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ORIGINAL INVESTIGATION

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

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**Abstract**
*Rationale* Psychosis susceptibility is mediated in part by the dopaminergic neurotransmitter system. In humans, individual differences in vulnerability for psychosis 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.

*Objectives* 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.

*Methods* 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, nucleus accumbens, and cornu ammonis 1 (CA1) area of the hippocampus, were isolated by laser microdissection and subjected to DNA microarray analysis 1 h after a challenge dose of amphetamine.

*Results* 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 real-time quantitative polymerase chain reaction 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 cAMP response element (CRE) family and targets of the glucocorticoid receptor (GR) and myocyte enhancer factor 2 (Mef2) transcription factors.

*Conclusion* 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.

**Keywords** Mouse · Brain · Psychosis · Amphetamine sensitization · Laser microdissection · Gene expression · Glucocorticoid receptor · Myocyte enhancer factor 2

N.A. Datson and N. Speksnijder contributed equally to this work.

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## Introduction

Psychosis is characterized by a gradual loss of contact with reality, progressing from emotional instability, acoustic and visual disturbances, and 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. 2006, 2008; 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 (DA) release in the striatum (Featherstone et al. 2007; Laruelle and Abi-Dargham 1999; Morrens et al. 2006). Moreover, the number of 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. 2005, 2007). 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 DA pathway, are associated with the expression of behavioral sensitization (Nestler 2005a). The mesolimbic DA 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 DA 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. In the NAc, induction of the transcription factors ΔFosB and cAMP response element binding (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 extracellular signal-regulated kinase (ERK) pathway and cAMP-independent activation of protein kinase B (Akt)-GSK-3 (glycogen synthase kinase 3) signaling 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 DA 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 and methods

*Drugs* D-Amphetamine ((+)-α-methylphenethylamine sulfate; Unikem A/S, Copenhagen, Denmark) was dissolved in 0.9% sodium chloride. Doses are listed as salt equivalents (in milligrams per kilogram).

*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 four mice per cage in a temperature- and humidity-controlled environment at a 12-h 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 into 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 five 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 Fig. 1). At the drug challenge (day 20), locomotor behavior 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 HR and LR were used for subsequent gene expression analysis.

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 of the amph/amph group ($n=10$ each) on day 20 were subsequently left undisturbed for an additional 7 days and rechallenged with 1.25 mg/kg on day 27 and locomotor behavior was measured again (Fig. 1). The HR and LR were used for revalidation of gene expression changes measured in experiment 1.

*Locomotor behavior* Animals were placed individually in Makrolon locomotor activity cages (20×35×18 cm; Lundbeck). Following a 60-min 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-min 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 pulverized dry ice) and stored at −80°C 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 Tomtec Autogizer. The supernatant was analyzed like plasma. Online sample preparation and LC were performed with turbulent flow chromatography (Cohesive Technologies, UK), using a dual-column configuration. MS/MS detection was done with an Applied Biosystems Sciex 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 at −18°C. According to the Mouse Brain Atlas (Franklin and Paxinos

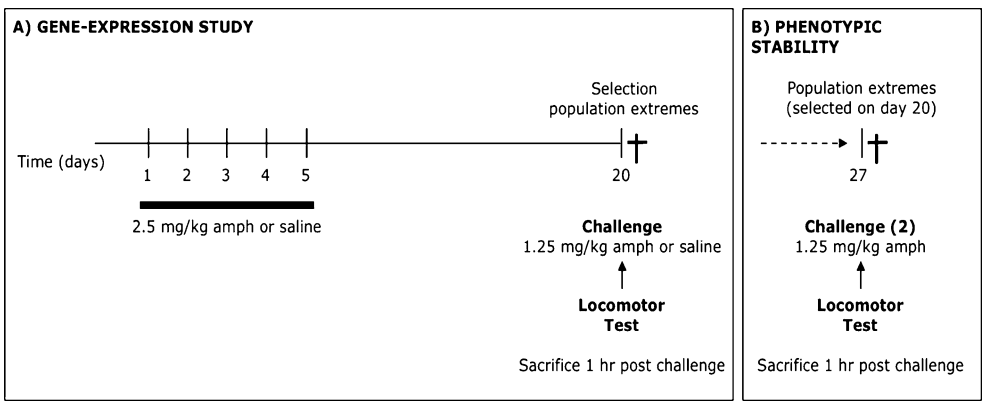


**Fig. 1** **a** Animals received either D-amphetamine (2.5 mg/kg) or saline for five consecutive days (days 1–5). After a 14-day withdrawal period (day 20), animals were given a low-dose amphetamine challenge (1.25 mg/kg) or saline and the 10% population extremes in the amph/amph group (LR and HR) were selected. In the expression profiling study, mice were sacrificed 1 h after the challenge on day 20 (experiment 1). **b** In the follow-up study (experiment 2), the LR and HR received an additional amphetamine (1.25 mg/kg) challenge on day 27 and were sacrificed 1 h later. Locomotor tests were performed on the indicated days

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 h at 180°C and subsequent UV irradiation for 30 min at 254 nm. After sectioning, the slices were kept at −80°C until further use. On the day of LMD, the slides were briefly stained with hematoxylin (10%), dehydrated in 70%, 95%, and 100% ethanol, briefly dipped in xylene, and dried at 40°C. Immediately afterwards, the slides were used for LMD on a PALM MicroLaser System (PALM, Bernried, Germany) 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 (Fig. 2). Per mouse, a total of four sections were dissected and pooled to constitute a sample for subsequent linear amplification and microarray hybridization.

*RNA isolation, linear amplification, and microarray hybridization* Immediately after LMD, RNA was isolated using Trizol (15596-026, Invitrogen Life Technologies, Carlsbad, CA, USA) using the manufacturer's protocol. Linear acrylamide was added as a carrier. RNA quality and quantity were 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 nanograms 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, CA, 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, Santa Clara, CA, USA).

*GeneChip hybridization* Twenty micrograms of biotinylated RNA was subsequently fragmented using RNA Fragmentation Reagents (No. AM8740, Ambion, Austin, TX, USA). The biotinylated 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, Leiden, 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 GeneChip Operating Software (Affymetrix, Foster City, CA, USA). For each brain region, the resulting CEL files containing probe-level information were then normalized and converted to gene intensity values by the GeneChip Robust Multi-array Average (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

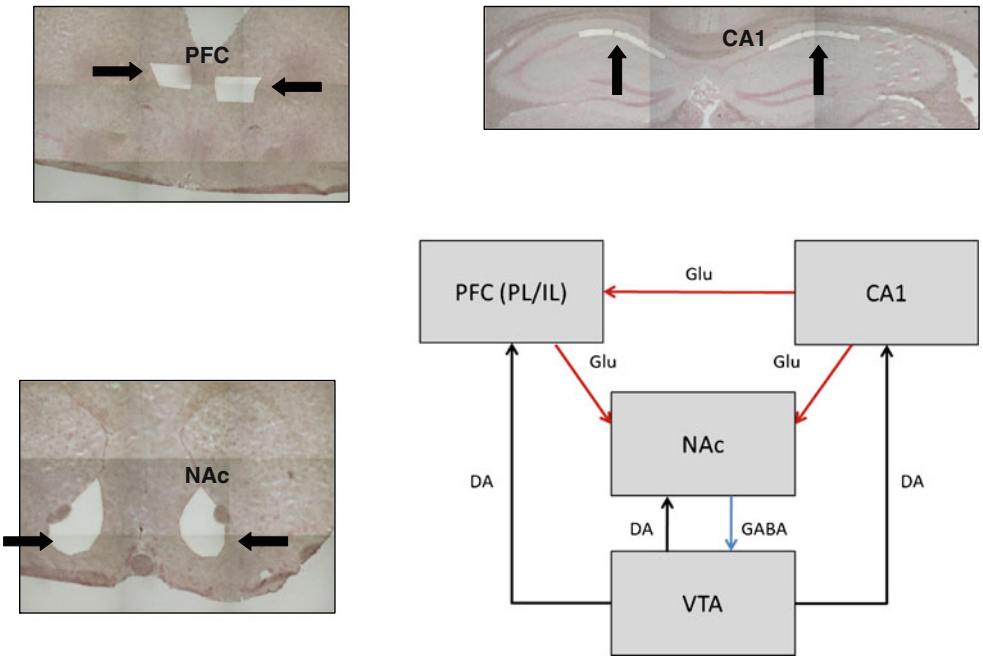


**Fig. 2** Scheme showing the connection between the selected brain areas including examples of LMD (indicated by *thick arrows*). *PFC* prefrontal cortex, *IL* infralimbic, *PL* prelimbic, *NAc* nucleus accumbens, *CA1* cornu ammonis 1 region of the hippocampus, *VTA* ventral tegmental area, *Glu* glutamate, *DA* dopamine, *GABA* gamma-aminobutyric acid. *Red arrows* indicate glutamatergic neurons, *black arrows* indicate dopaminergic neurons, and *blue arrows* indicate GABAergic neurons (Adapted from Thierry et. al., 2000)

*t* test (fold change>1.2 and *p* value cutoff of $p<0.01$) comparison between HR and LR. Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, http://www.ingenuity.com) version 7.5 was used to identify pathways, networks, and for gene list matching to published datasets of genes involved in specific transcription regulation systems (Mef2, cAMP response element [CRE], and 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* Primers for real-time quantitative polymerase chain reaction (RT-qPCR) validation were designed using Primer3 freeware within the target sequence used by Affymetrix for probe design. Primers were checked for specificity using BLAST (NCBI, Bethesda, MD, USA) and for hairpins and self-complementarity using oligo 4.0 (MBI, Cascade, CO, 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 iScriptTM cDNA Synthesis Kit (170-8897, Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. RT-qPCR was performed on a Lightcycler 2.0 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) using the Lightcycler FastStart DNA MasterPLUS SYBR Green I Kit (Roche). The standard curve method was used to quantify the expression differences (Livak and Schmittgen 2001). The nonparametric Mann–Whitney test was used to assess significant differential gene expression between LR and HR.

Brain tissue from follow-up experiment 2 was used to replicate the changes in gene expression between LR and HR found in the CA1 area in an independent experiment. For this purpose, the dorsal hippocampus was dissected from frozen brain and eight punches containing CA1 tissue were obtained from two 1-mm tissue sections. RNA was synthesized to cDNA without further amplification and RT-qPCR and data analysis were performed as previously reported (Christensen et al. 2010) 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 Fig. 3a. On average, animals that received amphetamine pretreatment on days 1–5 (amph/amph) were more responsive to the acute amphetamine challenge than saline pretreated mice (sal/amph), signifying the occurrence of sensitization. However, a large interindividual variability was observed in the amph/amph group. The 10% amph/amph animals with highest locomotor response to amphetamine on day 20 were designated HR ($n=10$), while the 10% animals with the lowest response were designated LR ($n=10$). In an independent follow-up study, it was demonstrated that the HR and LR phenotypes are stable until at least 1 week after the first drug challenge (Fig. 3b). The

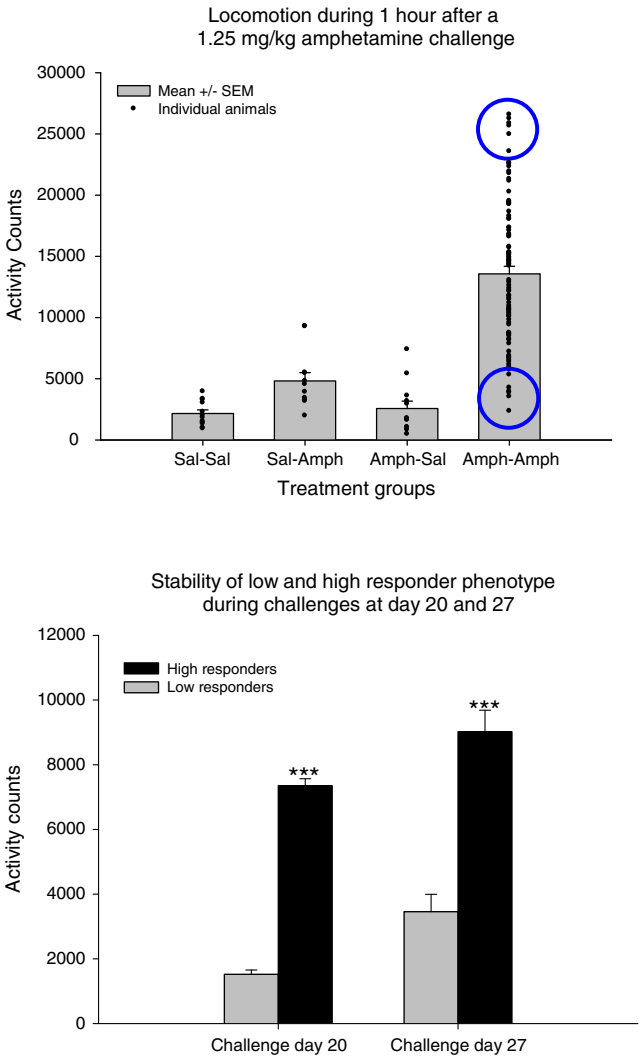


**Fig. 3** **a** Locomotor responses to the amphetamine (1.25 mg/kg) or saline challenge on day 20. Data are represented as total activity count over the 60-min treatment period. *sal/sal* $n=10$, *sal/amph* $n=10$, *amph/sal* $n=10$, *amph/amph* $n=100$. *Circles* indicate the 10% population extremes (LR, $n=10$; HR, $n=10$) 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-min treatment period. LR, $n=10$; HR, $n=10$. ***$p<0.001$ vs LR (Mann–Whitney rank sum test)

slight increase in both groups might signify further incubation of sensitization which is known to occur with prolonged withdrawal periods.

### Amphetamine exposure is not different in HR and LR

Amphetamine in total brain homogenates was measured in two groups ($n$=10 each) of mice with locomotor activity counts just below the highest (21,289±377 counts) and just above the lowest responders (4,387±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 behavioral 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 (Fig. 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 probe sets on the Affymetrix GeneChip 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; Fig. 4a). These gene lists are referred to as the primary lists (Supplementary material, Table SII). Comparison of the three primary lists revealed no overlapping genes (Fig. 4b). Moreover, pairwise correlation analysis of all expression values in the 60 samples showed a clear distinction in region-specific expression signatures (Fig. 4c). These specific molecular signatures of the analyzed 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%).

### 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 was 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 (Fig. 1). In contrast to the validation described above, the six genes were measured in non-amplified mRNA derived from manually dissected CA1 rather than LMD. 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 (Fig. 5).

### Validated genes overlap with several gene classes, including GR-, Mef2-, and CRE-regulated genes

The genes differentially expressed in CA1 were subjected to 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 repressor element 1 silencing transcription factor (REST) (see 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 2,000 randomly selected genes from the entire list of probe sets (~45,000 probe sets). This comparison indicated a clear overrepresentation of GR, CRE, and Mef2 promoter-regulated genes among the differentially regulated gene set in CA1 (Fig. 6). The comparison was repeated with a large number of randomizations of the R2K set and the differences shown in Fig. 6 were found to be stable.

## 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

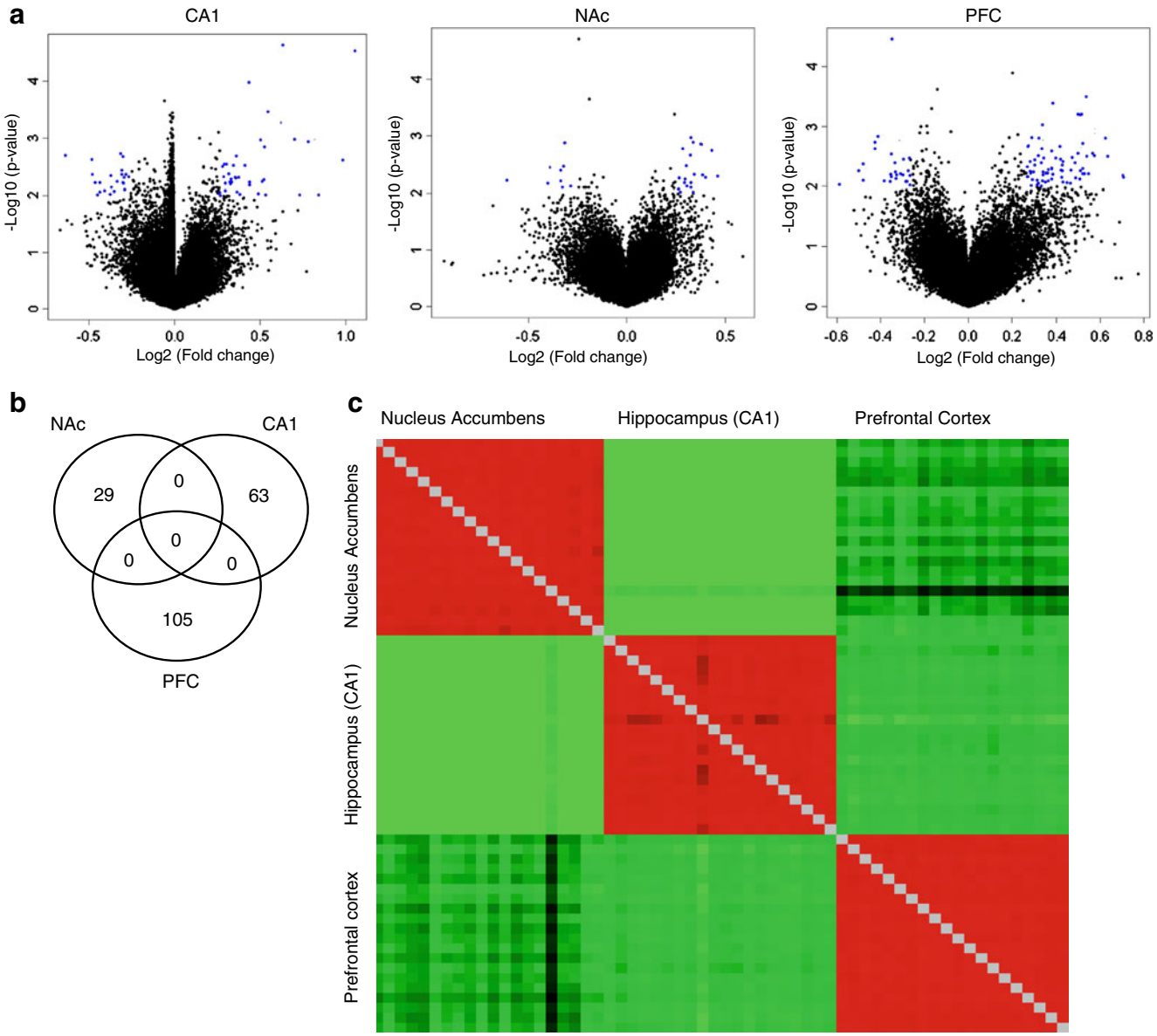


**Fig. 4** **a** Volcano plots of −log10 (*p* value) vs. log2 (fold change). The *blue points* in each graph indicate the Affymetrix probe sets that passed the *t* test $p<0.01$ and fold change>1.2 statistical requirements. **b** Venn diagram of genes differentially expressed between HR and LR. Genes meeting the fold change>1.2, $p<0.01$ criteria were included. No common genes were identified when comparing CA1, PFC, and NAc. **c** Correlation matrix of expression levels between all 60 samples in the experiment. Differential expression between tissues was clearly identified. Correlation analysis was not able to differentiate between HR and LR groups

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, 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 most likely reflects the underlying mechanism for sensitization and may point to why some individuals get schizophrenia whereas others do not.

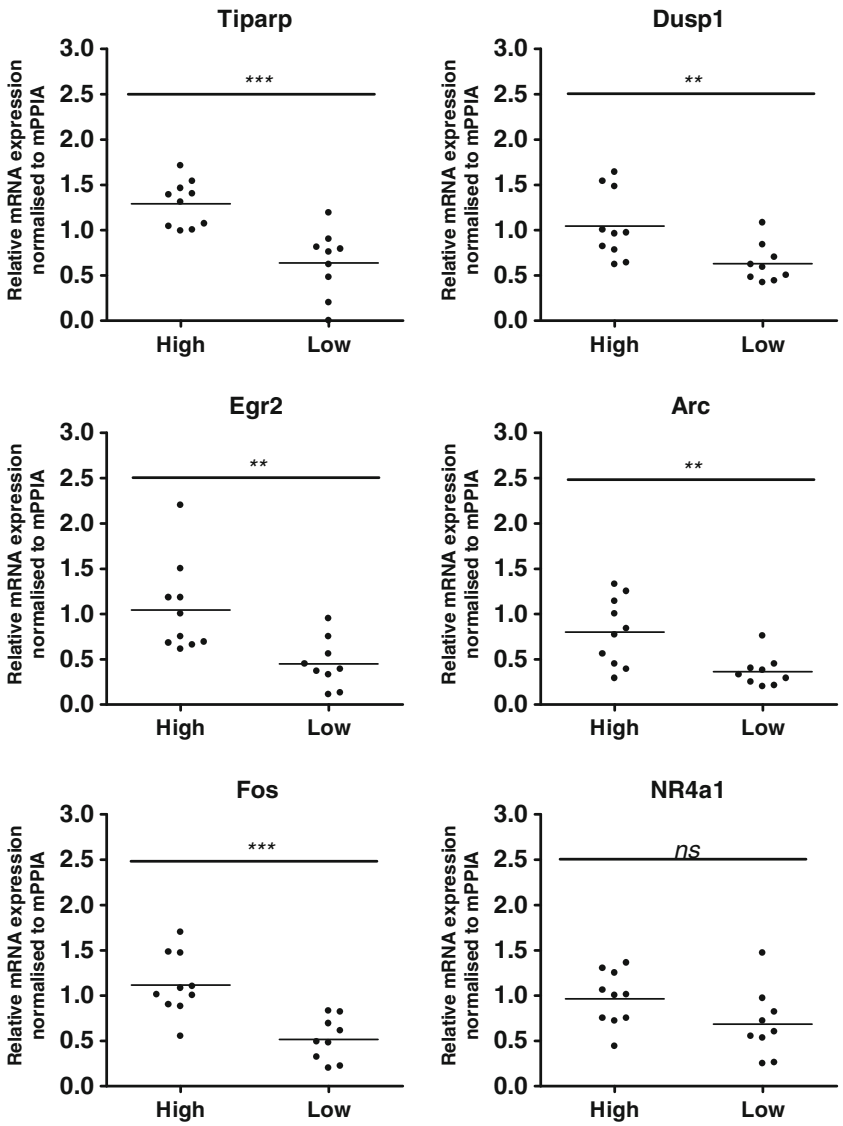


**Fig. 5** RT-qPCR validation results of gene expression differences between LR and HR in the CA1 region of the hippocampus in the second animal experiment. $^{**}p<0.01$ vs LR; $^{***}p<0.001$ vs LR (Mann–Whitney rank sum test)

The 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 h after amphetamine challenge. Gene expression signatures were highly brain region-specific, which is not surprising given that these brain regions differ so extensively in basal molecular makeup. We found the strongest differential expression between LR and HR 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) and not considered CA1 to play a key role in psychostimulant effects. However, our data are consistent with recent literature pointing to a prominent role of the hippocampus and DA 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 subregion of the 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. (2007) showing how the hippocampus controls DA neuron activity, possibly by increasing the number of DA neurons that can be activated by salient signals. In contrast, antipsychotic phenotype measured as reduced amphetamine-induced locomotion and release of DA in the 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).

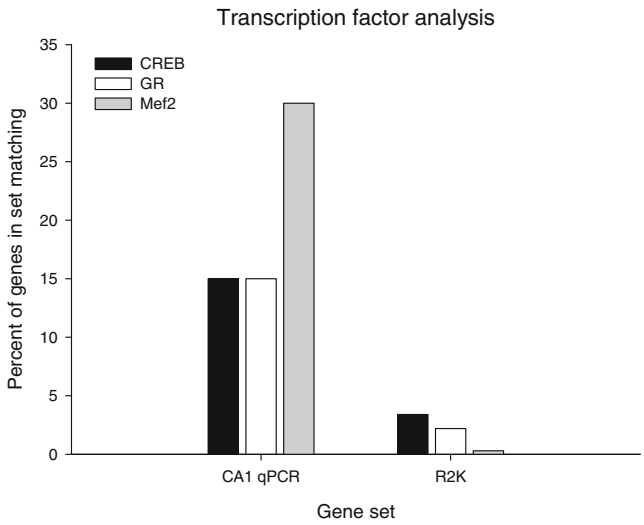


**Fig. 6** Comparison of genes regulated in CA1, NAc, and PFC to genes involved in specific transcriptional regulation as identified by ChIP/ChIP experiments. For each of the brain areas, the comparison was made for the genes identified in expression array and for those confirmed by qPCR. The R2K dataset represents 10×2,000 random probe sets, indicating background signal and size difference of ChIP/ChIP datasets used. Genes compared are those listed in Supplementary Table SI and shown in Fig. 4. (Supplementary Table SIII)

Furthermore, preventing synaptic transmission in the dorsal region of the hippocampus by local infusion of the anesthetic lidocaine is able to block the expression of behavioral sensitization to amphetamine (Degoulet et al. 2008). 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).

Since we only looked at NAc, PFC, and CA1 in the current study, we cannot exclude that gene expression differences in other dopaminergic brain regions may have contributed to the development of behavioral sensitization, e.g. the VTA or the amygdala (Yuferov et al. 2005). 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. Several other studies have also found IEGs to be responsive to amphetamine in the brain. Most studies show an upregulation of IEGs in the striatum in response to acute amphetamine, while chronic administration has been shown to blunt the effects of a single dose (for review, see McCoy et al. 2011). In contrast, Shilling et al. (2006) showed a downregulation of several IEGs in the PFC of HR 24 h after a single injection of methamphetamine. Downregulation 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 upregulated in the HR is c-*fos*. Interestingly, Zhang et al. (2006) found that c-*fos* downregulation in DA D1 receptor-containing neurons attenuates cocaine-induced behavioral sensitization. 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 h onwards in multiple brain regions, which can be blocked by giving a DA 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 (Fig. 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 HR and LR 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 psychostimulant 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 nonsensitized 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 sequence on the Pro-opiomelanocortin (POMC) promoter in the hypothalamus, preventing the GR-induced inhibition of Adrenocorticotropic hormone (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 others of the differentially expressed genes we identified are glucocorticoid responsive, such as Dusp1 (King et al. 2009). Hippocampal Dusp1 expression is known to be induced by glucocorticoids (Morsink et al. 2006), suggesting that HR 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 and Schmittgen 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 h 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 LR and HR.

*cAMP response element binding* We found that CRE family transcription factors overall can affect at least 15% of qPCR confirmed AMPH-regulated genes in CA1 (Fig. 6). In a random set of genes picked from the gene expression chip, this number is low (3.4%, see Fig. 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 et al. 2008). Genes relevant for amphetamine sensitization and DA function whose transcription is regulated by CREB include c-*fos*, brain-derived neurotrophic factor (BDNF), tyrosine hydroxylase, and many neuropeptides (such as somatostatin, enkephalin, VGF, and corticotropin-releasing hormone) (Purves et al. 2008). CREB has a well-documented role in neuronal plasticity and long-term memory formation in the brain (Silva et al. 1998). Altered cAMP signaling was previously identified to be one of the most consistent changes in the striatum in an extensive study using three genetic and one pharmacological mouse models of psychostimulant or DA supersensitivity (Yao et al. 2004). The molecular and cellular mechanisms underlying plasticity related to behavioral sensitization to psychostimulants and learning and memory may be similar (Yao et al. 2004). This fits well with our observations that CREB and GR, both known to be important for learning and memory, may be key transcription factors regulating behavioral sensitization to amphetamine.

*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 three 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 LR and HR have the same genetic background and received an identical sensitization protocol using exactly the same amphetamine dosing regimen. Nonetheless, our setup using LMD 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 twofold change is reported as the cutoff below which microarray and qPCR data begin to lose correlation. Dallas et al. (2005) reported decreased correlations for genes expressing <1.5-fold change using qPCR and oligonucleotide microarrays. 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. Detection of false positives may also have contributed to the relatively low success rate of validation. Using a microarray with 45,000 probe sets, a $p$ value of 0.01 is likely to yield 450 false positives.

While comparing gene expression differences between LR and HR with the same genetic background and an identical sensitization protocol is the strength of the current study, a limitation is that we did not perform gene expression measurements in response to amphetamine challenge in the nonsensitized saline group as a reference.

*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 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 remeasurement 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 HR or LR phenotype.

*Timing* The time at which the gene expression changes were measured in the current study, i.e. 1 h 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 LR and HR 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 LR and HR become evident, while under basal conditions there are no apparent differences. Furthermore, 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. (2001) found differential gene expression in the frontal cortex up to 16 h 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. Nonetheless, the success of our approach is evident since the changes in gene expression we identified in CA1 reproducibly discriminate HR from LR, as demonstrated in the independent follow-up experiment we performed.

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.

**Disclosure/conflicts of interest** I.E.M. de Jong, K. Vielsted Christensen, K. Potempa, J. Torleif Pedersen, J. Egebjerg, P. Kallunki, E.B. Nielsen, and M. Didriksen are all full-time employees of H. Lundbeck A/S, Copenhagen, Denmark.

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