulants and is accepted as a model for the positive symptoms observed in schizophrenia (Featherstone *et al*, 2007; Hermens *et al*, 2009; Peleg-Raibstein *et al*, 2008; Peleg-Raibstein *et al*, 2009; Tenn *et al*, 2003). Additionally, there is increasing evidence for long-lasting cognitive deficits in sensitized animals (Featherstone *et al*, 2007). In this study we used a unique setup based on genetically identical inbred mice, all receiving the same treatment yet still displaying differences in amphetamine-sensitization. This is an important divergence to most studies reporting on gene expression focusing on differences in outbred strains and/or differences in treatment (e.g. control vs. amphetamine or acute vs. chronic amphetamine) (Funada *et al*, 2004; Palmer *et al*, 2005; Shilling *et al*, 2006; Sokolov *et al*, 2003). By taking this approach we are ruling out changes in gene regulation due to variation in genetic makeup and different treatment paradigms. Thus, the differential gene regulation found in the present study is most like reflecting the underlying mechanism for sensitization and may point to why some individuals get schizophrenia whereas others do not.

Largest effect of sensitization on gene expression was found in the CA1 area of the hippocampus

We observed a considerable variation in sensitization to amphetamine in DBA/2 mice measured by locomotor output. Gene expression in CA1, NAc and PFC, all dopaminergic output brain areas, of the 10 lowest and 10 highest responders (LR and HR) was assessed 1 hour after amphetamine challenge. Gene expression signatures were highly brain region-specific, with the strongest differential expression between low and high responders in the CA1 subregion of the hippocampus. These findings are of interest since most research on amphetamine-induced gene expression so far has focused on PFC, Striatum, NAc and VTA (Mirnics *et al*, 2000; Palmer *et al*, 2005; Yuferov *et al*, 2005). However, our data are consistent with recent literature pointing to a prominent role of the hippocampus and dopamine in schizophrenia (Grace, 2010; Lisman and Grace, 2005; Lodge and Grace, 2007, 2008; Rossato *et al*, 2009), for review see (Shohamy and Adcock, 2010). In schizophrenic patients and high-risk individuals there is elevated regional cerebral blood volume (rCVB) in the CA1 sub-region of hippocampus, which correlates with positive symptoms and predicts clinical progression (Gaisler-Salomon *et al*, 2009b; Schobel *et al*, 2009). The increased hippocampal activity linked to psychotic symptoms is in line with data by Grace *et al.* showing how the hippocampus controls dopamine (DA) neuron activity, possibly by increasing the number of DA neurons that can be activated by salient signals (Grace *et al*, 2007). In contrast, antipsychotic phenotype measured as

reduced amphetamine-induced locomotion and release of dopamine in NAc is seen in an animal model with reduced glutaminase activity leading to a CA1/subiculum-specific decrease in rCVB(Gaisler-Salomon *et al*, 2009a).

Furthermore, preventing synaptic transmission in the dorsal region of the hippocampus by local infusion of the anaesthetic lidocaine is able to block the expression of behavioral sensitization to amphetamine (Beck *et al*, 2009). Finally, Crombag *et al*. showed that amphetamine self-administration leads to increased spine-density in the CA1 region of the hippocampus (Goeman *et al*, 2004). Although, not investigated in the current study changes in spine morphology may likely be present in our sensitized mice. The differences in expression of Mef2 target genes we identified fit well with a potential difference in spine-density, given that Mef2 is a key regulator of neuronal plasticity and that manipulating Mef2 expression and activity directly influences psychostimulant sensitization (Pulipparacharuvil *et al*, 2008).

We cannot draw any conclusions on the role of other dopaminergic brain regions that are of relevance to the development of behavioral sensitization, e.g. the VTA or the amygdala (Yuferov *et al*, 2005). It is possible that they may harbor bigger differences in gene expression than currently observed in CA1. However, that would need to be addressed in a follow-up study.

Immediate early genes Many of the validated genes are immediate early genes (IEGs), which are among the first genes to be expressed (hence the name) in a changing environment. Examples of IEGs identified in this study are c-fos, Dusp1, Nr4a1, Egr2, Arc and Tiparp. Other studies have also found IEGs to be responsive to amphetamine in the brain. For example, Shilling *et al* showed down-regulation of several IEGs in the PFC of HR 24 hours after a single injection of methamphetamine (Shilling *et al*, 2006). Down-regulation of IEGs at such a late time point may represent an adaptive response to counterbalance the earlier increase in IEG expression as observed in the present study. One of the IEGs we found to be up-regulated in the HR is c-fos. Interestingly, Zhang *et al* found that c-fos down-regulation in DA D1 receptor containing neurons attenuates cocaine-induced behavioral sensitization (Zhang *et al*, 2006). This might indicate that higher c-fos expression in the HR is a cause rather than a consequence of the observed increased locomotor response to amphetamine. In line with our findings for c-fos, two independent studies show that methamphetamine increases expression of IEG Arc from 1 hour onwards in multiple brain regions, which can be blocked by giving a D1 receptor antagonist (Kodama *et al*, 1998; Yamagata *et al*, 2000). Since many IEGs are regulated by multiple transcription factors, the question rises what the link is to the underlying mechanisms of amphetamine sensitivity.

GR, Mef2 and Creb are important regulators of sensitization We found a clear overrepresentation of GR, Mef2 and CRE promoter regulated genes among the differentially regulated gene set in CA1 (Figure 6). These transcription factors are interesting candidates linking the regulation of IEGs to mechanisms of behavioral sensitization and psychosis susceptibility.

Glucocorticoids GR, an important receptor for glucocorticoid stress hormones in the brain, is a transcription factor that is able to regulate many of the IEGs as well as some of the other validated genes that were differentially expressed between high- and low-responders in CA1. Stress and more particular glucocorticoids are factors influencing sensitization to psychostimulants (Antelman *et al*, 1980). We have previously shown that cocaine sensitization in DBA/2 mice relies in part on corticosterone (de Jong *et al*, 2007). Moreover it was shown that antagonizing GR attenuates the expression of amphetamine-induced sensitization (De Vries *et al*, 1996). Also in humans, many studies have shown that psycho-stimulant abuse and stressful life events are associated with later-life psychotic episodes, with odds ratios even increasing with cumulative traumas (Johns *et al*, 2004; Shevlin *et al*, 2008; Wiles *et al*, 2006).

In rodents a similar link between stress, glucocorticoids and behavioral sensitization was found. Chronic social stress increased amphetamine-induced locomotion (Mathews *et al*, 2008) and vice versa (Antelman *et al*, 1980; Myin-Germeys and van Os, 2007; Vanderschuren *et al*, 1999). Withdrawal from amphetamine leads to increased corticosterone levels in rats that show sensitization but not in non-sensitized animals (Scholl *et al*, 2009). DBA/2 mice are known for their vulnerability to stressful events (Weaver *et al*, 2004). Our findings indicate that several of the genes that are differentially expressed between LR and HR are involved in glucocorticoid signaling. For example, Nr4a1 was one of the IEGs we identified to have a higher expression in the CA1 of HR. Nr4al belongs to the family of orphan nuclear receptors and is also increased by amphetamine in the striatum (Levesque and Rouillard, 2007). Nr4a1 is known to bind to NGFI-B sites in addition to glucocorticoid response elements (GREs). It has been shown that Nr4a1 can compete with the GR for binding to a negative GRE (nGRE) sequence on the POMC promoter in the hypothalamus, preventing the GR-induced inhibition of ACTH (Okabe *et al*, 1998; Philips *et al*, 1997), which is part of the negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis and vital for proper functioning of the stress system. Several other of the differentially expressed genes we identified are glucocorticoid-responsive, such as for example Dusp1 (King *et al*, 2009). Hippocampal Dusp1 expression is known to be induced by glucocorticoids (Morsink *et al*, 2006), suggesting that high responders have an increased corticosterone response to the amphetamine challenge, corresponding to a sensitized HPA-axis.

MEF2 The transcription factor Mef2 plays a role in regulation of IEGs and behavioral sensitization. MEF2 is a key regulator of structural synapse plasticity and has recently been implicated in behavioral sensitization to cocaine (Flavell *et al*, 2008; Livak *et al*, 2001). Chronic cocaine treatment was shown to affect Mef2 phosphorylation in the NAc, thus altering its activity (Pulipparacharuvil *et al*, 2008). Mef2 is phosphorylated and consequently inhibited by Cdk5 in combination with its activators p35 and p25 (Gong *et al*, 2003). P25 protein level, responsible for a prolonged activation of Cdk5, was shown to be increased 4 hours after acute or chronic amphetamine treatment (Mlewski *et al*, 2008) and might explain the altered activity of Mef2 during psychostimulant sensitization. Expression of Cdk5 itself can be directly regulated by ΔFosB (Kumar *et al*, 2005), that in turn is increased after psychostimulant treatment and can remain elevated for weeks (Nestler, 2005b). Cdk5 not only phosphorylates Mef2 but was also found to phosphorylate GR in a dexamethasone-dependent manner (Kino *et al*, 2007). Consequently, amphetamine-induced changes in Cdk5 may affect both GR and Mef2 transcriptional activity. This suggests that the glucocorticoid stress system and Mef2-driven pathways converge, and would provide an explanation for how individual differences in stress can affect the sensitization process. Interestingly, Mef2 expression itself was not found to be different between low- and high-responders.

CREB (cAMP response element-binding). We found that cAMP response element (CRE)-family transcription factors overall can affect at least 15% of qPCR confirmed AMPH-regulated genes in CA1 (Figure 6). In a random set of genes picked from the gene expression chip this number is low (3.4%, see Figure 6). This CRE-family transcription factor overrepresentation is in line with the literature. The CREB protein is a transcription factor that binds to CRE DNA signature sequences and, thereby, increases or decreases the transcription of downstream genes (Purves D, 2008). Genes relevant for amphetamine sensitization and dopamine function whose transcription is regulated by CREB include: c-fos, BDNF, tyrosine hydroxylase (TH), and many neuropeptides (such as somatostatin, enkephalin, VGF, and corticotropin-releasing hormone) (Purves D, 2008) . CREB has a well-documented role in neuronal plasticity and long-term memory formation in the brain (Silva *et al*, 1998).

Environmental factors Since all mice from this inbred strain received an identical treatment, a plausible underlying cause for difference in sensitization may be that differences in handling, social hierarchy or maternal care underlie the differential expression of amphetamine sensitivity via effects on the glucocorticoid stress system (Badiani *et al*, 1992; Holmes *et al*, 2005; Lockwood and Turney, 1981). This fits well with the numerous studies pointing to an association between early childhood trauma, parental care and social adversity and the later development of psychotic illness (Janssen *et al*, 2004; Morgan and Fisher, 2007; Morris *et al*, 2006; Wicks *et al*, 2005). The stress-system may be

an important biological mechanism linking sensitization processes initiated by developmental stress exposures to an increased risk for psychosis. Recent studies have shown changes in cortisol secretion associated with smaller left hippocampal volume in first-episode psychosis patients (Mondelli *et al*, 2010b) and a blunted cortisol awakening response compared with controls (Mondelli *et al*, 2010a) and increased emotional reactivity to stress in daily life (Lataster *et al*, 2009).

Technical considerations In the current study we demonstrated that there are individual differences in gene expression in key dopaminergic output areas in the brain that reflect a differential sensitivity to amphetamine. Differences in gene expression in all 3 brain regions were subtle, with the majority of gene expression changes being below 1.5-fold. These modest changes in gene expression are not surprising, given that low and high responders have the same genetic background and received an identical sensitization protocol using exactly the same amphetamine dosing regimen. Nonetheless, our setup using laser microdissection in combination with DNA microarrays is evidently sensitive enough to detect these changes. Validation of the identified gene expression changes proved to be difficult, in particular in the NAc and PFC. Validation of subtle differences in gene expression by other methods such as RT-qPCR is notoriously difficult, due to limitations in sensitivity. Most commonly, a 2-fold change is reported as the cutoff below which microarray and qPCR data begin to lose correlation. Dallas *et al*. reported decreased correlations for genes expressing less than 1.5-fold change using qPCR and oligonucleotide microarrays (Dallas *et al*, 2005). Nonetheless, we were able to validate 22 out of 87 genes with RT-qPCR, with the highest success rate (50%) in the CA1 region of the hippocampus.

Sources of experimental uncertainty We have a high level of confidence in our CA1 array data for the following reasons. First, the genes identified here are based on strong statistical comparisons with ten biological replicates in each group, decreasing the probability of false negatives. This is in contrast to a majority of published reports where either small numbers of animals are used in each comparison group or technical replicates of pooled animals are applied to identify target genes (Pawitan *et al*, 2005). Second, rather than using a whole hippocampus homogenate we specifically isolated the CA1 pyramidal cell layer, resulting in a more homogeneous population of neurons highly enriched for CA1 pyramidal neurons and therefore more likely to yield a transcriptional response that is undiluted by effects in other parts of the hippocampus, non-neuronal cells such as glia and isolation artefacts. We have previously demonstrated that the different subregions of the hippocampus differ profoundly in basal transcriptome, demonstrating that in the brain specific isolation and analysis of homogeneous neuronal subpopulations is of utmost importance (Datson *et al*, 2004; Datson *et al*, 2009). Third, the validation rate was high considering the small differences in expression. Finally, RT-qPCR re-measurement of representative genes in an independently performed

follow-up experiment demonstrated that the changes in gene expression in CA1 were reliably reproduced and correlated with the high or low responder phenotype.

Timing The time at which the gene expression changes were measured in the current study, i.e. 1 hour after an amphetamine challenge, is a point of consideration. Our rationale for choosing this time point was that we wanted to investigate gene expression between low and high responders under challenged rather than baseline conditions, which we hypothesize is a prerequisite to identify pathways relevant for behavioral sensitization and thus susceptibility for psychosis. Under challenged conditions the phenotypic extremes between low and high responders become evident while under basal conditions there are no apparent differences. Further the current design is appropriate for detecting primary gene responses rather than secondary or even more downstream waves of gene expression. It could be argued that looking at a later time point would give more insight in the long-lasting changes in gene expression rather than in acute changes associated with the amphetamine challenge. Indeed, Cadet et al found differential gene expression in the frontal cortex up to 16 hours after a 40 mg/kg dose of methamphetamine, although this dose is much higher (32-fold higher) compared to the rather low doses given in our study (Cadet *et al*, 2001). Nonetheless, the success of our approach is evident since the changes in gene expression we identified in CA1 reproducibly discriminate high from low responders, as demonstrated in the independent follow up experiment we performed.

CONCLUSION

In conclusion, we show that inbred DBA/2 mice exhibit large differences in sensitization to amphetamine that is reflected at the transcriptional level in several dopaminergic output brain areas, but in particular in the CA1 area of the hippocampus. We have identified CRE, Mef2 and GR transcription factors as possible mediators of these differences. CRE, Mef2 and GR signaling appears to form a transcription regulation network involved in the amphetamine susceptibility response and thus may play an important role in psychosis susceptibility. To which extent these systems act as independent, linked or sequential programs is the target of future studies.

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SUPPLEMENTARY DATA

Table SI | Sequences of the primers used for validated genes in the three brain areas; CA1, Nac and PFC

Affymetrix Id	Gene description	Gene symbol	Forward primer sequence	Reverse primer sequence
1459698_at	Unknown		TCAGCTGTTGGGTGTGAAAG	TTACCCAGGGTGATCTCCAG
1426719_at/1426720_at	amyloid beta (A4) precursor protein-binding, family B, member 2	Apbb2	AACAGGACTTCCGAGCACAC	CCAGTCAAGAGGACAGCAAA
1418687_at	activity regulated cytoskeletal-associated protein	Arc	GCTCCTAGGCTGTTCCATGA	CAAGCAGCTACCAGCACAAG
1444667_at	Bromodomain, testis-specific	Brdt	AGCCTCTCCCTGACCTCACT	AGTAGCATGGAGCCCAACAC
1448272_at/1416250_at	B-cell translocation gene 2, anti-proliferative	Btg2	TGGCTTCGTCTCTCTTGCTT	GTGTGCGGACAAAACACAAG
1448830_at	Dual specificity phosphatase 1	Dusp1	CAACAATGACTTGACCGCAA	GCGAAGAAACTGCCTCAAAC
1423100_at	FBJ osteosarcoma oncogene	Fos	AGTCAAGGCCTGGTCTGTGT	TCCAGCACCAGGTTAATTCC
1416155_at	High mobility group box 3	Hmgb3	TGGCTAGCAATCCTGAGTTGT	GCCAAACAAGGAGCATCAAG
1417409_at	Jun oncogene	Jun	GGTGGGAGGGGTTACAAACT	GGGGAGTTCATCTGCAGTCT
1447308_at	longevity assurance homolog 5 (S. cerevisiae)	Lass5	TATTTAATTGGTGTGCTGGCTA	GCTCTATAGGCTTGCCCACT
1426850_a_at	mitogen activated protein kinase kinase 6	Map2k6	GCCCTGGTAACAAGGTGCTA	TCCAACCAAGCACTGAAACA
1436858_at	Muscleblind-like 2	Mbnl2	GCACCATGATCGACACAAAC	GTGTGCAGGAGGGTGAAAAT
1448645_at	male-specific lethal-3 homolog 1 (Drosophila)	Msl3l1	TACTTCCTGGGTGCCTGAAC	CCTGCGCTGTCTACCAGAAT
1416808_at	nidogen 1	Nid1	CTCCACCTCGACCTGCTTAC	GGGTGCATGAAAGAGTCACA
1416505_at	nuclear receptor subfamily 4, group A, member 1	Nr4a1	TATCCCTCCAGCTCAGTCCT	CCCCATCTCAACCTCTTCCT
1422707_at	phosphoinositide-3-kinase, catalytic, gamma polypeptide	Pik3cg	CGTGAAAAGTGGAGGTGACA	CAGCTAGCGACTTCCTGCTT
1425059_at	Protein arginine N-methyltransferase 6	Prmt6	GTTGCTGAACCTAGCCCAAG	GATTAGAGTGCTCGCGTTCC
1448401_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	Smarcd2	TTGCATGTTTACAGGCTCCA	GGCTTGTAGGAATGGCAAAA
1460557_at/1432623_at	Suppressor of var1, 3-like 1 (S. cerevisiae)	Supv3l1	TCTTGAAGCTGTCCATGACG	CTGGAGACTTCGAACAAGGC
1422570_at	YY1 transcription factor	YY1	GCCTGCCTTCTTTCTCATCA	GGACTGCACTGAGATTTCCTG

Figure SI | No correlation is observed in total brain exposure to amphetamine and locomotor activity of high and low responders to amphetamine

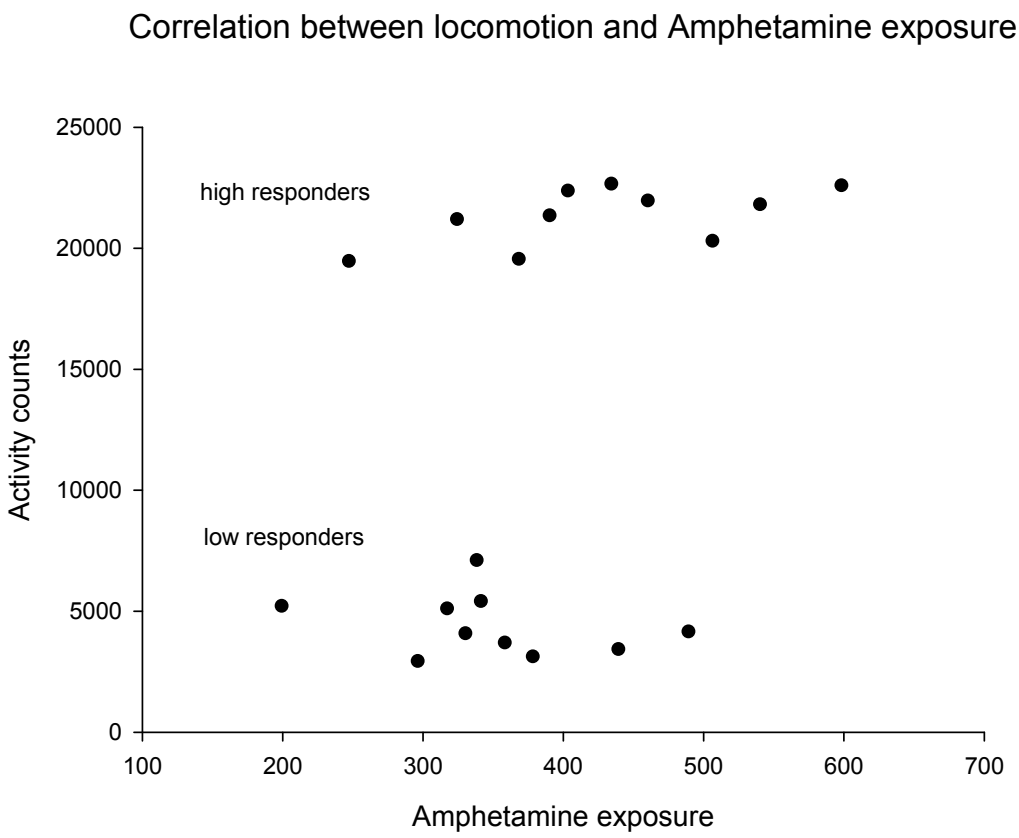

Table SII | CA1 differentially expressed genes sorted by FC of the univariate test. The 63 genes are significant at the nominal 0.01 level of the univariate test with a fold change cut-off of 1.2. 39 genes are upregulated and 24 downregulated. Note: Comparison is based on high to low responders group

Probe set	Description	Gene symbol	FC	Parametric p-value	DefinedGenelist	p-value (10K permutations)
1423100_at	FBJ osteosarcoma oncogene	Fos	2.1	2.85E-05	immunology, tsonc	< 1e-07
1416250_at	B-cell translocation gene 2, anti-proliferative	Btg2	2.0	0.0023899	BTG family proteins and cell cycle regulation	0.0017
1420136_a_at	NA	NA	1.8	0.0099056		0.0102
1418687_at	activity regulated cytoskeletal-associated protein	Arc	1.7	0.0011492		0.0013
1421811_at	thrombospondin 1	Thbs1	1.7	0.0097194	TSP-1 Induced Apoptosis in Microvascular Endothelial Cell , Cell Communication, ECM-receptor interaction, Focal adhesion, TGF-beta signaling pathway, angiogenesis, cell_signaling, immunology, metastasis	0.005
1415899_at	Jun-B oncogene	Junb	1.6	0.0010322	GATA3 participate in activating the Th2 cytokine genes expression, tsonc	0.0011
1448830_at	dual specificity phosphatase 1	Dusp1	1.6	2.25E-05	CD40L Signaling Pathway, NFkB activation by Nontypeable Hemophilus influenzae, Regulation of MAP Kinase Pathways Through Dual Specificity Phosphatases, TNFR2 Signaling Pathway, MAPK signaling pathway	3.00E-04
1416505_at	nuclear receptor subfamily 4, group A, member 1	Nr4a1	1.5	0.0005339	MAPK signaling pathway	6.00E-04
1452160_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	1.5	0.0003376		3.00E-04
1451332_at	zinc finger protein 521	Zfp521	1.4	0.0096931		0.0093
1426721_s_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	1.4	0.0014196		0.0012
1448384_at	protein O-fucosyltransferase 2	Pofut2	1.4	0.00532		0.0057
1418932_at	nuclear factor, interleukin 3, regulated	Nfil3	1.4	0.0057115	immunology	0.0064
1448272_at	B-cell translocation gene 2, anti-proliferative	Btg2	1.4	0.0010767	BTG family proteins and cell cycle regulation	4.00E-04
1434025_at	NA	NA	1.4	0.0092861		0.0108
1424517_at	coiled-coil domain containing 12	Ccdc12	1.4	0.0065704		0.0025
1417293_at	heparan sulfate 6-O-sulfotransferase 1	Hs6st1	1.4	0.0059311	Glycan structures - biosynthesis 1, Heparan sulfate biosynthesis	0.0077
1460257_a_at	5, 10-methenyltetrahydrofolate synthetase	Mthfs	1.4	0.0001039	One carbon pool by folate	2.00E-04
1452161_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	1.3	0.0029702		0.0016
1416122_at	cyclin D2	Ccnd2	1.3	0.0096607	Cyclins and Cell Cycle Regulation, Cell cycle, Focal adhesion, Jak-STAT signaling pathway, Wnt signaling pathway, cell_cycle	0.0093
1419522_at	zinc finger, MYND domain containing 19	Zmynd19	1.3	0.0048729		0.0027
1449851_at	period homolog 1 (Drosophila)	Per1	1.3	0.0088176	Circadian Rhythms, Circadian rhythm	0.0089
1427405_s_at	RAB11 family interacting protein 5 (class I)	Rab11fip5	1.3	0.0020378		0.0014
1428367_at	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	Ndst1	1.3	0.0050318	Glycan structures - biosynthesis 1, Heparan sulfate biosynthesis	0.0058
1441087_at	RIKEN cDNA 2810011L19 gene	2810011L19Rik	1.3	0.0048717		0.0052
1434013_at	actin binding LIM protein family, member 3	Ablim3	1.3	0.0030014	Axon guidance	0.0041
1428759_s_at	coiled-coil domain containing 49	Ccdc49	1.3	0.0058428		0.0076
1416110_at	solute carrier family 35, member A4	Slc35a4	1.3	0.0052264		0.0064
1425019_at	UBX domain protein 2A	Ubxn2a	1.3	0.0062819		0.0036
1417155_at	v-myc myelocytomatosis viral related oncogene,	Mycn	1.3	0.0058435	tsonc	0.0076

	neuroblastoma derived (avian)					
1429139_at	OTU domain containing 7B	Otud7b	1.2	0.004244		0.0016
1424214_at	RIKEN cDNA 9130213B05 gene	9130213B05Rik	1.2	0.0028285		0.0023
1438751_at	solute carrier family 30, member 10	Slc30a10	1.2	0.0082548		0.0079
1451264_at	FERM domain containing 6	Frmd6	1.2	0.0061425		0.0079
1429466_s_at	anterior pharynx defective 1c homolog (C. elegans)	Aph1c	1.2	0.0028677		0.003
1449886_a_at	translocase of inner mitochondrial membrane 9 homolog (yeast)	Timm9	1.2	0.004809		0.0023
1452179_at	PHD finger protein 17	Phf17	1.2	0.0031242		0.0029
1450027_at	syndecan 3	Sdc3	1.2	0.0099391	Cell adhesion molecules (CAMs), ECM-receptor interaction	0.005
1434034_at	ceramide kinase	Cerk	1.2	0.0088971	Sphingolipid metabolism	0.0108
1428918_at	SCY1-like 3 (S. cerevisiae)	Scyl3	0.8	0.0048004		0.0065
1432538_a_at	replication factor C (activator 1) 3	Rfc3	0.8	0.0043177	DNA_adducts, DNA_damage	0.005
1436447_at	RIKEN cDNA A630026N12 gene	A630026N12Rik	0.8	0.0079911		0.005
1428312_at	leucine rich repeat containing 57	Lrrc57	0.8	0.0048458		0.0047
1438873_at	zinc finger protein 389	Zfp389	0.8	0.0061628		0.0064
1456948_at	adaptor-related protein complex AP-4, epsilon 1	Ap4e1	0.8	0.0021178		0.001
1439884_at	nudix (nucleoside diphosphate linked moiety X)-type motif 16	Nudt16	0.8	0.0059877		0.0076
1422570_at	YY1 transcription factor	Yy1	0.8	0.0043526	The PRC2 Complex Sets Long-term Gene Silencing Through Modification of Histone Tails	< 1e-07
1435082_at	synaptophysin-like protein	Sypl	0.8	0.0036971		0.0054
1459900_at	expressed sequence C79468	C79468	0.8	0.0018875		0.0026
1455525_at	endo/exonuclease endonuclease G-like	Exog	0.8	0.0093459		0.009
1455460_at	predicted gene, 100040120	100040120	0.8	0.0057281		0.0071
1457680_a_at	transmembrane protein 69	Tmem69	0.8	0.0084538		0.009
1440264_at	NA	NA	0.8	0.0072561		0.01
1430382_at	RIKEN cDNA 4833413G10 gene	4833413G10Rik	0.8	0.0045134		0.0019
1453024_at	WD repeat domain 37	Wdr37	0.8	0.0094737		0.0083
1449910_at	RIKEN cDNA 2210418O10 gene	2210418O10Rik	0.8	0.0082338		0.0082
1441148_at	NA	NA	0.7	0.0086454		0.0034
1446840_at	NA	NA	0.7	0.0060192		0.0039
1449872_at	heat shock protein 3	Hspb3	0.7	0.009898		0.0087
1457757_at	TOX high mobility group box family member 2	Tox2	0.7	0.0060703		0.0059
1440222_at	superoxide dismutase 1, soluble	Sod1	0.7	0.0043737	Free Radical Induced Apoptosis, The IGF-1 Receptor and Longevity, Amyotrophic lateral sclerosis (ALS), Neurodegenerative Disorders, immunology, pharmacology	0.0042
1459958_at	arginine/serine-rich coiled-coil 1	Rsrc1	0.7	0.0023528		0.002
1426356_at	RIKEN cDNA 6330578E17 gene	6330578E17Rik	0.6	0.0020099		0.0026

Table SIII | Gene list sources for IPA Publist analyses

Study Type/ Database	Factor	Total IPA-mapped Genes	Reference
In silico	CRE	3445	(Zhang *et al.* 2005)
CHIP	CRE		(Tanis *et al.* 2008)
CHIP	MEF2	107	(Flavell *et al.* 2008, Pulipparacharuvil *et al.* 2008)
CHIP	NR-GR	445	(So *et al.* 2007, So *et al.* 2008) (Wang *et al.* 2004, Phuc Le *et al.* 2005)