Determinants of Psychosis Vulnerability

Focus on MEF2- and Glucocorticoid Signaling

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Preface

PREFACE

Schizophrenia is often inherited, but even in monozygotic twins one sibling can be more susceptible to schizophrenia than the other. This raises the question what the cause of this difference in susceptibility in genetically identical individuals might be. The objective of this thesis research was to identify novel susceptibility genes and pathways for psychosis in a psychostimulant mouse model which is considered a model for psychosis.

Using genome-wide micro-array analysis of transcripts expressed in discrete laser-dissected brain regions of mouse brain we found a large number of genes differentially expressed particularly in the hippocampal CA1, a region known to drive mesocortical dopaminergic activity which has a prominent role in the pathogenesis of schizophrenia. Profound differences were found in expression of target genes of Myocyte Enhancer Factor 2 (MEF2) and the Glucocorticoid Receptor (GR), suggesting that this gene network is involved in sensitivity to amphetamine. In primary hippocampal neuronal cultures knockdown of MEF2 not only reduced the expression of its target gene c-Jun, but also abolished its regulation by GR. Moreover, activation of MEF2 by depolarization of these neurons was found to be attenuated by glucocorticoids suggesting a complex mutual feedback regulation of the two transcription factors. Finally, *in vivo* in the mouse MEF2 and GR appeared to be active in the induction rather than in the expression phase of amphetamine sensitization.

Taking our data together, the findings suggest that in the hippocampus the effect of stress, via glucocorticoid activation of GR, can modulate the role of MEF2 target genes in induction of behavioral sensitization. This finding points to the hippocampus as an exciting target for further studies on the role of MEF2 and GR in the precipitation of psychosis susceptibility.

Chapter 1 | General Introduction

Contents of Introduction

1.1 Schizophrenia and Psychosis

Schizophrenia has a prevalence of approximately 1% and is one the most life-debilitating psychiatric disorders. It is also a very costly disorder since the onset of the disease is typically during young adulthood, leaving the patients with a reduced ability to contribute to society, while life expectancy is high (McEvoy 2007, Wu *et al.* 2005, Mangalore & Knapp 2007).

Schizophrenia is characterized by a combination of positive, negative and cognitive symptoms. Positive symptoms, such as hallucinations, delusions, disordered thought and speech, are not normally experienced by most individuals, but are present in schizophrenics as manifestations of psychosis. Negative symptoms are deficits in emotional and cognitive processes and become manifest as depressed mood, blunted affect and lack of motivation. Cognitive impairment can already be observed at young age and may serve as a prodromal factor to monitor children with a high risk of developing schizophrenia in later life.

The diagnosis of schizophrenia is set in most cases when the patient is suffering from overt psychosis. A psychotic episode is defined, according to DSM-IV, as a state of two or more of the following symptoms: delusions, hallucinations, disorganized speech, disorganized behavior and negative symptoms for at least 1-6 months, with significant negative pressure on social life (e.g. work) (American Psychiatric Association. & American Psychiatric Association. Task Force on DSM-IV. 1994).

Psychoses can be triggered by several factors such as exposure to a severe stressor or psychostimulant drugs. The moment of the first psychotic episode is generally considered to be preceded by several neurodevelopmental deficits that can be divided in several factors that will be described below (Velakoulis *et al.* 2000, Brown 2011)

1.2 Development of schizophrenia

Over the years numerous causes and correlates for schizophrenia have emerged, ranging from paternal age, prenatal events like bacterial and viral infections, obstetric complications to postnatal experiences, and even the season of birth and urbanicity have been suggested as risk factors. All these possible contributors have been extensively studied and reviewed elsewhere (Brown 2011, Velakoulis et al. 2000, Rapoport *et al.* 2005, Fatemi & Folsom 2009). More generally, the developmental cascade precipitating full-blown schizophrenia can be divided in 3 steps: 1. genetic vulnerability; 2. adverse environmental factors; and 3. a trigger (Cannon *et al.* 2003). This is also called the two- or three-hit model of schizophrenia depending on whether a distinction is made between genetic vulnerability and adverse environmental factors (Maynard *et al.* 2001) (Fig. 1).

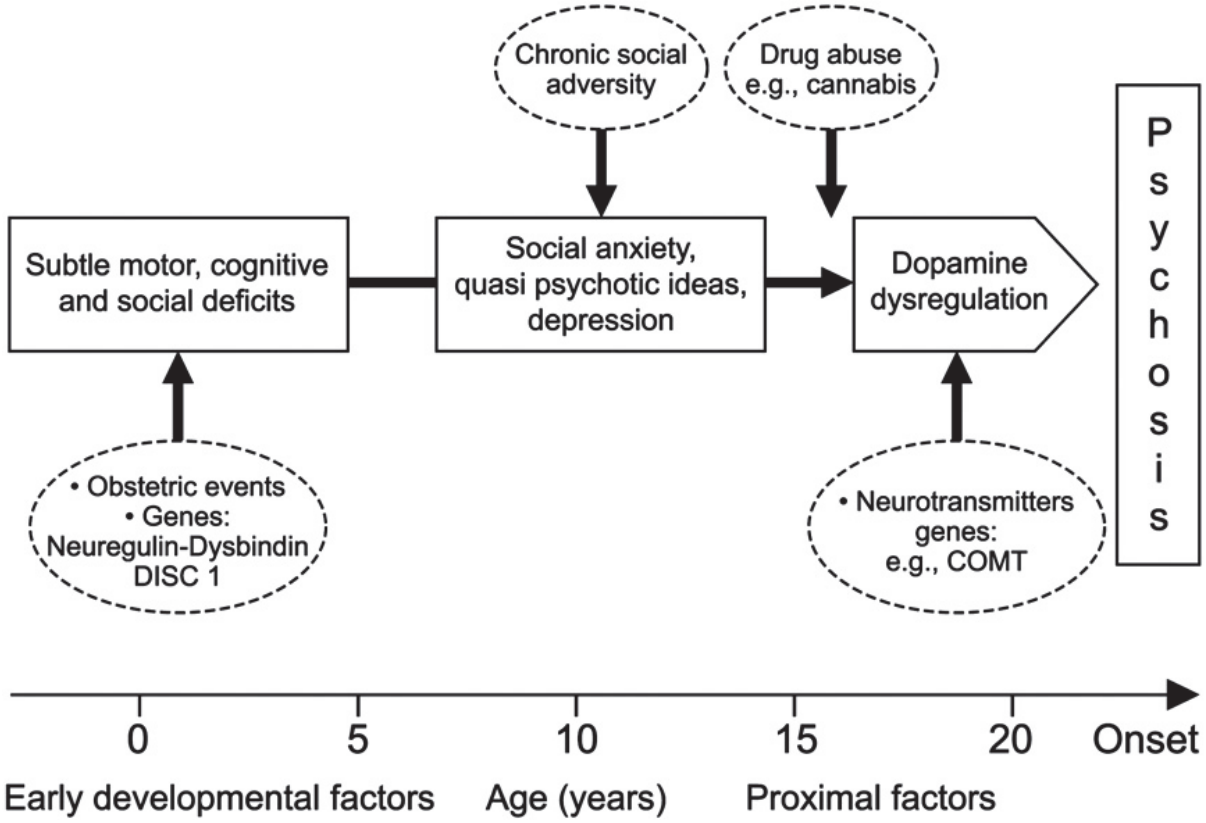

Figure 1| Developmental cascade of schizophrenia. Genetic predisposition for schizophrenia may lead to prodromal symptoms such as cognitive deficits. Combined with chronic social adversity this predisposition may give rise to an altered neuronal development giving rise to quasi psychotic ideas that at this point are difficult to distinguish from normal childhood fantasies. Drug abuse or another stressor during adolescence might then trigger a full-blown psychotic event, the first stage of schizophrenia (Murray *et al.* 2008). Reprinted with permission.

1.3 Genetics of schizophrenia

Twin studies are invaluable for studying the relative contribution of genetic predisposition to polygenic disorders. Especially the comparison of dizygotic and monozygotic twins, who share ~50% and 100% of their genetic makeup respectively, provides information on the extent of the genetic contribution to a disorder (Rahmioglu & Ahmadi 2010). For schizophrenia the concordance rate is 50% in monozygotic twins, while as genetic homogeneity with the schizophrenic individual decreases the risk of developing schizophrenia is concomitantly reduced, reaching approximately 1% in the general population (Cardno & Gottesman 2000). Hence it is clear that genetic make-up plays a major role in susceptibility to schizophrenia. Many different genetic studies have been conducted in order to gain more insight into which genes or chromosomal loci contribute to schizophrenia susceptibility, including linkage studies, genome-wide association studies (GWAS), copy number variation studies (CNV) and candidate gene approaches. These different approaches will be briefly described below, including their best validated susceptibility genes/loci.

1.3.1 Genetic linkage studies

Genetic linkage studies are designed to find susceptibility genes within pedigrees of families with a higher than average disease prevalence. With regard to schizophrenia, linkage studies were the first genetic studies designed to understand schizophrenia vulnerability. The basis of this type of studies is to find the common denominators of genetic make-up within a family where several members might suffer from schizophrenia while others do not. Together with other linkage studies, several chromosomal loci have been found and replicated that are connected to schizophrenia such as 1q42, 6p22-24, 1q21-22 and 13q32-34 (Brzustowicz *et al.* 2000, Straub *et al.* 2002, Blouin *et al.* 1998, Riley & Kendler 2006, Lewis *et al.* 2003, Millar *et al.* 2001, Ng *et al.* 2009).These chromosomal regions harbor many genes that were the first likely candidates to study in schizophrenia development such as Disrupted in Schizophrenia 1 (DISC1), Regulator of G-protein Signaling 4 (RGS4), Dystrobrevin DTNBP1, D-Amino Acid Oxidase (DAAO/G72).

1.3.2 Genome-wide association studies

Genome-wide association studies (GWAS) compare occurrence of single nucleotide polymorphisms (SNPs) between unrelated groups of patients and (matched) controls. SNPs are heritable genetic changes that occur frequently (>1%) in the general population and might result in altered expression or functionality of the protein. GWAS studies are relatively new for schizophrenia, with the first study published only 7 years ago (Mah *et al.* 2006). Within this short timespan the studies have evolved enormously, both in design and number of subjects. For example, the previously mentioned study by Mah et al compared over 300 patients with matched controls, while one of the more recent studies

by Lee et al included almost 9,000 patients and 12,000 controls (Mah et al. 2006, Lee *et al.* 2012). The number of SNPs screened increased as well from ~26,000 to almost 1 million. Among the replicated results are the MHC region (Major Histocompatibility Complex), DTNBP1 and TCF4 (Transcription Factor 4) (Bergen & Petryshen 2012). The MHC region is the region most strongly linked to schizophrenia in all genetic studies and harbors genes that play an important role in the immune system, of which the role in schizophrenia is largely unknown (de Jong *et al.* 2012, Girard *et al.* 2012). DTNBP1 plays a role in glutamate release from synaptic vesicles and is reportedly downregulated in the hippocampus and prefrontal cortex of schizophrenia patients (Coyle 2006) and enhanced expression of TCF4 has been found in brains of schizophrenia patients as well as in the brains of psychostimulant-treated animals (Kurian *et al.* 2011, Mudge *et al.* 2008)..

1.3.3 Copy-number variations

Copy-number variations (CNVs) are large deletions or multiplications of DNA sequences and give rise to enhanced or repressed expression of certain genes or splice variants. Well known examples are Down syndrome, where subjects have an additional copy of chromosome 21, and velocardiofacial syndrome (VCFS) were patients have a deletion of a part of chromosome 22.

CNVs are more frequently apparent in schizophrenia patients compared to healthy controls (Walsh *et al.* 2008), although it is estimated that only 2-4% of the genetics of schizophrenia can be linked to CNVs (St Clair 2009). CNV studies in schizophrenia patients found changes at loci such as 22q11.2, the locus where the DISC1 gene is located (Xu *et al.* 2008) and which is also part of the deletion found in VCFS. Approximately 1% of the schizophrenic population has the deletion of this locus but up to 25% of the people carrying the deletion have schizophrenia (Bassett *et al.* 2005). It is therefore considered one of the major genetic vulnerability factors linked to schizophrenia. Although it is difficult to link the CNV to certain genes, because CNVs often span multiple genes, recent advances in CNV studies implicate a role specifically for postsynaptic glutamate signaling underlying neural plasticity (Kirov *et al.* 2012, Walsh et al. 2008).

Table I | Chromosomal locations identified in schizophrenia genetic studies.

Chromosome	Region/genes	Study Type[a]	Cases/controls[b]	P/OR/LOD[c]	Reference
1q21-q22	Regulator of G-protein signaling 4 (RGS4)	Linkage	22 /304	6.5 (LOD)	(Brzustowicz et al. 2000)
1q21.1	Regional deletions	CNV	Original: 1433/33250 Follow-up: 3285/7951	14.83 (OR) $P = 2.9*10^{-5}$	(Stefansson et al. 2008)
1q21.1		CNV	11372/47311	9.5 (OR) $P = 8.5*10^{-6}$	(Levinson *et al.* 2011)
1q42	Disrupted in Schizophrenia 1 (DISC1)	Linkage	1 /87	3.6 (LOD)	(Blackwood *et al.* 2001)
2q32.1	Zinc Finger Protein 804A (ZNF804A)	GWAS	Original: 479/2937 Follow-up: 6829/9897	1.12 (OR) $P = 1.61*10^{-7}$	(O'Donovan *et al.* 2008)
6p21.32	Major Histocompatibility (MHC) Region	GWAS	3322/3587	0.82 (OR) $P = 4.79*10^{-8}$	(Purcell *et al.* 2009)
		GWAS	Original: 2663/13498 Follow-up: 4999/15555	1.21 (OR) $P = 2.1*10^{-8}$	(Stefansson et al. 2009)
6p21.3-p22.1	Major Histocompatibility (MHC) Region	GWAS meta-analysis	2681/2653	0.88 (OR) $P = 9.54*10^{-9}$	(Shi *et al.* 2009)
		GWAS	Original: 9394/12462 Follow-up: 8442/21397	1.15 (OR) $P = 2.18*10^{-12}$	(Ripke et al. 2011)
6p22.3	Dystrobrevin binding protein 1 (DTNBP1)	Linkage	270 /1425	2.2 (LOD)	(Straub et al. 2002)
8p22-p21	Neuregulin 1 (NRG1)	Linkage	54 /363	3.6 (LOD)	(Blouin et al. 1998)
		Linkage	33 /110	2.5 (LOD)	(Stefansson *et al.* 2002)
13q22-34	D-Amino Acid Oxidase (DAAO/ G72)	Linkage	54 /363	4.2 (LOD)	(Blouin et al. 1998)
		Linkage	Original: 213/241 Follow-up: 183/183	P = <0.05 P = <0.05	(Chumakov *et al.* 2002)
15q13.3	Regional deletion	GWAS	Original: 1433/33250 Follow-up: 3285/7951	11.54 (OR) $P = 5.3*10^{-4}$	(Stefansson et al. 2008)
	Regional deletion	CNV	Meta-analysis: 10866/45913	12.1 (OR) $P = 6.9*10^{-7}$	(Levinson *et al.* 2011)
16p11.2	Regional duplications	CNV	9859/29589	9.5 (OR) $P = 2.6*10^{-8}$	(Levinson et al. 2011)
		CNV	Original: 1906/3971 Follow-up: 2645/2420	14.5 (OR) $P = 4.8*10^{-7}$	(McCarthy *et al.* 2009)
18q21.2	TCF4	CNV	Original: 2663/13498 Follow-up: 4999/15555	1.23 (OR) $P = 4.1*10^{-6}$	(Stefansson et al. 2009)
		GWAS	Original: 9394/12462 Follow-up: 8442/21397	1.23 (OR) $P = 1.05*10^{-6}$	(Ripke et al. 2011)
22q11.2	Regional deletions	CNV	Meta-analysis: 11365/45361	20.3 (OR) $P = 7.3*10^{-13}$	(Levinson et al. 2011)
		CNV	695 patients	unknown	(Karayiorgou *et al.* 1995)

Chromosomal locations and allocated genes found to be associated with the diagnosis of schizophrenia. Note that some studies included a two-stage approach, where the original findings where followed up by an additional set of cases and controls. In that case, odds ratios are combinations of both the original and the follow-up study.

[a] Study type: CNV: Copy-number variation; GWAS: Genome-wide association study

[b] Linkage studies often don't include controls. The numbers given in that case refer to the number of pedigrees or child-parents trios vs total members included in the study.

[c] Differences are calculated based on study type. LOD scores are defined as the Logarithm of ODds; for example: a LOD of 2 means 100 to 1 odds that the observed linkage did not occur by chance. OR is Odds Ratio and is defined as the fold difference in risk of a schizophrenia patient of carrying a specific CNV or SNP vs healthy controls. P-values are given when provided in the study and define the strength of the give OR.

1.3.4 Gene expression studies

In gene expression studies an underlying hypothesis or genes and pathways that emerged from genetic studies or from mRNA expression analysis are the central focus. For example, by studying

gene expression in post-mortem brain material of schizophrenic patients and controls, DISC1 was identified as a candidate gene that correlated with a high degree of schizophrenia, as well as DTNBP1, NRG1, Calcineurin (CaN) and members of the CaN pathway (Yamada *et al.* 2007, Norton *et al.* 2006, Stefansson et al. 2002) and many others. However, post mortem studies generally consist of small sample sizes and confounders such as cause of death, medication history and postmortem delay. This has significant effects on gene expression, making it hard to draw conclusions on causality. Nevertheless, candidate gene studies, performed almost exclusively in animals and cell culture, have provided enormous insight in the pathways of candidate genes and the underlying mechanisms (for references to all known target genes see Heimer 2012, Carter 2012).

Overall, genetic studies have provided insight on possible genes and pathways that are involved in schizophrenia. New methods are still being developed to integrate results from different genetic studies to find common genes and pathways that are linked in the development of schizophrenia (Ayalew *et al.* 2012). However, it remains challenging to determine the causality of the observed changes in relation to the development of the disorder. Research is still very much needed to pinpoint which genes contribute to the disorder and also in which phase of the development. Nonetheless, the development of schizophrenia can only partly be explained by genetics, and understanding the environmental factors that contribute to disease onset is equally important.

1.4 Environmental effects

Throughout the years, a growing number of environmental effects have been pinpointed as possible contributors to schizophrenia development. Some examples are in utero infections, famine, in utero vitamin D deficiency, obstetrical complications (Cannon *et al.* 2002, St Clair *et al.* 2005, Buka *et al.* 2008, McGrath *et al.* 2010), urbanicity, migrant status, social isolation, cannabis use and early-life stress (van Os *et al.* 2010, van Os *et al.* 2003, Scheller-Gilkey *et al.* 2004, Myin-Germeys & van Os 2007, Morgan & Fisher 2007, McGrath et al. 2004, Allardyce *et al.* 2005). However, many controversies exist in these studies and the need for prospective studies is becoming increasingly important (Oh & Petronis 2008). In the next sections of this thesis the focus will be on the stress system and the effect of early-life adverse events.

1.4.1 Adverse Life Events and the role of the stress system

Stressful life events during childhood, like physical or sexual abuse, potentiate the susceptibility to a wide variety of physical and mental disorders, including psychotic disorders (Shevlin *et al.* 2008, Agid *et al.* 1999). There are large individual differences in the impact of stressors, but generally the most stressful experience is uncertainty, lack of information and the inability to predict and control situations perceived as fearful (Dickerson & Kemeny 2004, Jones & Fernyhough 2007). Interestingly, several studies failed to show differences in the severity of adverse life events to which schizophrenia patients and healthy controls are exposed (Devylder *et al.* 2012, Rabkin 1980), but rather suggested that schizophrenic patients may perceive events as being more stressful and fearful than healthy controls. It has therefore been suggested that stressful life events in themselves are not enough to evoke schizophrenia but may contribute to the onset of schizophrenia in combination with an enhanced stress vulnerability. This is called the "*diathesis-stress model*" or "*stress-vulnerability hypothesis of schizophrenia*" (Zubin & Spring 1977). Adverse life events have been linked to relapse (Hirsch *et al.* 1996, Bebbington *et al.* 1996, Norman & Malla 1993) and predict worse outcome in schizophrenia patients (Rosenberg *et al.* 2007, Gil *et al.* 2009).

Besides the psychological reaction, the body also rapidly responds to a stressor by activation of the sympathetic nervous system, which triggers the release of adrenaline from the adrenal medulla. Adrenaline activates the fight-or-flight response and directs the blood flow, and hence the energy expenditure, away from systems that are not needed, such as the gastro-intestinal and reproductive systems, to organs such as the brain and the muscles. This response happens within seconds and is normalized after several minutes. At this time the hypothalamic-pituitary-adrenal (HPA)-axis is activated (Figure 2). The activity of the HPA axis is initiated by signals of limbic brain regions such as the amygdala, hippocampus and prefrontal cortex and by pathways ascending from the brain stem

that regulate the release of corticotrophin releasing hormone (CRH) and vasopressin from the paraventricular nucleus (PVN) of the hypothalamus. CRFand vasopressin trigger the synthesis of pro-opiomelanocortin (POMC) and the release of one of its cleavage products, adrenocorticotropic hormone (ACTH) by the anterior pituitary corticotrophs. ACTH is released in the bloodstream and subsequently triggers the release of the glucocorticoids (GCs) cortisol (man) and corticosterone (rodents) by the adrenal cortex. GCs are agonists of the mineralocorticoid and glucocorticoid receptors (MR and GR) (Reul & de Kloet 1985). GCs feedback via GR on the HPA-axis at the level of the pituitary and the hypothalamus to inhibit the system (De Kloet et al. 1998, Sarabdjitsingh et al. 2010). In addition trans-synaptic inputs to the PVN from higher brain regions in the limbic system, e.g. hippocampus, amygdala and prefrontal cortex, that are themselves under control of MR and GR mediated actions, can modulate HPA axis activity. In a healthy individual this negative feedback loop will result in a rapid normalization of the stress response and corticosterone will reach pre-stress levels within a few hours, depending on the initial height of the corticosterone response (de Kloet *et al.* 2005).

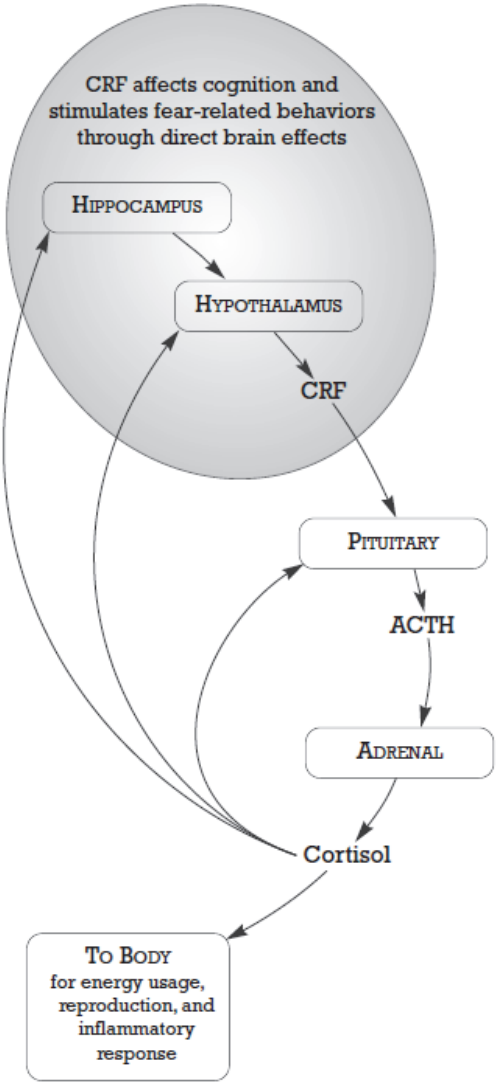

Figure 2 | Graphical representation of the HPA-axis and how cortisol feeds back on the pituitary, hypothalamus and hippocampus (Corcoran *et al.* 2001). Reprinted with persmission.

1.4.2 Glucocorticoid Receptor

The GR is ubiquitously expressed throughout the brain, but occurs most abundantly in the hippocampus, hypothalamus and anterior pituitary corticotrophs (De Kloet *et al.* 1998). The GR belongs to the group of nuclear receptors. Under basal conditions it resides in the cytoplasm, associated with chaperone proteins. Upon binding of GCs, GR dissociates from the chaperones and translocates to the nucleus where it binds to the DNA and initiates transcription of target genes (Datson *et al.* 2001).

Upon binding of the ligand to GR, several other proteins bind to GR such as cyclin-dependent kinase 5 (CDK5). CDK5 phosphorylates GR at serine 211 (220 in mice and 232 in rat (Beck *et al.* 2009)) and thereby attenuates its transcriptional potential (Kino *et al.* 2007, Wang *et al.* 2002). The amount of phosphorylation is correlated to the transcriptional activity induced by the ligand, with the synthetic glucocorticoid dexamethasone (DEX), inducing the strongest effects (Wang et al. 2002). In absence of CDK5 or in the case that GR is mutated, replacing serine 211 with an alanine group, the transcriptional activity of GR is significantly enhanced (Kino et al. 2007). In vivo in rats both acute and chronic stress enhance phosphorylation at the corresponding serine, suggesting that increased CDK5 activity is responsible for the increase in phosphorylation of GR (Adzic *et al.* 2009).

Within several minutes after treatment with a GR agonist, GR proteins dimerize and translocate to the nucleus where the dimers bind to the DNA. GR homodimer bind either directly to the DNA to so-called GR response elements or GREs or are tethered indirectly to the DNA by binding as monomers to other transcription factors such as NFkB or AP1 (Wei *et al.* 1998, King *et al.* 2009), thereby modulating the expression of other sets of genes (Morsink *et al.* 2006). Binding to GREs is generally considered to promote gene transcription, while binding via other transcription factors will indirectly lead to transrepression of genes. Much more information exists regarding factors that can influence GR-regulated gene transcription such as different GR isoforms, GR phosphorylation, the type of agonist binding to the GR and even the exact DNA sequence to which GR binds (Lu & Cidlowski 2005, Meijsing *et al.* 2009, Kino et al. 2007).

1.4.3 HPA-axis, glucocorticoids and psychotic symptoms

In untreated schizophrenic patients baseline HPA-axis activity is enhanced, resulting in elevated cortisol levelscompared to healthy controls (Ryan *et al.* 2004), although some inconsistencies have been observed (Yeap & Thakore 2005). Immediately preceding a psychotic episode, cortisol levels can rise up to 250% on top of the already elevated baseline levels (Sachar *et al.* 1973). In agreement with these results, post mortem studies in schizophrenic patients revealed reduced GR mRNA and protein levels in several brain regions like CA1, CA3 and DG of the hippocampus (Suchecki *et al.* 1995, Webster *et al.* 2002) as well as a decreased volume of the total hippocampus (Wright *et al.* 2000). GR downregulation is hypothesized to be a compensatory mechanism to protect against chronic hypercortisolism. As a consequence of GR downregulation , the HPA-axis becomes less responsive to negative feedback, resulting in prolonged cortisol secretion after exposure to a stressor. Approximately 25% of schizophrenia patients vs 5% of controls show this decreased negative feedback, which also can be demonstrated by a reduced ability of the synthetic glucocorticoid DEX to block the secretion of endogenous cortisol release (Duval *et al.* 2000, Yeragani 1990, Sharma *et al.* 1988).

In line with these findings in schizophrenia, GR blockade with the antagonist mifepristone has been reported to have beneficial effects in the treatment of psychotic depression, in particular in relation to the positive symptoms (Blasey *et al.* 2009, DeBattista *et al.* 2006). Furthermore mifepristone was found to enhance spatial working memory in bipolar patients (Watson *et al.* 2012).

1.5 Glucocorticoids and Dopamine

The stress system is hyperactive in schizophrenia. This has consequences for many neurotransmitter systems in the brain including the dopamine system which is the neurotransmitter system first implicated in the etiology of schizophrenia. How the dopamine system is involved in schizophrenia and how the interaction between dopamine and GCs proceeds will be described below.

1.5.1 Dopamine dysregulation in schizophrenia

Dopamine (DA) is released from dopaminergic neurons residing mainly in two locations, the Substantia Nigra (SN), or A9 and the Ventral Tegmental Area (VTA), or A10. DAergic efferents from the SN project to the striatum in the nigrostriatal pathway. DAergic neurons originating in the VTA project to the Prefrontal Cortex (PFC) via the mesocortical pathway, while VTA neurons projecting to the Nucleus Accumbens (NAc) constitute the mesolimbic pathway (Figure 3). In addition DA is released from a number of other cell groups among which the arcuate/periventricular A12 and A14 DA tubero-infundibular pathway controling pituitary prolactin release is the most prominent.

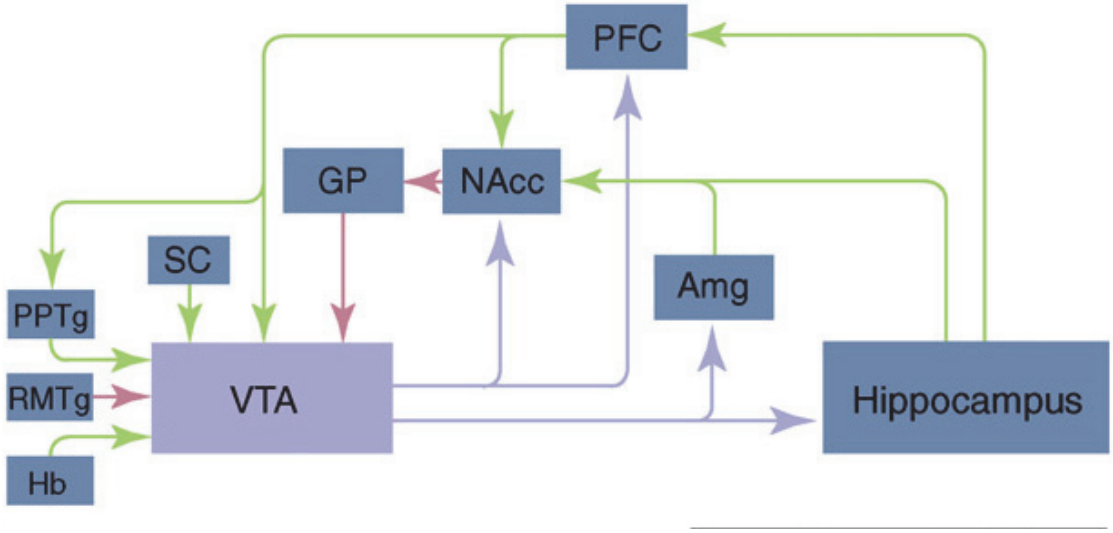

Figure 3 | Schematic representation of neuronal connections to and from the ventral tegmental area (VTA). Purple lines indicate dopaminergic connections to the nucleus accumbens (NAcc), prefrontal cortex (PFC), amygdala and hippocampus. Green represents excitatory glutamatergic connections and red inhibitory GABAergic connections (Shohamy & Adcock 2010). Reprinted with permission.

The first antipsychotic, chlorpromazine, as well as the other classical antipsychotics, were all found to target the Dopamine D_2 receptor (Stone *et al.* 2007). Moreover, clinical efficacy was found to correlate with the ability of antipsychotics to bind to the D_2 receptor (Seeman *et al.* 1975). This observation gave rise to the "*dopamine theory of schizophrenia*". Key components of this theory are that the mesolimbic dopamine pathway is hyperactive while the mesocortical dopamine pathway is hypoactive. The positive and negative symptoms of schizophrenia are attributed to these respective

changes in activity. In agreement with this, *invivo* imaging studies in humans revealed that pre-synaptic dopamine synthesis was significantly enhanced especially in acutely psychotic patients (Hietala *et al.* 1995) showing a marked correlation between positive symptoms and dopamine levels (Hietala *et al.* 1999). Moreover, post-mortem studies showed that D_2-receptors in schizophrenic individuals were significantly more sensitive to dopamine compared to non-schizophrenic persons (Seeman *et al.* 2006).

1.5.2 Interaction between glucocorticoids and dopamine signaling

Since stress and GCs are likely involved in the development of schizophrenia and preceding a psychotic episode GC levels rise, the question can be raised: do GCs influence the DA system?

As a first indication that GCs have an effect on the DA system, in rodents corticosterone was shown to enhance extracellular DA levels in the NAc, resulting in enhanced locomotor activity (LA), which is considered to be a reliable readout to measure dopaminergic effects. Subsequently dividing the animals in high responders (HR) and low responders (LR) to novelty based on their LA response, revealed that only the HR showed enhanced LA upon a corticosterone injection (Piazza *et al.* 1996). This suggests that corticosterone is able to enhance DA output in the HR. Furthermore, HR to novelty showed higher endogenous corticosterone levels, indicating that a hyperactive DA system is correlated with enhanced HPA-axis activity. Moreover, blocking GR with mifepristone (RU486) in amphetamine-sensitized animals (the process of repeated stimulations with amphetamine resulting in an enhanced response to the drug), prior to an amphetamine challenge, completely prevented the behavioral effect, showing that the GR plays an important role in the expression of sensitization (De Vries *et al.* 1996, Deroche *et al.* 1992, Piazza et al. 1991). Finally, both acute and chronic treatment with DEX significantly attenuated amphetamine-induced LA (Wrobel *et al.* 2005). Since DEX poorly enters the brain and is given preceding the amphetamine injection, it likely shuts down the HPA-axis at the level of the pituitary. This results in lower levels of corticosterone that are insufficient to enhance the amphetamine-induced LA, further reinforcing the notion that GCs are required for an enhanced dopaminergic function in the brain.

Many of the above-mentioned studies make use of a well-known animal model of schizophrenia, also known as the "*amphetamine sensitization model*". In the next chapter this model is discussed in more detail.

1.6 Modeling Vulnerability

In an attempt to model the changes in schizophrenia and search for neurobiological underpinnings and treatments, many different animal models have emerged. These models can roughly be divided in 4 different groups; 1. Genetic models, 2. Lesion-induced models, 3. Pharmacological models and 4. Neurodevelopmental models (Jones *et al.* 2011). Despite the wealth of information and new drug targets that have emerged from these models a general weakness of schizophrenia animal models is that features like delusions, hallucinations and poverty of speech cannot be modeled. Therefore most models fitting one of the four above-mentioned categories focus only on some of the symptoms of schizophrenia or on so-called endophenotypes, hereditary changes that underlie the overt symptomatology and are brought about after stimulation of the animal with e.g. a stressor or psychostimulant and/or specific testing (Feifel & Shilling 2010). To model the positive symptoms of schizophrenia the amphetamine model is often used since repeated amphetamine administration inducesdopaminergic changes and behaviors comparable to psychosis (Peleg-Raibstein *et al.* 2008, Featherstone *et al.* 2007, Shilling *et al.* 2006, Tueting *et al.* 2006). This includes enhanced LA and decreased sensorimotor gating, defined as the reduced ability of an individual to filter out irrelevant stimuli (Tenn *et al.* 2003). These endophenotypes are easier to model since their underlying pathophysiology is more comparable to humans.

1.6.1 Amphetamine Sensitization

The most accepted theory for the working mechanism of amphetamine is called the "*weak base"* or "*vesicle depletion model*". This model implies that amphetamine inhibits the dopamine transporter and the vesicular monoamine transporter, thereby increasing the dopamine concentration in the synaptic cleft (Figure 4). Increased dopamine release is found in the shell of the NAc (Gambarana *et al.* 1999) and seems to be the result of so-called dopamine supersensitivitiy (Seeman *et al.* 2005). Enhanced sensitivity to amphetamine is mediated by enhanced expression of a more sensitive isoform of the D2 receptor, also known as the D_2L isoform, in the dorsal striatum. The upregulation of the D_2L isoform was found to be significantly correlated with enhanced sensitivity towards amphetamine (Giordano *et al.* 2006).

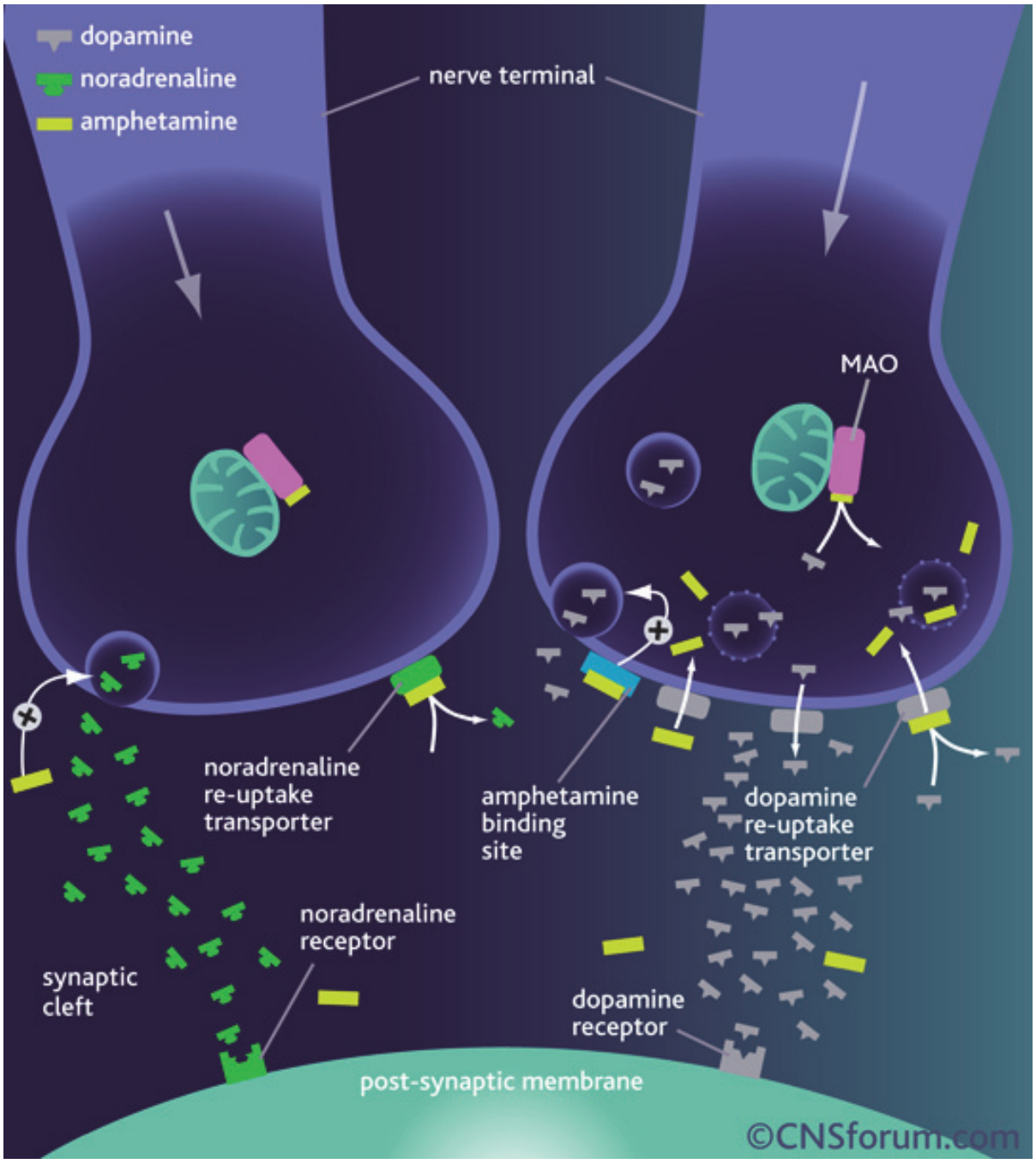

Figure 4 | Working mechanism of amphetamine. For explanation, see chapter 1.6.1. First, amphetamine releases newly synthesized dopamine into the synaptic cleft. Second, it enhances vesicle fusion at the membrane, Third, it blocks reuptake of dopamine and fourth it blocks MAO-mediated breakdown of dopamine. Reprinted with permission.

For an animal to become sensitive to a psychostimulant it has to be treated repeatedly with the drug. Several theories exist on how sensitivity arises, but this goes beyond the scope of this thesis. Generally a sensitization paradigm consists of three stages (Figure 5) (Peleg-Raibstein et al. 2008, Robinson & Becker 1986). First the animal is injected for several days with the psychostimulant, ranging from three times daily to three times a week across a variable time period. This period is called the initiation of sensitization. Despite the fact that different time periods, dosages and times in

between injections are common, they cannot be considered equal. It was shown that a short-term escalating dose regimen of amphetamine resulted in diminished latent inhibition, defined as the ability of a subject to filter out irrelevant information. Long-term, intermittent injections of amphetamine however also resulted in disrupted PPI, the ability of an organism to attenuate a startle response to a stimulus by sensing a (weaker) prestimulus (Peleg-Raibstein et al. 2008, Tenn et al. 2003, Peleg-Raibstein *et al.* 2006). Disrupted PPI is a hallmark of the inability to filter out irrelevant stimuli and is often found in schizophrenic patients as well.

The second stage is the actual sensitization or withdrawal period (Pierce & Kalivas 1997). It has been shown that up to a year or longer after the last psychostimulant injection the sensitivity remains, both in rodents and humans (Vanderschuren *et al.* 1999, Sato *et al.* 1983). The third stage, often several days or weeks after the last injection at the first stage, is called the expression of sensitization and is brought about by a single low dose injection of the psychostimulant. The extent to which the animal reacts to this injection is a measure of its sensitivity to the drug.

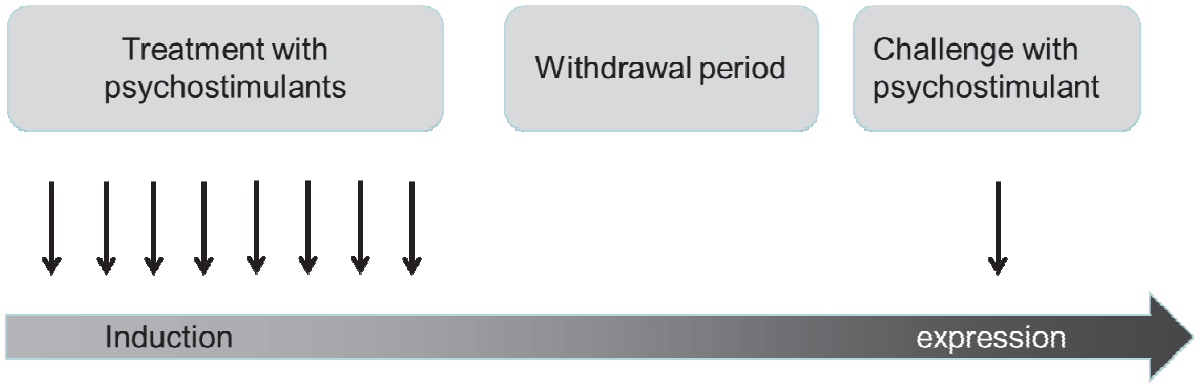

Figure 5| Graphical representation of the sensitization paradigm. In short, an animal is repeatedly injected with a psychostimulant, the induction period. The animal is then withdrawn from psychostimulant treatment during which the animal is building up sensitivity towards the psychostimulant, the withdrawal period. Upon challenging the animal again with the psychostimulant, often with a lower dose compared to the induction period, the animal shows sensitized behavior, the expression of sensitization.

1.6.2 Glucocorticoids and Amphetamine Sensitization

Since stress and GCs can exacerbate dopamine signaling and psychotic symptoms, do they also influence amphetamine sensitivity? As a first indication that GCs are implicated in the sensitization process it was shown that a strong stressor, applied 20 minutes before a cocaine injection, enhanced cocaine-induced locomotor activity as well as the dopamine release in the ventral striatum (Sorg & Kalivas 1991). The effect of cocaine on locomotor activity was attenuated by adrenalectomy (ADX),

which depletes the animals of endogenous GCs. Subsequent replacement with corticosterone pellets reinstated the cocaine-induced locomotor response in a dose-dependent fashion (Marinelli *et al.* 1997), pointing to a key role for GCs in the response to cocaine. Interestingly, in DBA/2J mice it was found that corticosterone alone is not enough to reinstate sensitization since both adrenalin (also released from the adrenals) together with corticosterone were necessary for the locomotor response to cocaine (de Jong *et al.* 2009). This study also revealed an important genetic component in psychostimulant sensitivity since C57Bl/6 mice do not show the same magnitude of sensitization, whether adrenalectomized or not (de Jong *et al.* 2007).

Conversely, it was also observed that amphetamine treatment changes GR mRNA expression in the hippocampus. Acute amphetamine treatment upregulates GR expression while chronic amphetamine downregulates GR expression (Shilling et al. 1996). Another study pinpointed GR mRNA downregulation, after chronic methamphetamine treatment, specifically in the CA1 region of the hippocampus (Kabbaj *et al.* 2003). It was also observed that blocking the GR in the hippocampus flattens the dose-dependent sensitization to cocaine, indicating that the loss of GR expression by chronic amphetamine is a compensatory mechanism for the psychostimulant sensitization (Deroche-Gamonet *et al.* 2003).

Other studies in HR and LR to novelty found higher expression of the GR in the hippocampus of LR rats (Kabbaj *et al.* 2000). Novelty-seeking behavior of the LR rats becomes similar to that of the HR animals when they are treated with a GR antagonist. Conversely, when HR rats are exposed to isolation stress, which is considered more stressful than novelty exposure, they are not different anymore from LR rats. Whether the response to amphetamine also becomes similar in LR and HR was not studied.

1.7 Molecular Basis of Vulnerability

Vulnerability to psychostimulants such as amphetamine has been the subject of many studies and, as explained in chapter 1.5, also has consequences for disorders of the dopaminergic neurotransmitter system. In order to study the molecular basis of vulnerability, animal models have been developed where animals are subjected to regimes of psychostimulant injections. This section briefly describes the most common molecular pathways derived from these studies.

Psychostimulant treatment first leads to cyclic AMP-regulated induction of CREB, a pathway that is also implicated in long-term memory formation (Impey *et al.* 1998). CREB is an activity-regulated transcription factor and binds to cAMP/calcium response element (CRE) sites which in turn lead to the expression of multiple genes. One of the most well-known molecular targets of CREB is delta-FosB (Conversi *et al.* 2008, Levine *et al.* 2005, McClung & Nestler 2003, McClung *et al.* 2004). Delta-FosB poorly responds to a single psychostimulant injection but slowly builds up in concentration after repeated psychostimulant injections as well as after exposure to chronic stress (Perrotti *et al.* 2004). This build-up is partly due to its slow degradation as opposed to other Fos genes such as c-FOS (Ulery *et al.* 2006).

Delta-FosB acts both as a transcriptional repressor by recruiting histone deacetylase 1 (HDAC1) as well as a transcriptional enhancer (Kumar et al. 2005, Renthal et al. 2008). Two important genes that are regulated by delta-FosB are cyclin dependent kinase 5 (CDK5) and p35 (Kumar et al. 2005). CDK5 is a kinase that plays an important role in psychostimulant sensitization since blockade of CDK5 by the inhibitor roscovitine potentiates the behavioral effect of the psychostimulant cocaine (Bibb *et al.* 2001). P35 is known as the activator of CDK5 and the expression of p35 is transiently enhanced by amphetamine (Mlewski *et al.* 2008). CDK5 can phosphorylate GR and myocyte enhancer factor 2 (MEF2), both known to play major roles in psychostimulant sensitization (Pulipparacharuvil *et al.* 2008, Adzic *et al.* 2009, Gregoire *et al.* 2006)

1.8 Myocyte Enhancer Factor 2

MEF2 was first found to enhance transcription of muscle-specific genes and was hence designated as myocyte-specific enhancer factor 2 (Gossett *et al.* 1989). However, subsequent studies revealed that the expression of MEF2 is not specific for muscle tissue, but is also expressed in other cells that are subject to differentiation such as endothelial cells, T-cells and neurons (Potthoff & Olson 2007). Several years after MEF2 was identified, MEF2C was the first of the four MEF2 proteins found to bind to MEF2 specific sites within the cerebral cortex (Leifer *et al.* 1993) and thus the name was changed to myocyte enhancer factor 2. Apart from MEF2B the other three proteins are highly expressed throughout the brain. However, the expression patterns of the different proteins are quite distinct and change throughout development. MEF2D is ubiquitously expressed, as is MEF2A, albeit to a lesser extent than MEF2D. MEF2C is mainly expressed within the amygdala and MEF2B in cerebral cortex. MEF2A, C and D expression is high in hippocampus (Lin *et al.* 1996), while both MEF2A and –D are highly expressed in the striatum (Neely *et al.* 2009).

MEF2 functions as a transcription factor that is preferentially regulated by posttranslational modification. The most studied modification is phosphorylation of serine 408 in MEF2A, which is structurally similar to serine 444 in MEF2D. This serine resides in the transactivation domain and it is therefore not surprising that phosphorylation of this site mainly revolves around its transcriptional activity. Multiple studies showed that MEF2 is inactivated when it is phosphorylated at this site, but that phosphorylated MEF2 is still capable of binding to the DNA (Gregoire *et al.* 2006). It is therefore suggested that MEF2 represses transcription in an active way, by recruiting transcriptional repressors such as HDACs 4, 5, 7 and 9 (Chawla *et al.* 2003, Nebbioso *et al.* 2009).

Functioning of MEF2 has been implicated in neuronal plasticity, formation of dendrites and spines, neuronal survival and behavioral sensitization (Flavell *et al.* 2006, Flavell *et al.* 2008, Pulipparacharuvil *et al.* 2008, Shalizi *et al.* 2006, Tian *et al.* 2010, Gong *et al.* 2003). *In vitro* studies in primary neurons showed that KCl-induced neuronal depolarization significantly enhances the transcriptional activity of MEF2 (Flavell et al. 2006), in correlation with an increase in dendrite formation (Fiore *et al.* 2009). Knockdown of both MEF2A and –D results in a concomitant decrease of dendrites, clearly showing the effect of MEF2 on neuronal plasticity. On top of this, MEF2A and –D knockdown enhances cocaine-induced spine formation in the NAc (Pulipparacharuvil et al. 2008), thereby resulting in a strengthening of existing synapses.

Surprisingly, at a behavioral level MEF2 overexpression in the NAc, which blocks the cocaine-induced increase in dendritic spine density, resulted in an accelerated sensitization to cocaine, while

knockdown had the opposite effect. Although animals reached the same level of behavioral sensitization compared to scrambled shRNA transfected animals, they did so in a significantly slower way (Pulipparacharuvil et al. 2008). This indicates that expression and activity of MEF2 contributes to the process of sensitization and a change in the activity of the protein may lead therefore to differences in psychostimulant vulnerability.

1.9 Scope and outline of the thesis

The pathogenesis of schizophrenia is extremely complex and involves an intricate combination of genetic vulnerability and adverse environmental events. Each gene and event contributes only to a small extent to the overt symptomatology of schizophrenia. The aim of this thesis was to determine the individual vulnerability of inbred DBA/2 mice to amphetamine sensitization, which is considered to be a model for psychosis. For this purpose HR and LR to an amphetamine sensitization paradigm were selected. These extremes in amphetamine sensitization were then used to generate transcriptional profiles in several key brain areas removed by laser dissection technology. Next susceptibility pathways for amphetamine sensitization were identified and examined for interaction with GR. The study was concluded with validation of the novel targets *in vivo* in the amphetamine sensitization paradigm.

1.9.1 Objective

The objective of this thesis was to identify genes and pathways involved in psychosis susceptibility

1.9.2 Specific aims

- To generate transcriptional profiles in several dopaminergic brain areas of genes that are differentially expressed between HR and LR in an amphetamine sensitization paradigm
- To study the effect of GCs mediated by GR on MEF2 function *in vitro* in neuronally differentiated PC-12 cells
- To study the context-dependent effect of the GR on MEF2 function *in vitro* in a model of depolarized neurons.
- To manipulate the activity of MEF2 *in vivo* and to study its effects in the amphetamine sensitization paradigm

1.9.3 Outline

In **chapter 2** transcriptional profiles are presented that were generated in three dopaminergic brain areas from animals that were selected for either a low or high locomotor response to amphetamine. We show that the largest differences in gene expression between HR and LR can be found in the CA1 area of the hippocampus and that target genes of the
transcription factors GR and MEF2 are overrepresented among the differentially expressed genes.
In **chapter 3** we demonstrate that GR and MEF2 signaling pathways converge at multiple levels in the control of their shared target gene c-JUN.
In **chapter4** the cooperation is reported of GR and MEF2 signaling pathways under depolarizing conditions, using the MEF2 target gene NR4A1 as a proof-of-principle.

In **chapter 5** studies are described aimed at measuring the effect in vivo of manipulating MEF2 and GR activity using roscovitine, a potent inhibitor of CDK5, on the behavioral reaction to amphetamine. In **chapter 6** the results are discussed and a model is presented of MEF2 and GR regulation by GCs to explain vulnerability to psychostimulants.

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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.

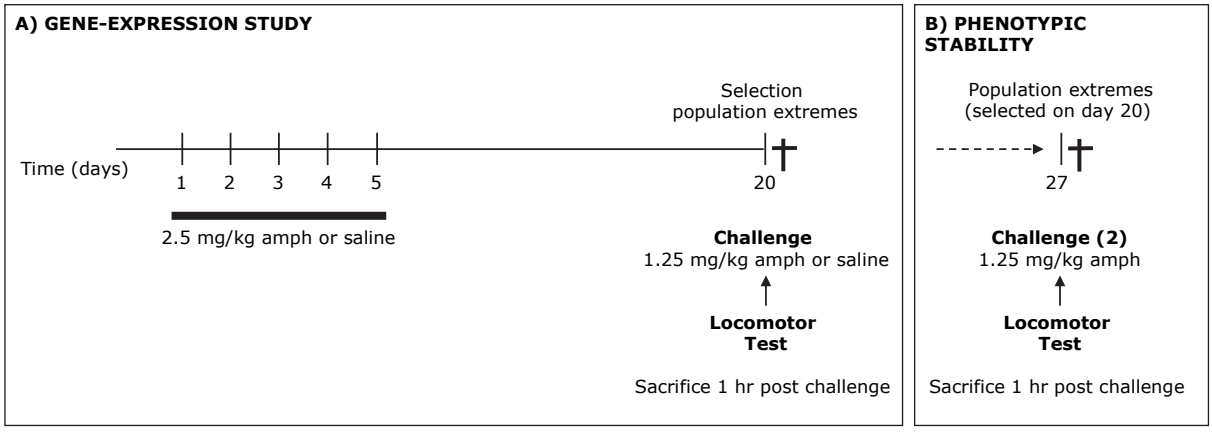

Figure 1A | Animals received either d-amphetamine (2.5 mg/kg) or saline for 5 consecutive days (days 1-5). After a 14-day withdrawal period (day 20) animals were given a low dose amphetamine challenge (1.25 mg/kg) or saline and the 10% population extremes in the AMPH/AMPH group (low and high responders) 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.

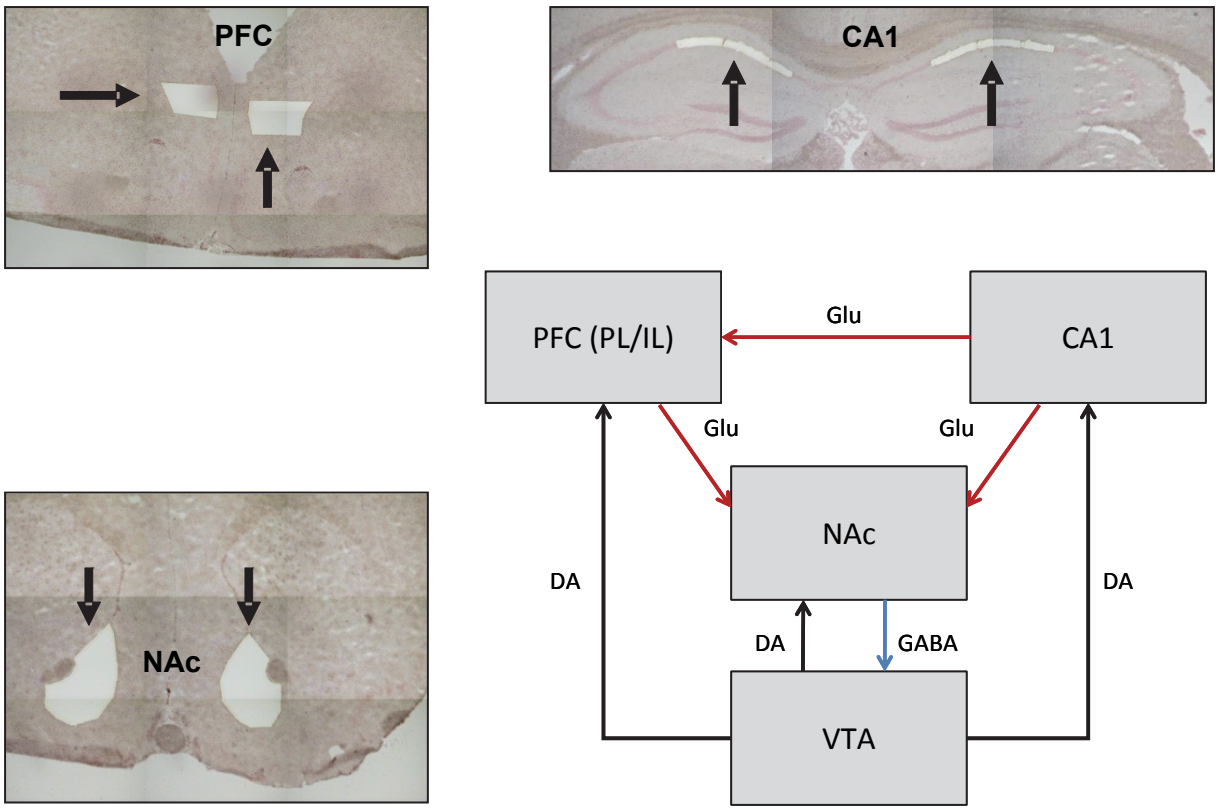

Figure 2 | Scheme showing connection between the selected brain areas including examples of laser microdissection. PFC: prefrontal cortex; IL: infralimbic; PL: prelimbic; (NAc) nucleus accumbens; CA1: cornu ammonis 1 region of the hippocampus; VTA: ventral tegmental area. Red arrows indicate glutamatergic 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.

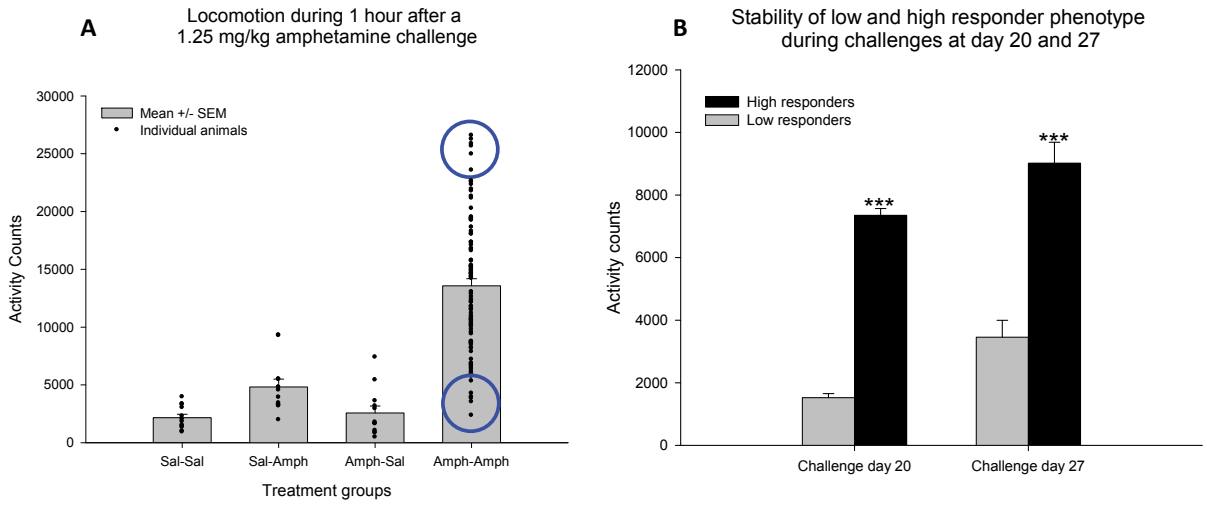

Figure 3A| Locomotor responses to the amphetamine (1.25 mg/kg) or saline challenge on day 20. Data are represented as total activity count over the 60 minute treatment period. SAL/SAL n=10, SAL/AMPH n=10, AMPH/SAL n=10, AMPH/AMPH n=100. Ovals indicated the 10% population extremes (low responders n=10, 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%).

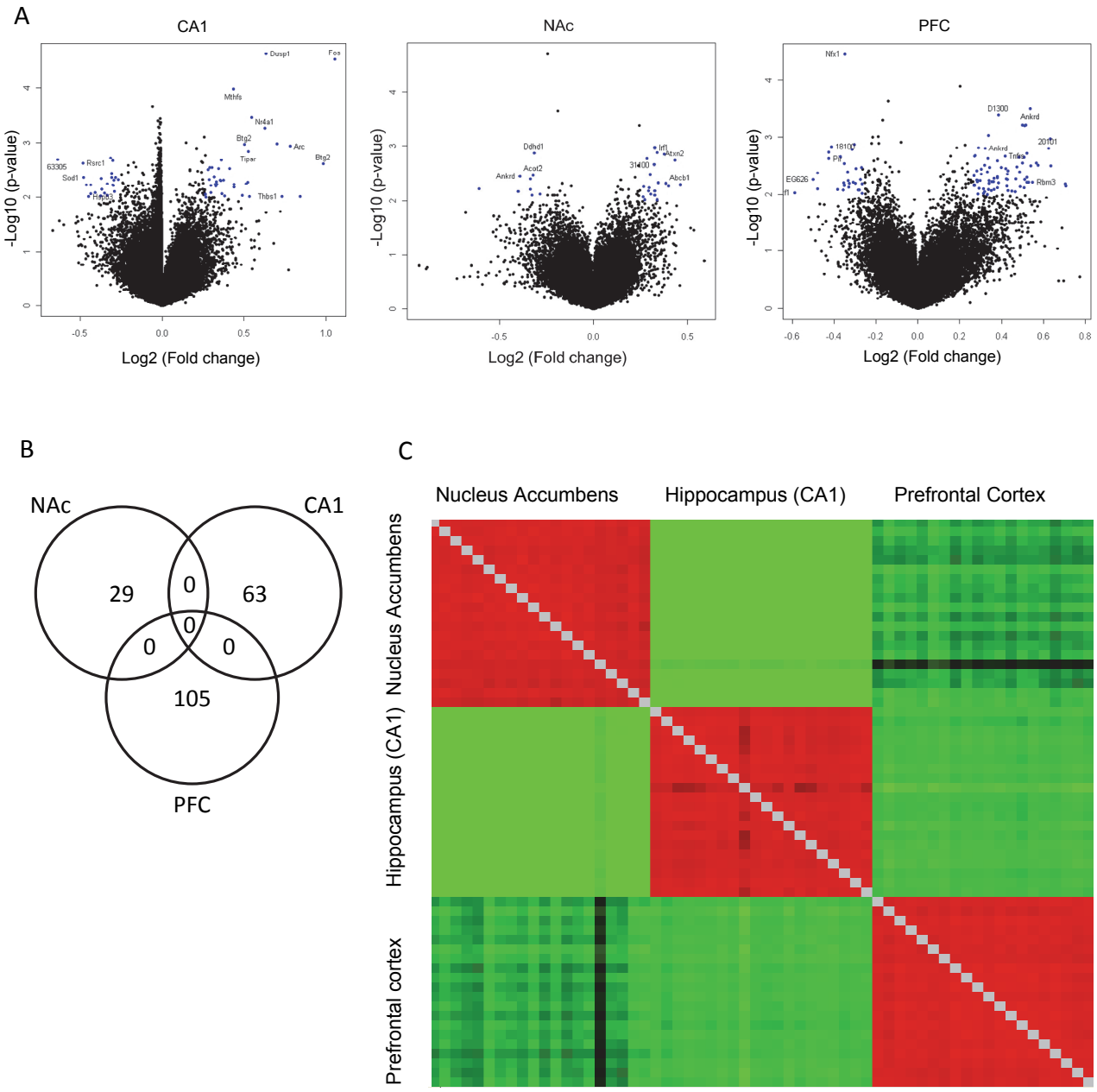

Figure 4A |Volcano plots of -log10 (p-value) vs. log2(Fold change). Labeled are largest fold change and lowest p-value genes. The blue points in each graph indicate the Affymetrix probesets that passed the t-test p<0.01 and FC>1.2 statistical requirements. B: Venn diagram of genes differentially expressed 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).

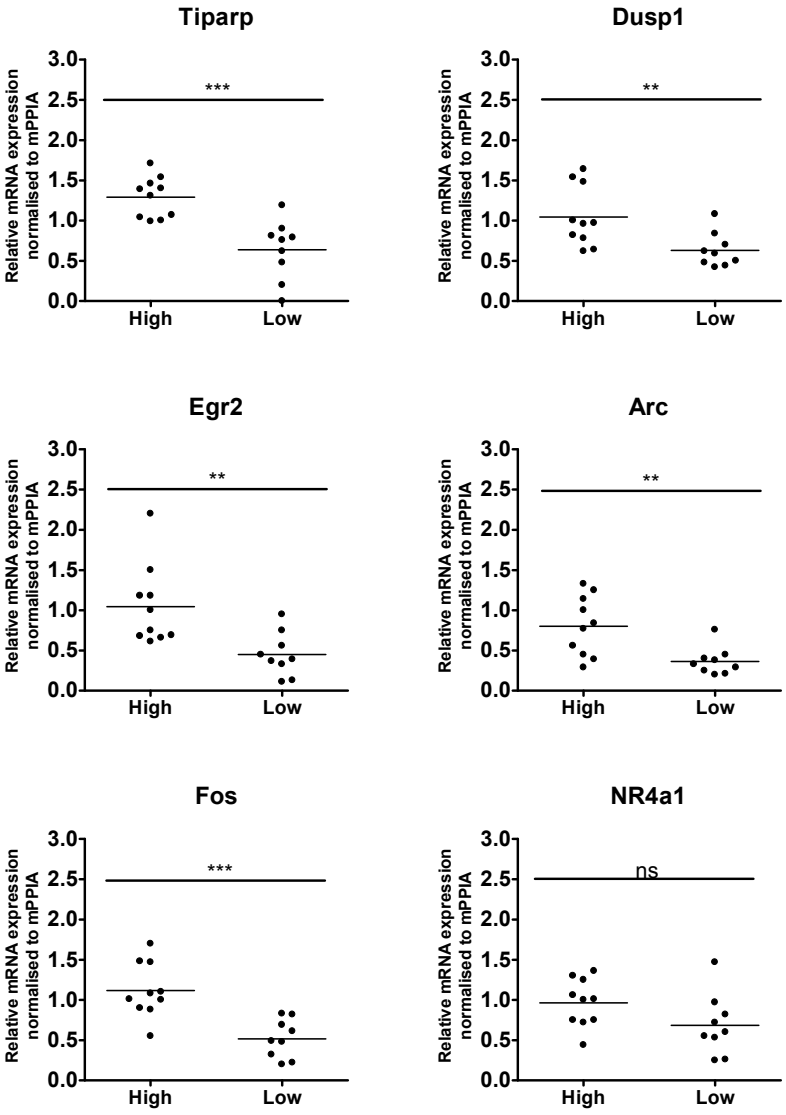

Figure 5 |RT-qPCR validation results of gene expression differences between low and high responders in the CA1 region of the hippocampus in the second animal experiment. ** p<0.01 vs low responders; *** p<0.001 vs low responders (Mann-Whitney Rank Sum Test)

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.

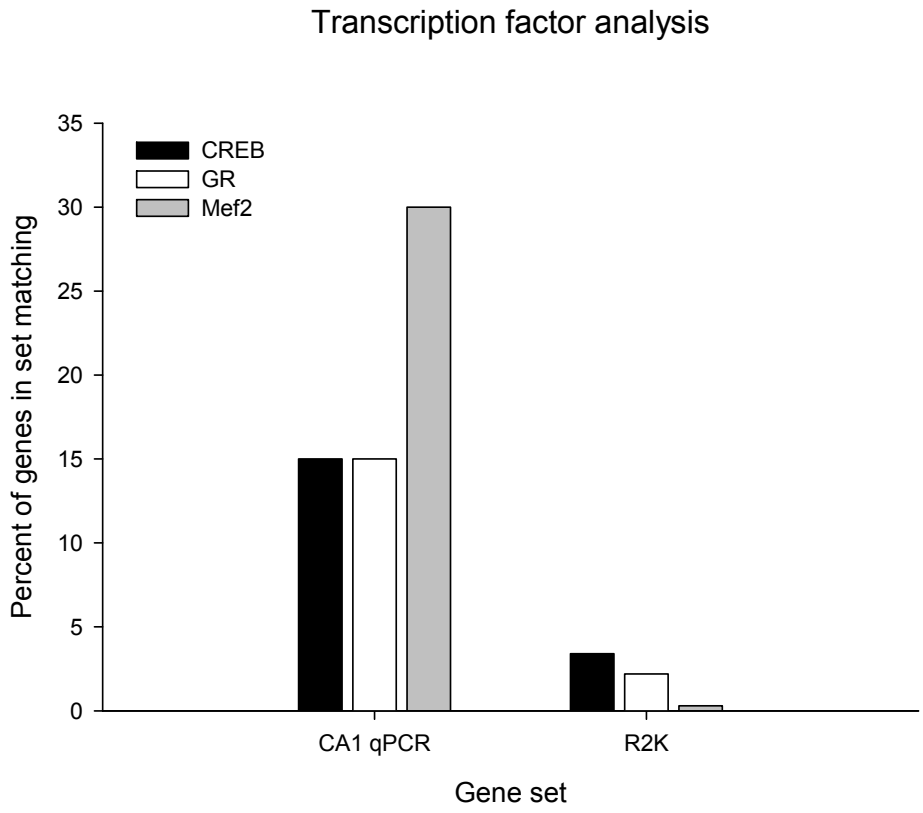

Figure 6 | Comparison of genes regulated in CA1, Nucleus Accumbens (Acc) and Prefrontal Cortex (PFC) to genes involved in specific transcriptional regulation as identified by ChIP/ChIP experiments. For each of the brain areas the comparison is made for the genes identified in expression array and for 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

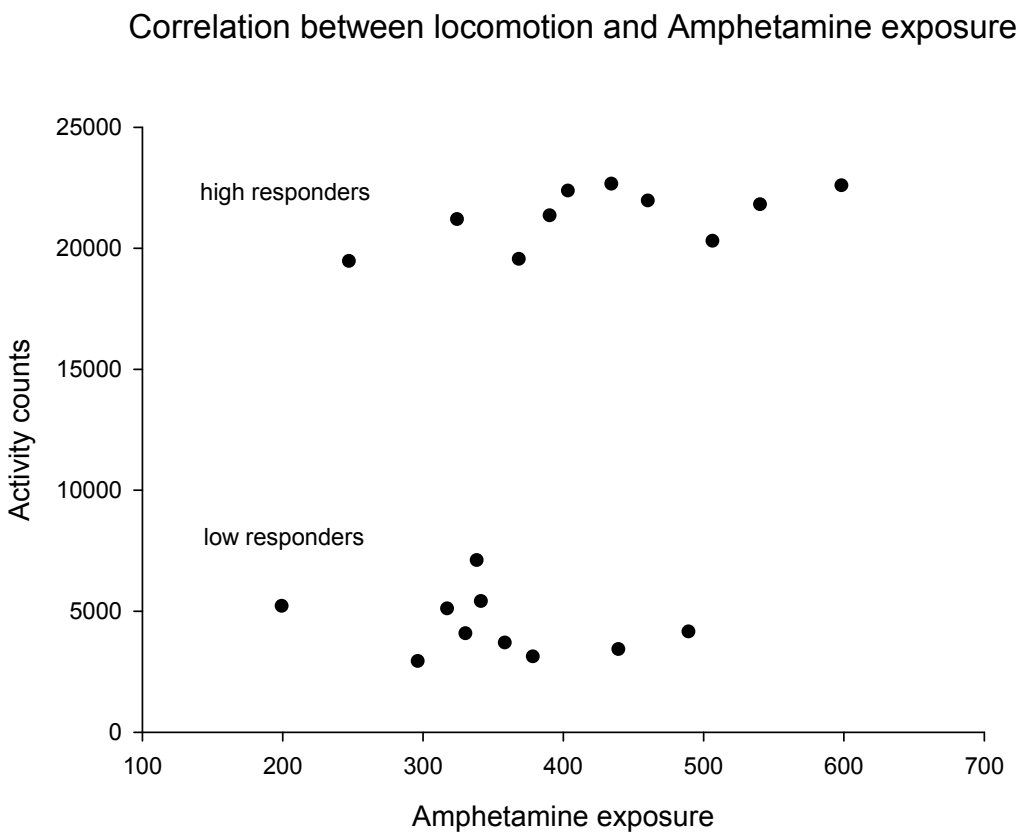

Table SII | CA1 differentially expressed genes sorted by FC of the univariate test. The 63 genes are significant at the nominal 0.01 level of the univariate test with a fold change cut-off of 1.2. 39 genes are upregulated and 24 downregulated. Note: Comparison is based on high to low responders group

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)

Chapter 3 | Glucocorticoid Receptor and Myocyte Enhancer Factor 2 cooperate to regulate the expression of c-JUN in a neuronal context

ABSTRACT

The Glucocorticoid Receptor (GR) and Myocyte Enhancer Factor 2 (MEF2) are transcription factors involved in neuronal plasticity. C-JUN, a target gene of GR and MEF2, plays a role in regulating both synaptic strength and synapse number. Aim of this study was to investigate the nature of this dual regulation of c-JUN by GR and MEF2 in a neuronal context. First we showed that GR mediates the dexamethasone-induced suppression of c-JUN mRNA expression. Next, we observed that GR activation resulted in an increase in phosphorylation of MEF2, a post-translational modification known to change MEF2 from a transcriptional enhancer to a repressor. In addition, we observed an enhanced binding of MEF2 to genomic sites directly upstream of the c-JUN gene upon GR activation. Finally, in primary hippocampal neuronal cultures, knockdown of MEF2 not only reduced c-JUN expression levels, but abolished GR-regulation of c-JUN expression. This suggests that MEF2 is necessary for GR-regulation of c-JUN. In conclusion, for the first time we show that activated GR requires MEF2 to regulate c-JUN. At the same time, GR influences MEF2 activity and DNA binding. These results give novel insight into the molecular interplay of GR and MEF2 in the control of genes important for neuronal plasticity.

INTRODUCTION

Neuronal plasticity, a change in the structure, function, and organization of neurons in response to environmental stimuli, underlies many key processes such as learning and memory, adaptation and behavioral sensitization. Changes in gene expression, governed by key transcription factors, such as the Glucocorticoid Receptor (GR) and Myocyte Enhancer Factor 2 (MEF2), underlie neuroplasticity. GR is activated by glucocorticoid stress hormones, released by the hypothalamic-pituitary-adrenal (HPA) axis in response to stress. Upon activation, GR acts as a ligand-activated transcription factor to influence expression of a wide variety of genes, including genes involved in neuronal plasticity (Datson *et al.* 2008). MEF2 comprises a family of four members, MEF2a-d, showing distinct but partly overlapping expression patterns and is activated by neuronal activity. Upon activation, MEF2 regulates the expression of genes that control dendritic remodeling, resulting in the inhibition of synapse formation. Conversely, a decrease in MEF2 activity increases spine density (Flavell et al. 2006, Shalizi et al. 2006).

We previously showed that GR and MEF2 have several target genes in common, including the c-JUN gene (Datson et *al.* 2011). c-JUN is a subunit of the transcription factor AP-1 and is an ubiquitously expressed immediate-early gene (IEG) with important functions in cell death, differentiation and inflammation (Beck *et al.* 2009, Sun *et al.* 2005). The AP-1 family of transcription factors is recruited in the activation of neuronal circuits leading to long-term changes, such as long-term memory formation (Alberini 2009). MEF2 is known to induce transcription of c-JUN (Kato *et al.* 1997, Aude-Garcia *et al.* 2010, Han & Prywes 1995), while GR on the other hand is known to repress the expression of c-JUN in vitro in AtT-20 cells and mouse fibroblast cells (Autelitano 1994, Wei *et al.* 1998). Aim of this study was to investigate the molecular interplay of GR and MEF2 in a neuronal context, using the shared target gene c-JUN as a proof-of-principle.

MATERIALS & METHODS

Cell culture and treatment Rat pheochromacytoma (PC-12) cells (passage # 15-29) were cultured as described earlier (Morsink *et al.* 2006b). In short, cells were grown in DMEM medium, supplemented with 0-10% fetal bovine serum and 0-10% horse serum, dependent on the stage of neuronal differentiation. For mRNA and protein analysis cells were seeded at a confluency of 30-50% in pre-coated 6-well plates (356400, BD Biosciences, San Jose, CA, USA). For ChIP experiments the cells were seeded at 50% confluency in pre-coated 175 cm^2 plates (356478, BD Biosciences). Neuronal differentiation was achieved by giving 50 ng/ml NGF-ß (N2513, Sigma-Aldrich, St. Louis, MO, USA) every other day for 10 days. Medium at day 9 of the differentiation stage was supplemented with charcoal stripped serum to deprive the medium of endogenous steroids (Sarabdjitsingh *et al.*). At day 10 the cells were treated for 30, 60, 90 or 180 minutes, dependent on the experiment, with either vehicle (VEH) (0.1% ethanol) or 100 nM dexamethasone (DEX) (D1756, Sigma-Aldrich). For GR blockade, cells were pretreated with VEH (0.1% ethanol) or 1 mM RU486 (M8046, Sigma-Aldrich) for 60 minutes before addition of DEX or VEH.

Hippocampal cultures Newborn pups from NMRI mice were decapitated at postnatal day 1 (P1). Brains were isolated and kept in Hank's Balanced Salt solution (HBSS) on ice until dissection. Hippocampi were dissected in ice-cold dissection solution consisting of Krebs Buffer supplemented with 3 mg/ml BSA, 1.2 mM MgSO4 and 2mM HEPES. Hippocampi (n=12) were transferred to a conical tube containing 1.5ml of dissection solution supplemented with 184µg/ml trypsin. The tissue was incubated at 37°C for 6 minutes. Subsequently, 3.5 ml of dissection solution supplemented with 0.65 mg/ml Soyabean Trypsin Inhibitor, 10µg/ml DNAse and 0.19 mM MgSO4 were added. The trypsinated and DNase treated hippocampi were centrifuged at 100g for 3 min. The supernatant was discarded and the conical part of the tube was filled with 1.5ml of dissection solution supplemented with 5.2 mg/ml Soyabean Trypsin Inhibitor, 80µg/ml DNAse and 1.5 mM MgSO4. The cells were dissociated by pipetting and left for 5 minutes at RT allowing remaining tissue to settle. The supernatant was transferred to a new tube containing 3.5 ml of dissection solution supplemented with 132 µM CaCl2 and 120 µM MgSO4 and centrifuged for 10 minutes at 100g. The cell pellet was resuspended in 3.5 ml of MEM II + B27 (MEM buffer supplemented with 0.5% d-glucose, 0.22% Bicarbonate, Penicillin-Streptomycin, 2mM l-Glutamate, 10% NU-serum and 2% B27). After resuspension the concentrated cell solution was diluted to 7.5 ml MEM II + B27. Cells were plated at a density of 50.000 live cells/well in poly-d-lysine coated 96-well dishes. Yield from one pup (two hippocampi) is approximately 400.000 living cells. The day after plating, media was changed to MEM

II + B27 buffer supplemented with 1 μM AraC (Cytosine Arabinoside). The cells were left for 14 days in vitro before assaying.

Lentiviral shRNA transduction, stimulation and RNA purification High titer batches (>5x10*8 TU/ml) of lentiviral particles harboring gene specific shRNAs targeting MEF2A (Sigma, TRCN0000095959) as well lentiviral particles harboring control shRNA (Sigma, SHC002V) were purchased from Sigma. The day after plating, hippocampal cultures were transduced with lentiviral particles at the following concentrations: 150.000 lentiviral particles/well, 75.000 lentiviral particles/well and 37.500 lentiviral particles/well. At day 14 the hippocampal cultures were stimulated for 90 minutes with 100nM dexamethasone diluted in astrocyte conditioned media. The latter to avoid glutamate induced excitotoxicity by the media change. Subsequently, cells were processed for RNA isolation using the Aurum Total RNA 96 Kit (BioRad).

Real time quantitative PCR (RT-qPCR) Total RNA was isolated using Trizol (15596, Invitrogen) according to the manufacturer's instructions. RNA was diluted to 50 ng/ul and cDNA was synthesized using the iScript cDNA synthesis kit (170-8897, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. RT-qPCR was performed on a Lightcycler 2.0 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) in combination with the Lightcycler FastStart DNA Master^{PLUS} SYBR Green I Kit (03515885001, Roche). As a control for genomic contamination, samples without reverse transcriptase were used. The standard curve method was used to quantify the expression differences (Livak and Schmittgen, 2001). Expression of TUBB2a (Tubulin, beta 2a) was used to normalize the RNA input.

RT-qPCR primer design Primers were designed using primer-BLAST (NCBI, Bethesda, USA). Apart from the built-in feature of selecting primers that do not cross-hybridize, an additional check for primer hairpins was performed using Oligo 7.0 (MBI Inc. Cascade, USA). Primers were tested with RT-qPCR on a standard curve to check the efficiency of the PCR reaction. After a visual check for single melting peaks the primer products were put on a 2.0-2.5% agarose gel to check for single products and absence of primer-dimers. The primer sequences used are listed in supplementary table S1.

Chromatin Immunoprecipitation (ChIP) The exact procedure is described in (Sarabdjitsingh *et al.* 2010).In short, cells were fixed with 1% formaldehyde for 10 minutes at RT to crosslink DNA-protein interactions. DNA was sonicated for 15-25 pulses to obtain DNA fragments between 200 and 500 basepairs and checked visually on a 1.2% agarose gel. Pre-cleared DNA (20-60 ug per antibody) was incubated overnight (o/n) with 6 ug of either anti-MEF2 (sc-313X; Santa Cruz), anti-GR (sc-8992X;

Santa Cruz) antibodies or normal rabbit IgG (Santa Cruz; sc-2027). The next day, 20 µl sepharose A beads were added to the DNA-protein-antibody complexes. The samples were washed 1x with low salt buffer (0.1% SDS; 2mM EDTA pH 8.0; 20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1% Triton-X-100), 1x with high salt buffer (as low salt except 500 mM NaCl), 1x with LiCL buffer (0.25 M LiCl; 1 mM EDTA pH 8.0; 20 mM Tris-HCl pH 8.0; 1% NP-40; 1% NaDOC) and 2x with TE buffer (1mM EDTA pH 8.0; 10 mM Tris-HCl pH 8.0). Subsequently the DNA complexes were eluted from the beads with 0.1M $NaHCO_3$ and 1% SDS and the DNA was reverse-crosslinked o/n at 4°C in 0.2 M NaCL. The samples were then treated for 1 hour with RNAse at 37°C and the DNA purified using Nucleospin columns. The DNA was eluted in TE buffer for RT-qPCR analysis. RT-qPCR on ChIP material was performed directly on purified DNA. ChIP results were obtained by performing 3 individual ChIP replicates. IgG ChIP was used as a negative control for aspecific precipitation while RT-qPCR of myoglobin was used as a negative control for specific precipitation of DNA.

ChIP primer design Primers were designed spanning a published MEF2 Binding Site (MBS) upstream of the c-JUN transcription start site (TSS) (Han & Prywes 1995, Haberland *et al.* 2007). GR binding sites were identified in neuronally differentiated PC-12 cells and rat hippocampus by GR ChIP-sequencing (unpublished data). This resulted in identification of three GR binding sites located ~300 bp upstream and ~2 and ~8 kb downstream the c-JUN TSS (Table 1). All three binding sites were screened for putative Glucocorticoid Response Element (GRE) sequences using an in-house screening method to identify evolutionary conserved GREs (Datson *et al.* 2011). Binding of MEF2 and GR to Myoglobin was used as a negative control as it is generally considered to be inaccessible for transcription factor binding. Primer sequences used are listed in Table S1.

Binding site	Chr	Peak start	Peak end	Distance from TSS	GRE sequence	Origin
GBS 1	5	115361507	115361560	-275	none	hippocampus
GBS 2	5	115359115	115359210	2097	GAACGGGCTGTGCC	hippocampus
GBS 3	5	115353332	115353445	7871	GAACCAAATGTTCA	PC-12 cells

Table 1 | ChIP-seq results showing chromosomal locations of the three peaks where increased GR binding was found. The three sites are designated GBS 1, GBS 2 and GBS 3. 'Origin' refers to which ChIP-sequencing experiment the GBS was first observed. 'Distance to gene' refers to the distance between the center of the peak and the transcription start site of c-JUN. A negative value indicates upstream the TSS. The MEF2 binding site (MBS 1) was previously identified (Han & Prywes 1995). The Myglobin site is used as a negative site of transcription factor binding.

Western Blotting Protein was harvested in ice-cold RIPA buffer containing Protease Inhibitors (#04693124001, Roche) and phosphatase inhibitors ($NaVO_3$ and B-glycerophosphate). The cell lysate was incubated on ice for 30 minutes, spun down and the supernatant transferred to a new tube. Protein concentration was measured using the Pierce BCA Protein Assay kit (23225, Thermo Scientific, Rockford, IL, USA), according to the manufacturer's protocol. Diluted samples were supplemented with 1:2 v/v of sample buffer (including 2.5% ß-mercaptoethanol and BromoPhenol Blue). Twenty µg of each sample was loaded on a 10% polyacrylamide gel. After sufficient separation of the proteins, they were transferred o/n at 4°C to a PVDF (Polyvinylidene fluoride) membrane. The membrane was subsequently blocked in 5% low fat milk for 1 hour at RT or 5 hours at 4°C for phospho-proteins. Primary antibodies were added in the blocking buffer and incubated for 1 hour at RT or at 4°C o/n for phospho-proteins with either one of the following primary antibodies: Anti-phospho S408 MEF2 rabbit monoclonal (ab51151, Abcam, Cambridge, UK), anti-MEF2a rabbit polyclonal (sc-313X, Santa Cruz) or anti-α-Tubulin DM1A mouse monoclonal antibody (T6199, Sigma). Blots were incubated for 1 hour at RT with the appropriate secondary antibody: goat-anti rabbit IgG HRP secondary antibody (sc-2054, Santa Cruz) or goat-anti mouse IgG HRP secondary antibody (sc-2055, Santa Cruz). Signals were quantified using ImageJ (v1.42; National Institute of Health, USA). α-Tubulin protein expression was used as input normalization and pMEF2a levels were normalized against total MEF2 levels.

Statistics Statistical analysis was performed with Sigmaplot 11.0 using independent t-tests. In the gene expression studies with/without RU486 pre-treatment. A two-way ANOVA was used with Tukey's post-hoc t-tests.

RESULTS

MEF2a is highly expressed in PC-12 cells As a first step to study GR and MEF2 interaction, the endogenous expression of MEF2 transcripts was determined in neuronally differentiated PC-12 cells. MEF2a was most abundantly expressed followed by MEF2d (Fig. 1). MEF2b had a very low expression while MEF2c was not reliably detected in PC-12 cells. Since MEF2a is most ubiquitous, the following experiments focused on this gene.

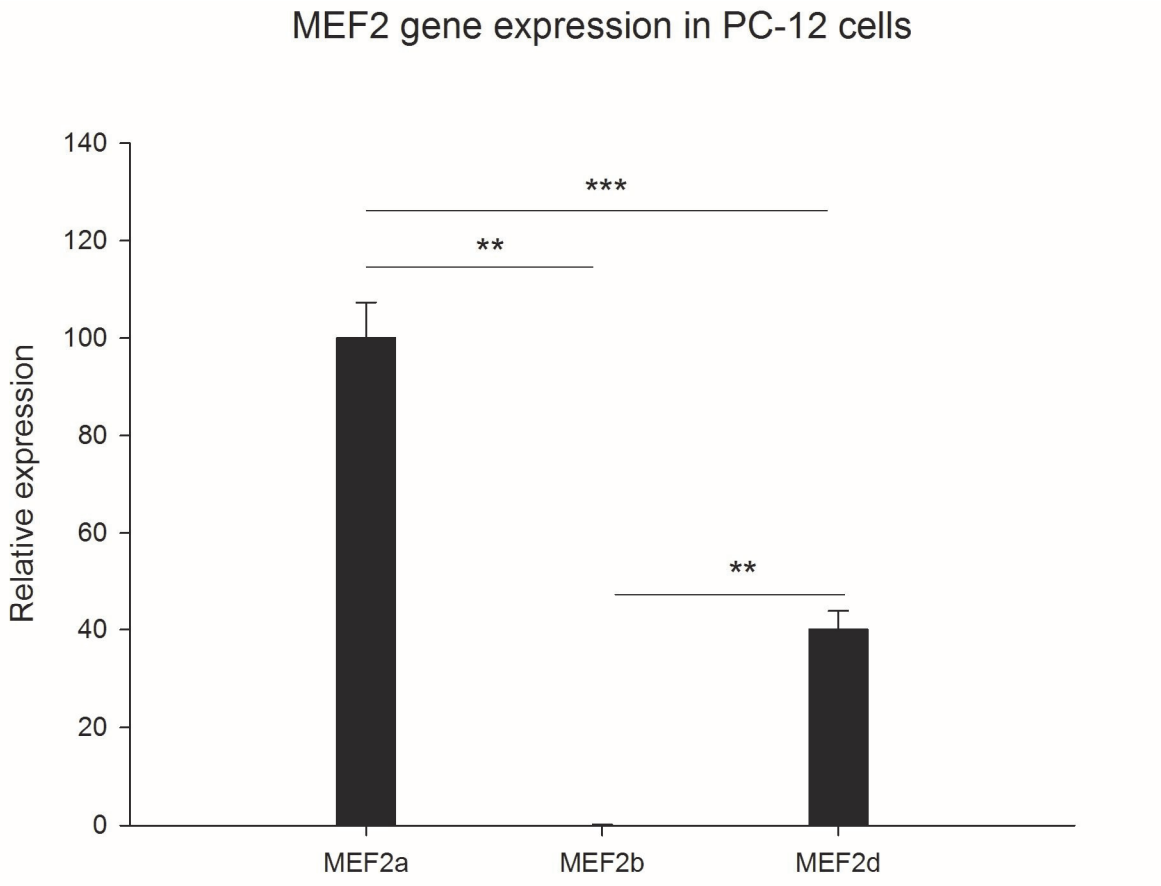

Fig. 1 |Relative expression levels of transcripts MEF2a, -b and -d in neuronally differentiated PC-12 cells under VEH conditions (n=6 per group). Expression is relative to MEF2a, which is set at 100%. Expression is normalized to TUBB2a. (p<0.01; *** p <0.001 sign. between transcripts).**

GR activation by DEX downregulates c-JUN expression Previous studies showed that GR is highly expressed in neuronally differentiated PC-12 cells (Morsink *et al.* 2006a). To study the GR-regulation of c-JUN in a neuronal context, neuronally differentiated PC-12 cells were treated for several time-points with 100 nM of the synthetic glucocorticoid dexamethasone (DEX). Expression of c-JUN mRNA was significantly downregulated after 90 minutes DEX treatment (36% $p<0.001$). After 180 minutes, c-JUN expression was significantly higher compared to 90 minutes DEX (33% $p<0.001$) but still significantly downregulated (16% $p<0.01$) compared to the VEH control (Fig. 2A).

c-JUN downregulation by DEX is mediated by GR To check whether the DEX effect on c-JUN expression is mediated via GR, PC-12 cells were pretreated with the GR antagonist mifepristone (RU486). Since 90 minutes DEX treatment showed the largest decrease in c-JUN mRNA expression, PC-12 cells were treated for this period with 100 nM DEX, after being pretreated for 60 minutes with 1mM RU486. Again, DEX treatment resulted in a significant downregulation (25% p<0.01) of c-JUN mRNA expression. However, pretreatment with RU486, having no significant effect on its own, completely prevented this effect, showing that the DEX-induced downregulation is mediated via GR (Fig. 2B).

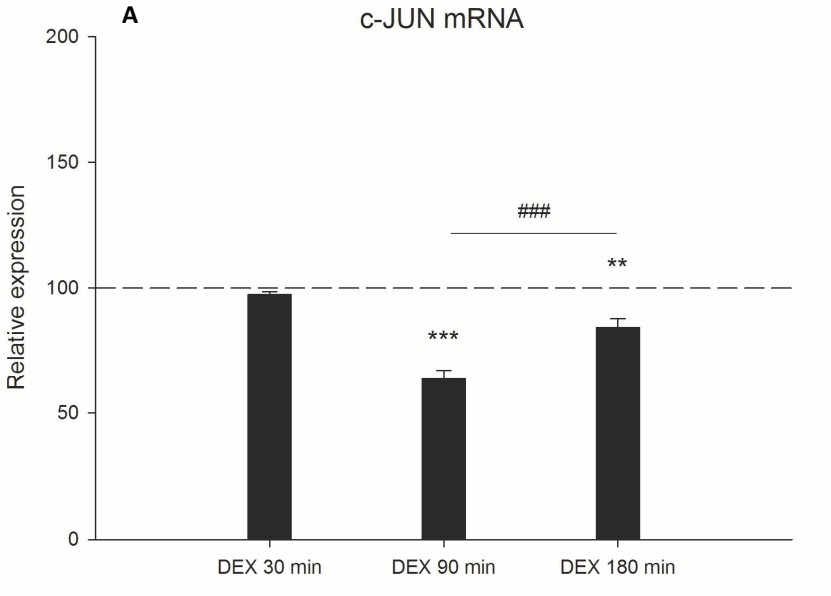

Fig. 2A |c-JUN mRNA expression at 30, 90 or 180 minutes of DEX treatment (n=6 per group). For each timepoint, expression level is relative to its VEH counterpart which is set at 100% and indicated by the dashed line. Expression is normalized to TUBB2a. (p<0.01; *** p<0.001 sign. vs corresponding VEH treatment) (### p<0.001 sign. between timepoints)**

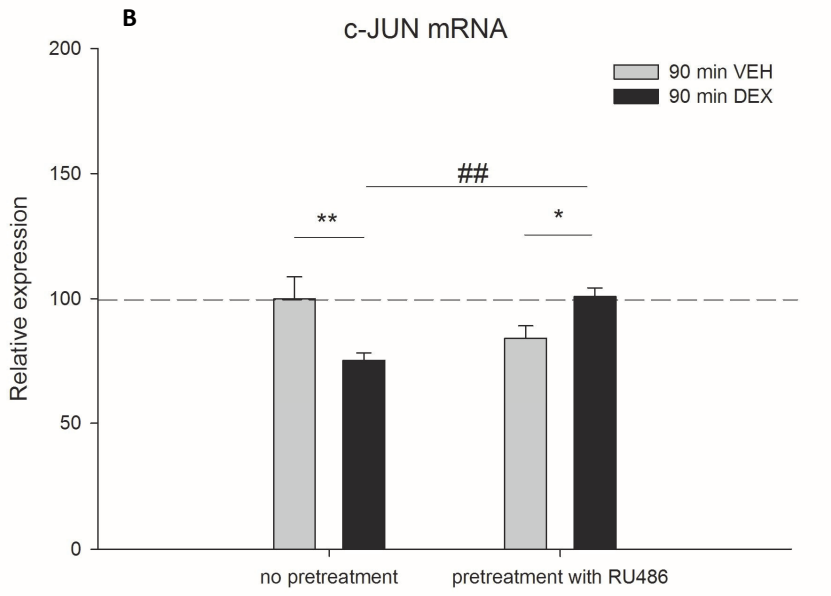

Fig. 2B |c-JUN mRNA expression at 90 minutes of DEX treatment with and without 60 minutes RU486 pretreatment (n=6 per group). Expression level is relative to VEH treated cells without pretreatment, set at 100%. Expression is normalized to TUBB2a. (* p<0.05; ** p<0.01 sign. vs VEH treatment) (## p<0.01 sign. vs DEX without pre-treatment).

MEF2a expression is not changed by GR Since c-JUN is also a known MEF2 target gene, we tested whether DEX treatment changed the expression of MEF2a in PC-12 cells. MEF2a expression showed no change following DEX treatment at the timepoints studied (Fig. 3A).

MEF2a is necessary for the GR-mediated effect on c-JUN To examine whether MEF2a is necessary for the DEX effect on c-JUN expression, we aimed to knock down MEF2a in PC-12 cells before treatment with DEX. Although MEF2a could be knocked down in non-differentiated PC-12 cells, it failed when cells have a neuronal phenotype (data not shown). Since MEF2 proteins are involved in regulation of the neuronal phenotype (Shalizi et al. 2006, Lin *et al.* 1996, Tian *et al.* 2010), as well as in neuronal viability (McKinsey *et al.* 2002), we did not consider knocking down of MEF2a before differentiation to be a good alternative. Instead, the involvement of MEF2a in DEX-mediated effects on c-JUN gene expression was evaluated in primary hippocampal cultures using lentiviral shRNA-mediated MEF2a knockdown. Hippocampal cultures were transduced and incubated with lentiviral particles harboring either negative control shRNA (scrambled sequence) or a gene-specific shRNA targeting MEF2a followed by a 90 minute 100 nM DEX treatment. Gene expression measurements revealed a significant knockdown of MEF2a (78% $p<0.001$) in VEH treated cells compared to cells transduced with negative control shRNA (Fig. 3B). DEX treatment did not influence MEF2a expression, neither in the control condition nor in MEF2a shRNA transduced cells. C-JUN expression showed a significant downregulation after DEX in control cells (44% $p<0.01$) (Fig. 3C), in accordance with our findings in PC-12 cells (Fig 2B). Knockdown of MEF2a, however, resulted in a downregulation of c-JUN comparable to the effect of DEX in control cells (38% $p<0.001$). Surprisingly, DEX treatment on top of knocked down MEF2a did not result in any additional knockdown.

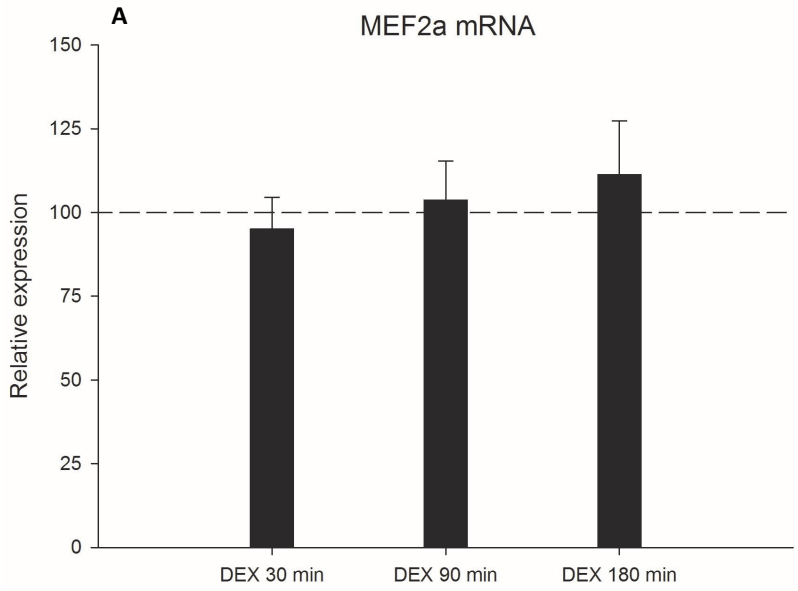

Fig. 3A | MEF2a mRNA expression in neuronal PC-12 cells at 30, 90 or 180 minutes of DEX treatment (n=6 per group). For each timepoint, expression level is relative to its VEH counterpart which is set at 100% and indicated by the dashed line. Expression is normalized to TUBB2a.

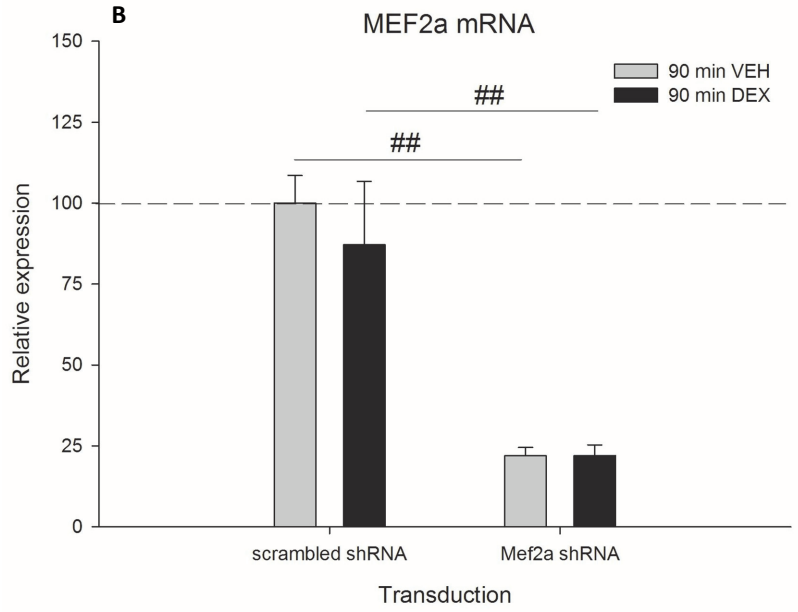

Fig 3B | MEF2a mRNA expression at 90 minutes of DEX treatment in lentiviral transduced primary hippocampal neurons expressing either scrambled shRNA or MEF2a shRNA (n=3 per group). Expression level is relative to scrambled shRNA transduced and VEH treated cells, set at 100%. Expression is normalized to TUBB2a (## p<0.01 sign. vs scrambled shRNA).

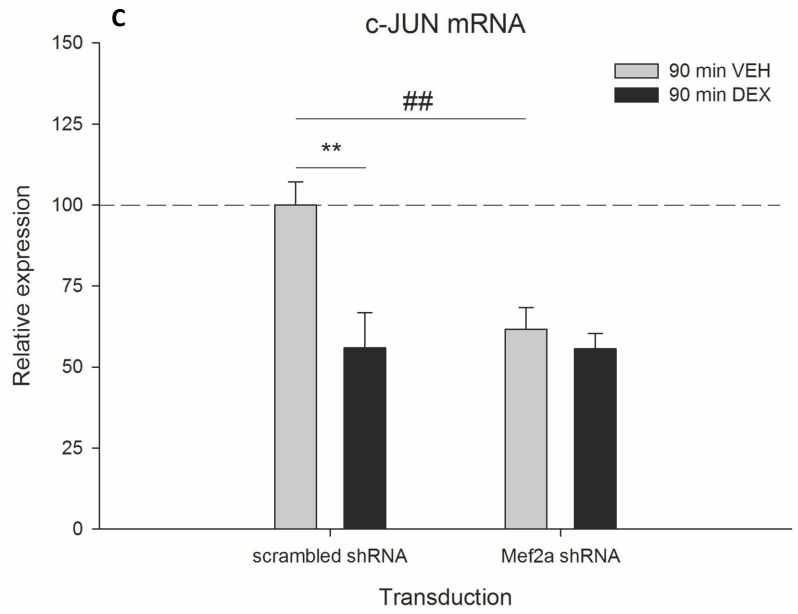

Fig 3C | c-JUN mRNA expression at 90 minutes of DEX treatment in lentiviral transduced primary hippocampal neurons expressing either scrambled shRNA or MEF2a shRNA (n=3 per group). Expression level is relative to scrambled shRNA transduced and VEH treated cells, set at 100%. Expression is normalized to TUBB2a (p<0.01 sign. vs VEH treatment) (## p<0.01 sign. vs scrambled shRNA).**

MEF2a phosphorylation is increased by GR Many studies have shown the importance of phosphorylation of Serine 408 in MEF2a for the activity of MEF2 (Shalizi et al. 2006, Gregoire *et al.* 2006, Li *et al.* 2001). An increased ratio of phosphorylated vs dephosphorylated MEF2a has been shown to decrease MEF2 transcriptional activity. Therefore we measured this important hallmark after 60 minutes of 100 nM DEX treatment (Fig. 4A and 4B) in neuronally differentiated PC-12 cells. DEX treatment induced a marked increase in phosphorylation of MEF2a compared to VEH treated cells (125% $p<0.05$). An independent experiment showed comparable changes in phosphorylation while at 180 minutes no difference in phosphorylation was detected anymore (results not shown).

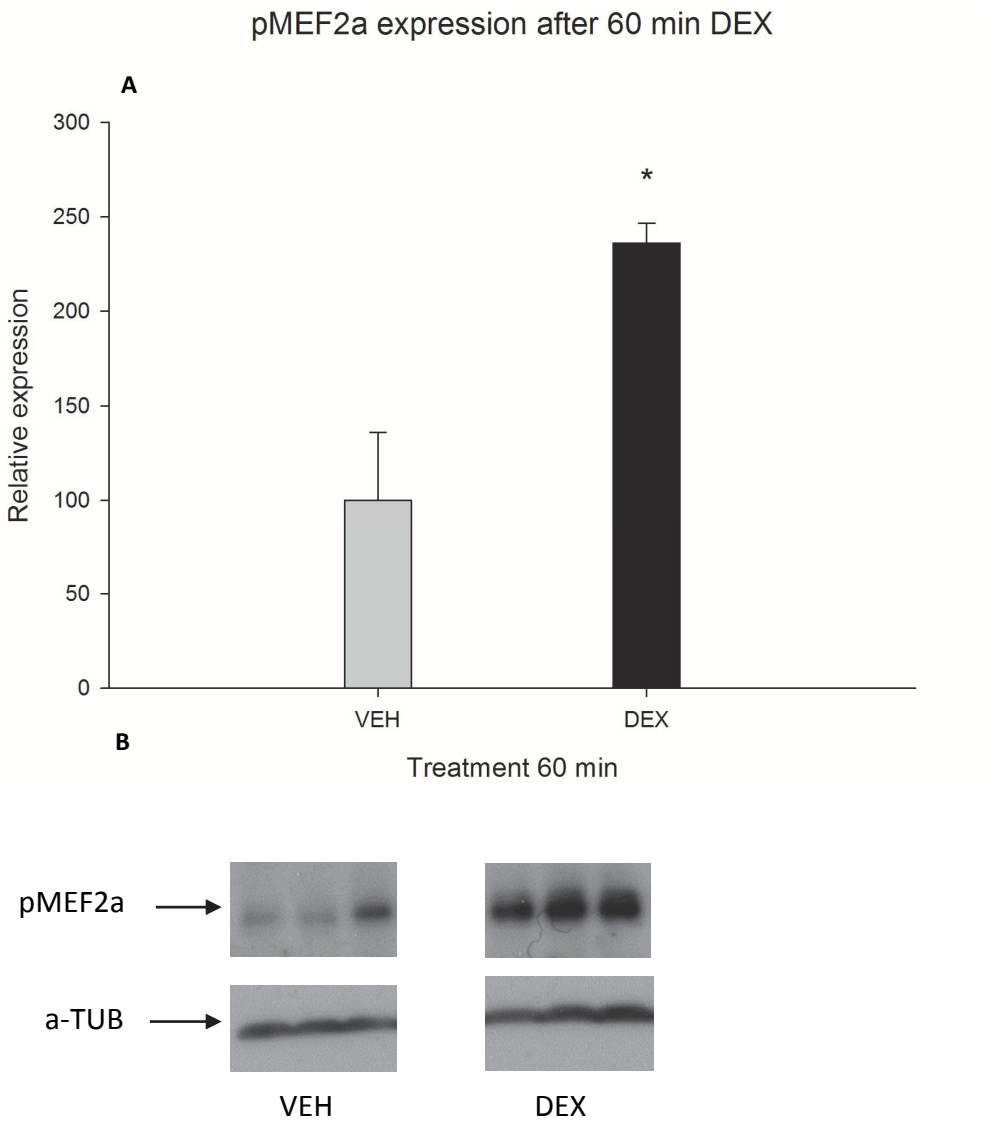

Fig. 4 | MEF2a S408 phosphorylation level after 60 minutes of DEX treatment (n=3 per group). A. Phosphorylation level is relative to 60 minutes VEH treatment, set at 100%. Expression is normalized to total MEF2a and alpha-tubulin. (* $p<0.05$ sign. vs VEH treatment). B. Representative example of protein expression.

GR- and MEF2-DNA binding are increased around the c-JUN gene MEF2 and GR are well known transcription factors and exert their action by binding directly or indirectly to the DNA. First, we investigated whether GR activation changed the binding of MEF2 to a previously described MEF2 Binding Site (MBS) upstream of c-JUN (Han *et al.* 1992) using Chromatin Immunoprecipitation (ChIP). In addition, we identified 3 novel GR binding sites (GBS) based on ChIP-Seq data for GR in PC-12 cells as well as rat hippocampus (unpublished data): a GBS 300 bp upstream to c-JUN (GBS1) and located within a short distance (< 100 bp) from the MBS and another two GBS ~2kb and ~8kb downstream of the c-JUN transcription start site (TSS) (GBS2 and 3 respectively) (Table 1 and Fig. 5A). We investigated whether activated GR showed binding to these sites. To this end, neuronally differentiated PC-12 cells were treated for 60 minutes with 100 nM DEX and DNA-protein complexes were immunoprecipitated using GR or MEF2 antibodies. MEF2 binding to the upstream MBS was increased after 60 min DEX treatment (2.26-fold $p<0.01$) (Fig. 5B). Moreover, DEX-treatment increased GR-binding to both GBS1 and 3 ($p<0.05$ for both GBS1 and 3) but not to GBS2 (Fig. 5B). Binding of GR and MEF2 to a control region (Myoglobin) was not enhanced after DEX treatment (results not shown). Screening of the GBS for putative glucocorticoid response elements (GREs) revealed presence of a GRE at the downstream GBS 3, but not in GBS1.

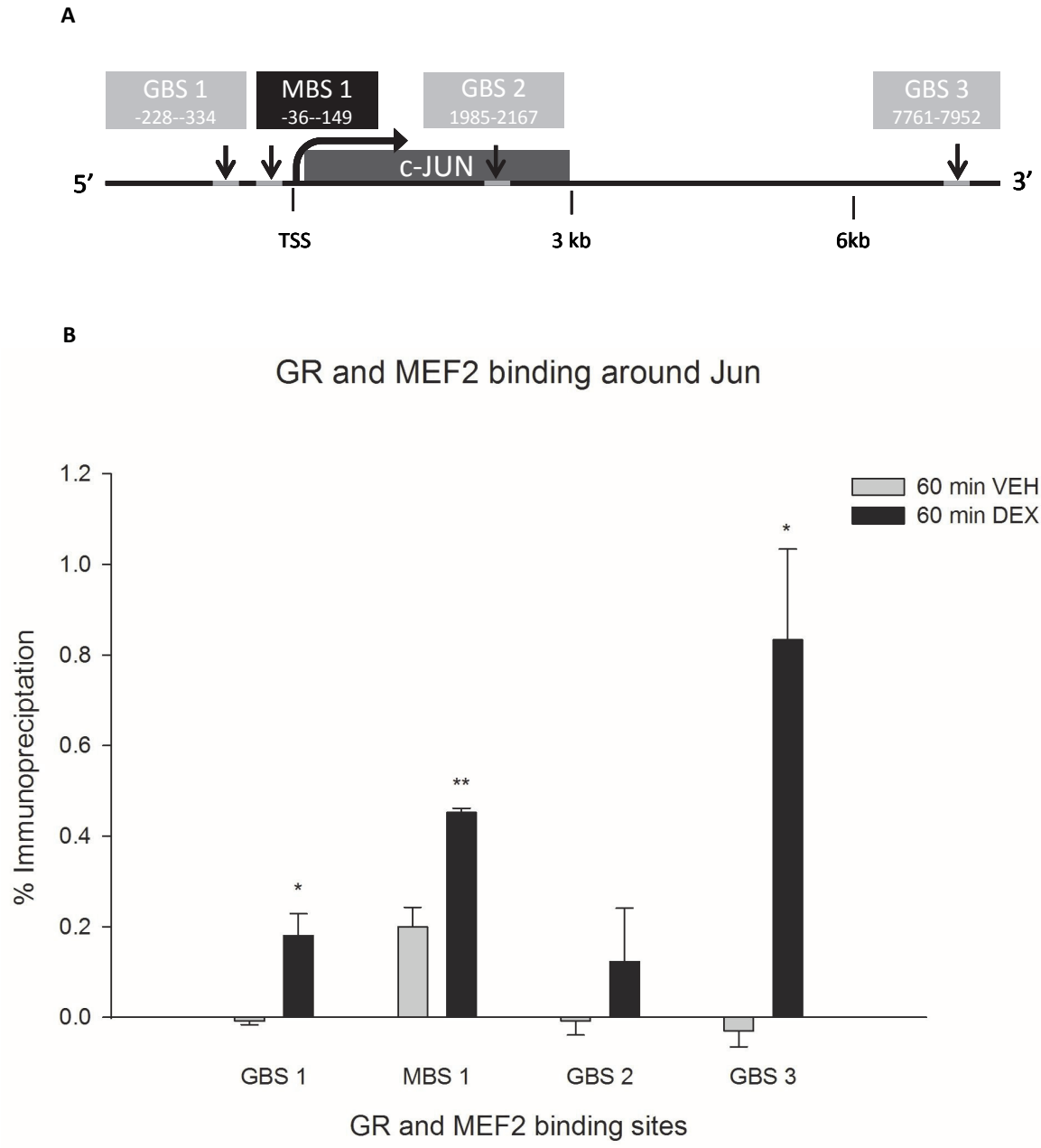

Fig. 5 |Binding levels of GR and MEF2a in the vicinity of the c-JUN gene (n=3 per group). A. Schematic overview of the c-JUN gene and surrounding sites. GR binding sites 1, 2 and 3 are depicted in red and the MBS1 is depicted in yellow. Numbers inside the boxes indicate distance from the beginning and end of the peak to the TSS. B. ChIP results representing DNA-binding of GR at three distinct binding sites designated GBS1, GBS2 and GBS3, and DNA-binding of MEF2a at the MEF2-binding site designated MBS1. Results are immunoprecipitated fractions plotted as percentage of total input DNA. The immunoprecipitated fraction is normalized to IgG binding. (* p<0.05, ** p<0.01 sign. vs VEH treatment).

DISCUSSION

GR and MEF2 are both transcription factors known to influence neuronal plasticity. We previously observed that GR and MEF2 have several target genes in common, including c-JUN and hypothesized that both transcription factors may cooperate in a neuronal context in the regulation of genes important for plasticity. Here we present evidence that there is an interplay of GR and MEF2 in the regulation of c-JUN at multiple levels. Our results show that activation of GR regulates phosphorylation, and hence transcriptional activity, of MEF2a as well as MEF2a-DNA binding upstream of target gene c-JUN.

In vitro model To study GR and MEF2 effects on target gene c-JUN we used neuronally differentiated PC-12 cells, a frequently used neuronal cell model. Previous studies showed that both GR and MEF2d are highly expressed in this cell line (Morsink et al. 2006a, Kim *et al.* 2011). Here we show here that the MEF2a isoform, which is highly expressed in the limbic system, has even higher expression levels than Mef2d. Therefore, we considered neuronal PC12 cells to be a good model system to study the interaction of MEF2 and GR signaling in a neuronal context. Since lentiviral or siRNA mediated knockdown of MEF2a proved to be difficult in this cell line after differentiation to a neuronal phenotype we decided to use primary hippocampal neurons to study the effect of MEF2a knockdown on c-JUN expression. Note that DEX downregulates c-JUN to the same extent in both cell lines.

c-JUN mRNA regulation as proof-of-principle for MEF2 and GR interplay To study the effect of GR on MEF2 activity and DNA-binding we focussed on the AP-1 transcription factor subunit c-JUN for several reasons. AP-1 is an ubiquitously expressed transcription factor and an important mediator of activity-induced dendritic growth (Hartwig *et al.* 2008). MEF2 is also a mediator of dendritic growth (possibly via c-JUN) and enhances the expression of c-JUN in an activity dependent manner (Flavell *et al.* 2008). Indeed, we show that acute activation of GR by DEX downregulates the expression of c-JUN, which is possibly mediated by decreased transcriptional activity of MEF2a due to phosphorylation.

MEF2a is necessary for the GR mediated effect on c-JUN transcription Knockdown of MEF2a led to a decrease in expression of c-JUN, implying that expression of c-JUN is mediated by MEF2a under vehicle conditions. This idea is strenghtened by our ChIP results which indicate that MEF2 is already bound to the MBS under VEH conditions. Other transcription factors than MEF2a likely also play a role, since the downregulation of c-JUN was relatively small compared to the knockdown of MEF2a . Indeed, MEF2d and MEF2c are also able to regulate c-JUN expression and are therefore possible

candidates controlling c-JUN expression. Interestingly, DEX treatment on top of MEF2a knockdown had no additional effect whatsoever, suggesting that the GR cannot exert its effect after knockdown of (phosphorylated) MEF2a.

Transcriptional machinery is repressed by phosphorylated and DNA-bound MEF2a Several studies have shown that post-translational modificiation of MEF2 significantly alters its activity (Molkentin *et al.* 1996, Shalizi et al. 2006, Gregoire et al. 2006). Phosphorylation of Serine 408 in MEF2a has an inhibitory effect on MEF2 transcriptional activity (Flavell et al. 2006, Gong *et al.* 2003, Shalizi et al. 2006, Potthoff & Olson 2007). Conversely, dephosphorylation of serine 408 in MEF2a is induced by neuronal activity, leading to activation of calcineurin, a potent phosphatase of MEF2 (Flavell et al. 2006, Shalizi et al. 2006). We show here for the first time that DEX treatment increases phosphorylation of MEF2a.

The DEX-effect on phosphorylation and hence decreased activity of MEF2a and increased DNA-binding at the same time, described in this study, seem contradictory at first. However, results on Phospoinositide-3-kinase, catalytic, gamma polypeptide (PIK3CG) binding and expression, reveal a similar response pattern. Pulipparacharuvil et al showed that increased phosphorylation of MEF2a at S408 correlated with increased binding of MEF2 close to the TSS of PIK3CG and decreased expression of the transcript (Pulipparacharuvil et al. 2008). In agreement with this, McKinsey et al showed that activated MEF2 is able to recruit histone acetyl transferases (HATs) such as p300, while phosphorylated MEF2 recruits histone deacetylases (HDACs) such as HDAC 4, 5, 7 and 9. MEF2 thus operates as a switch and is therefore able to directly activate or repress the transcriptional machinery (McKinsey et al. 2002).

A similar mechanism may be involved in regulation of c-JUN (Figure 6). At 60 minutes of DEX treatment MEF2a is phosphorylated and bound to the DNA to a higher extent than under VEH conditions. This may implie that the transcriptional machinery is repressed, probably by attracting HDACs. At the same time DNA binding of GR to GBS1, in the vicinity of MEF2a, is also increased, likely due to indirect binding via an intermediate transcription factor, since this site was not shown to contain a putative GRE. It was recently shown that increased HDAC7-DNA binding within exactly the same region as the GBS1 results in c-JUN downregulation in a deacetylase-independent manner (Ma & D'Mello 2011), suggesting that HDAC attraction may repress the transcriptional machinery without preventing other transcription factors to bind to the DNA.

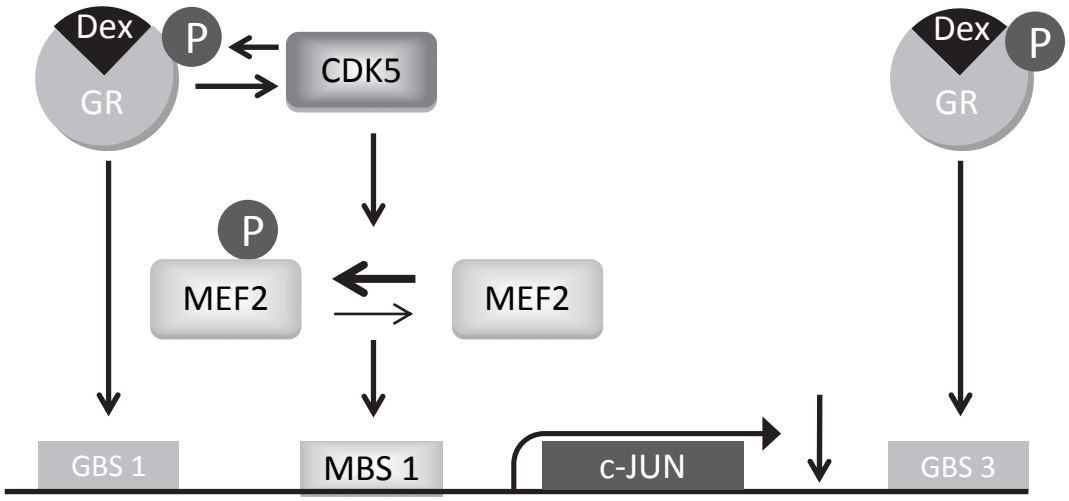

Fig. 6 | Schematic overview of the obtained results. Activation of GR by agonist DEX activates the kinase CDK5, responsible for phosphorylation of its target proteins, including GR and MEF2. MEF2, now phosphorylated, is increasingly bound to the DNA where it is suggested to act as a transcriptional repressor. GR is also bound to the DNA under this condition. However, to what extent GR itself is responsible for c-JUN downregulation remains to be studied.

GC-effects on the transcriptional machinery may represent a more general phenomenon underlying some of the long-term changes in neuronal expression that have been observed in response to acute GR activation. For example, in the CA1 area of the hippocampus, long-term potentiation (LTP) was found to be enhanced up to 24 hours after a brief stress-induced rise in corticosterone, accompanied by an enhanced expression of GR, that was still present 24 hours after termination of the stress response (Ahmed *et al.* 2006). Another study, focussing directly on the plasticity related gene neuropsin (NP), found that corticosterone readily upregulates this gene in the hippocampus, which remains elevated for over 24 hours (Harada *et al.* 2008). Even weeks after stress a persistent overexpression of the stress-associated splice variant of the neuronal acetylcholinesterase gene was observed, likely caused by long-term expression of the SC35 splicing factor in response to stress (Meshorer *et al.* 2005)

Exactly how activated GR leads to increased phosphorylation of MEF2a is not known. The mainly neuron specific kinase CDK5 phosphorylates MEF2a at serine 408 (Gong et al. 2003). Since GR is known to recruit CDK5 for its own phosphorylation upon DEX binding (Kino *et al.* 2007), we hypothesize that MEF2a is recruited at the same time by GR and hence is phosphorylated by CDK5. Alternatively, CDK5 activity may be enhanced upon binding to GR and subsequently, after detaching from GR, starts to phosphorylate other target proteins like MEF2a. Furthermore, it has been shown that calcineurin (CaN) mRNA expression, a phosphatase responsible for reducing phosphorylation at serine 408 in MEF2a, is significantly reduced by corticosterone treatment (Morsink et al. 2006b), which might also lead to increased phosphorylation levels. However, downregulation of CaN was

only observed at 180 minutes of corticosterone treatment, while the present study indicates a phosphorylation difference already at 60 minutes of DEX treatment.

Another possibility of decreased transcriptional activity by MEF2 might be the downregulation of transcriptional enhancers like the previously mentioned p300. This HAT is a direct target of microRNA-132 which was found to be extensively upregulated under stressful conditions (Shaltiel *et al.* 2012). MEF2 plays an important role in neuronal differentiation (Shalizi & Bonni 2005). Since miR-132 was also recently found to play an important role in neuronal differentiation of PC-12 cells as well as of hippocampal neurons, it is possible that elevated corticosterone levels influence MEF2 function via this pathway as well (Luikart *et al.* 2011, Magill *et al.* 2010).

CONCLUSION

This study provides new insights into the molecular interplay at multiple levels of two transcription factors that are central to neuronal plasticity, GR and MEF2a.To our knowledge this is the first report showing a direct effect of GR on the activity and DNA-binding of MEF2a. An interesting avenue for future studies will be to determine how stress and subsequent glucocorticoid release influences MEF2 in several brain areas and how this might affect plasticity-based processes such as learning and memory.

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SUPPLEMENTARY DATA

Table S1 Primer sequences used for gene expression measurements or immuno-precipitated DNA fragments bound by GR or MEF2.

NCBI accession #	Gene name	Species	Gene Symbol	purpose	BP from TSS	Forward primer	Reverse primers
NC_005104.2	Jun oncogene	Rat	c-Jun	GR DNA-binding 1	-275	CGCGAAGGCTCACGGGATGA	CGGGAACACAAGCCGGAGCA
	Jun oncogene	Rat	c-Jun	GR DNA-binding 2	2097	GGTCCATGCAGTTCTTGGTCA	TGGACTGGGTTGCGACCTGA
	Jun oncogene	Rat	c-Jun	GR DNA-binding 3	7871	TCTGATAACCCAATTTTCTGAAGCA	TGCCCATGTCTCAGCAGTGT
	Jun oncogene	Rat	c-Jun	Mef2 DNA-binding	-36	AGTCTCTGCCACACTCAGTGCAA	TGGGAAGGCCTTGGGGTGACA
NC_005106.2	Myoglobin	Rat	Mb	Negative control GR and MEF2 DNA-binding	3504	TAGTGTGCATCCAGCAGAGG	ACACTGTGGCCTTTTTGTCC
NM_021835.3	Jun oncogene	Rat	c-Jun	Expression	NA	GCTGGAAGAAGAGGGTGTTG	CACAGCGCATGCTACTTGAT
NM_010591.2	Jun oncogene	Mouse	c-Jun	Expression	NA	GGTGGGAGGGGTTACAAACT	GGGGAGTTCATCTGCAGTCT
NM_001014035.1	Myocyte enhancer factor 2a	Rat	Mef2a	Expression	NA	TCAAGCCACACAACCTCTTG	GTGTTGTAGGCTGTCGGCAT
NM_001017507.1	Myocyte enhancer factor 2b	Rat	Mef2b	Expression	NA	GAACAGCCACTCTGCACAAC	TTCTTCATCAGCCCGAACTT
XM_574821.3	Myocyte enhancer factor 2c	Rat	Mef2c	Expression	NA	AGCAGCAGCACCTACATAACA	GAAGGCAGGGAGAGATTTGA
NM_030860.2	Myocyte enhancer factor 2d	Rat	Mef2d	Expression	NA	GGAGGCTGTGCATAGGTGTT	TGATCAGGAGCCTCACACTG
NM_001109119.1	Tubulin beta 2A	Rat	Tubb2a	Expression	NA	GAGGAGGGCGAGGATGAGGCTT	GACAGAGGCAAACTGAGCACCAT
NM_009450.2	Tubulin beta 2A	Mouse	Tubb2a	Expression	NA	TCCTTAGCCCTCTGTCCACGCA	ACCTCCCAAAACTTAGCGCCGATCT

Chapter 4 | Depolarization-induced binding of MEF2 to the promoter region of NR4A1 is prevented by GR activation

ABSTRACT

Glucocorticoid Receptor (GR) and Myocyte Enhancer Factor 2 (MEF2) are transcription factors with important functions in synaptic plasticity. MEF2 activity is strongly induced by neuronal depolarization, resulting in increased expression of target genes. The most well-known MEF2 target gene regulated in this way is the immediate-early-gene Nuclear Receptor subfamily 4, group A, member 1 (NR4A1), known to play a role in neurite outgrowth. We previously showed in neuronal PC-12 cells that GR activation by dexamethasone leads to phosphorylation of MEF2, the transcriptionally repressive form of MEF2. Here we show in the same cell line that GR activation significantly attenuates NR4A1 expression underKCl-induced neuronal depolarization. While neuronal depolarization resulted in increased MEF2-binding to the NR4A1 promoter region, concomitant GR activation reduced MEF2-binding to levels observed under baseline non-depolarizing conditions. This points to a contextual action mediated by GR, in which activation of GR under depolarizing conditions abrogates the depolarization-induced effects of MEF2 on regulation of its target gene NR4A1, likely by interfering with MEF2 DNA binding. A similar modulatory effect via GR under depolarizing conditions could also be demonstrated for other MEF2 target genes, including immediate-early response 2 (IER2) and c-FOS. In conclusion we show that in a depolarizing environment, the modulatory activity of GR has direct consequences for MEF2-DNA binding and expression of its target genes. Counterbalancing MEF2-effects on gene expression upon neuronal depolarization may represent a novel mechanism via which glucocorticoids regulate neuronal plasticity.

INTRODUCTION

Neuronal activity plays a major role in neuronal plasticity, survival and long-term potentiation (LTP) (Wiegert & Bading 2011, Leslie & Nedivi 2011). Several processes such as learning, memory and behavioral sensitization depend on neuronal activity and its downstream effects, including induction of dendritic outgrowth and enhancement of synaptic strength.

Upon neuronal activation, neurons depolarize, leading to influx of Ca^{2+}-ions and activation of the phosphatase calcineurin (CaN) (Flavell *et al.* 2006, Lam *et al.* 2009, Youn *et al.* 2000). In turn CaN dephosphorylates the transcription factor myocyte enhancer factor 2 (MEF2) (Blaeser *et al.* 2000, Flavell & Greenberg 2008, Youn et al. 2000, Mao & Wiedmann 1999). It has been shown that dephosphorylation at serine 408 by CaN changes the transcriptional potential of MEF2 from repressive to active (Gregoire *et al.* 2006, Gong *et al.* 2003, Mao & Wiedmann 1999). Depolarization also decreases the activity of cyclin-dependent kinase 5 (CDK5), which is responsible for the phosphorylation of MEF2 at serine 408 (Schuman & Murase 2003). A recent genome-wide analysis in a depolarizing context, revealed that many MEF2 target genes have important functions in synaptic plasticity and neurotransmitter release (Flavell *et al.* 2008, Cole *et al.* 2012, Akhtar *et al.* 2012). One of these MEF2 target genes is the immediate-early gene nuclear receptor family 4, group A, member 1 (NR4A1) (also known as NGFI-B or NUR77) (Youn & Liu 2000) known to play a key role in neurite outgrowth (14,15).

The glucocorticoid receptor (GR), like MEF2, has also been implicated in neuronal plasticity (Liston & Gan 2011, Karst *et al.* 2010). GR acts as a ligand-activated transcription factor and is widely expressed throughout the brain. GR regulates a wide variety of functional gene classes including plasticity-related genes, in a context-dependent manner. GR mediates opposite effects of corticosterone on neuronal plasticity (Joels & Krugers 2007). In amygdala slices for instance, it was observed blockade of GR by mifepristone corticosterone enhances LTP via voltage-dependent calcium channels (VDCC) while it suppresses LTP via NMDA receptors (Krugers *et al.* 2005, Karst *et al.* 2000). These results suggest that a delicate balance exists between pathways that strengthen and pathways that weaken plasticity and that GR plays a key role in controlling this process that is so instrumental for neuronal functioning in a changing environment.

Using PC12 cells we recently showed for the first time that GR has a modulatory effect on MEF2 under baseline non-depolarizing conditions (Speksnijder *et al.* 2012). Since GR activation modulates depolarization-induced Ca^{2+}-influx, which is itself the activator of MEF2, the aim of this study was to investigate the effect of GR activation on MEF2 under depolarizing conditions. For this purpose we used NR4A1 as target gene because it is significantly induced following treatment with depolarizing

concentrations of KCl, while it is blocked when MEF2 is knocked down, both in PC-12 cells and hippocampal neurons [12, 16]. Constitutively active MEF2 was found to enhance NR4A1 expression in PC-12 cells via binding to two MEF2 binding sites, upstream of the transcription start site [17, 18]. In the current study we find in neuronally-differentiated PC12 cells that GR activation attenuates the expression of MEF2 target gene NR4A1 and interferes with MEF2-DNA binding to the NR4A1 promoter region in the context of KCl-induced neuronal depolarization.

MATERIALS & METHODS

Cell culture and treatment Rat pheochromocytoma (PC-12) cells (passage # 15-29) were cultured as described earlier (Morsink *et al.* 2006). In short, cells were grown in DMEM medium, supplemented with 0-10% fetal bovine serum and 0-10% horse serum, dependent on the stage of neuronal differentiation. For mRNA analysis, cells were seeded at a confluency of 30-50% in pre-coated 6-well plates (356400, BD Biosciences, San Jose, CA, USA). For chromatin immunoprecipitation (ChIP) experiments the cells were seeded at 50% confluency in pre-coated 175 cm^2 plates (356478, BD Biosciences). Neuronal differentiation was achieved by giving 50 ng/ml NGF-ß (N2513, Sigma-Aldrich, St. Louis, MO, USA) every other day for 10 days. At day 9 of the differentiation stage the culture medium was supplemented with charcoal stripped serum to remove endogenous steroids (Sarabdjitsingh *et al.*). At day 10 the cells were treated for 60 minutes (ChIP) or 90 minutes (mRNA expression), dependent on the experiment, with either vehicle (VEH) (0.1% ethanol), 100 nM dexamethasone (DEX) (D1756, Sigma-Aldrich), 55mM KCl (60128, Sigma-Aldrich) or a combination of these treatments.

Chromatin Immunoprecipitation (ChIP) At the end of the treatment time, the cells were fixed with 1% formaldehyde (344198, Merck, Darmstadt, Germany) for 10 minutes at room temperature (RT) to crosslink DNA-protein interactions. Cells were scraped from the plates in 10 ml ice-cold phosphate-buffered saline containing protease inhibitors (PI; 11836153001, Roche). The DNA was sonicated for 15-25 pulses (Bioruptor UCD-200, Diagenode, Sparta, NJ, USA). For immunoprecipitation, 20-60 µg of input DNA was used per antibody. Pre-cleared DNA was incubated overnight with 6 µg of anti-GR (H-300) antibody (sc-8992X, Santa Cruz, CA, USA), 6 µg of anti-MEF2 (C-21) antibody (sc-313X, Santa Cruz) or normal rabbit IgG (sc-2027, Santa Cruz). The next day, sepharose A beads were added to the DNA-protein-antibody complexes and incubated for 90 minutes at 4°C. The samples were subsequently washed at RT 1x with low salt buffer (0.1% SDS; 2mM EDTA pH 8.0; 20 mM Tris-HCl pH 8.0; 150 mM NaCl; 1% Triton-X-100), 1x with high salt buffer (as low salt except 500 mM NaCl), 1x with LiCL buffer (0.25 M LiCl; 1 mM EDTA pH 8.0; 20 mM Tris-HCl pH 8.0; 1% NP-40; 1% NaDOC) and 2x with TE buffer (1mM EDTA pH 8.0; 10 mM Tris-HCl pH 8.0) respectively. The DNA complexes were subsequently eluted from the beads and the DNA was reverse-crosslinked overnight in 0.2 M NaCl solution at 65°C. The next day the samples were treated with RNAse and the DNA purified using nucleospin columns (740609, Machery-Nagel, Duren, Germany) according to the manufacturer's protocol. The DNA was eluted in 50 µl of TE buffer for RT-qPCR analysis.

RT-qPCR primer design Primers were designed as described before (Speksnijder et al. 2012). In short, primers for RT-qPCR of immunoprecipitated DNA fragments were designed surrounding DNA regions

bound by GR as shown in glucocorticoid (GC) treated neuronally differentiated PC-12 cells (unpublished results by Polman et al). Primer-BLAST (NCBI, Bethesda, USA) was used to obtain primer specific sequences and an additional check for primer hairpins was performed using Oligo 7.0 (MBI Inc. Cascade, USA). Primers were tested with RT-qPCR on a standard curve to check the efficiency of the PCR reaction. Primer sequences are listed in supplementary table S1.

Real time quantitative PCR (RT-qPCR) Total RNA was isolated using Trizol (15596, Invitrogen) according to the manufacturer's instructions. RNA was diluted to 50 ng/ul and cDNA was synthesized using the iScript cDNA synthesis kit (170-8897, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. RT-qPCR was performed on a Lightcycler 2.0 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) in combination with the Lightcycler FastStart DNA Master^{PLUS} SYBR Green I Kit (03515885001, Roche). As a control for genomic contamination, samples without reverse transcriptase were used. The standard curve method was applied to quantify the expression differences (Livak and Schmittgen, 2001). Expression of TUBB2a (Tubulin, beta 2a) was used to normalize the RNA input. Primer sequences used are listed in supplementary table S1.

Western Blotting Protein was harvested in ice-cold RIPA buffer containing Protease Inhibitors (#04693124001, Roche) and phosphatase inhibitors ($NaVO_3$ and B-glycerophosphate). The cell lysate was incubated on ice for 30 minutes, spun down and the supernatant transferred to a new tube. Protein concentration was measured using the Pierce BCA Protein Assay kit (23225, Thermo Scientific, Rockford, IL, USA), according to the manufacturer's protocol. Diluted samples were supplemented with 1:2 volume of sample buffer (including 2.5% ß-mercaptoethanol and BromoPhenol Blue). Twenty µg of each sample was loaded on a 10% polyacrylamide gel. After sufficient separation of the proteins, they were transferred overnight at 4°C to a PVDF (Polyvinylidene fluoride) membrane. The membrane was subsequently blocked in 5% low fat milk for 1 hour at RT or 5 hours at 4°C for phospho-proteins. Primary antibodies were added in the blocking buffer and incubated for 1 hour at RT or at 4°C overnight for phospho-proteins with either one of the following primary antibodies: Anti-phospho S408 MEF2a rabbit monoclonal (ab51151, Abcam, Cambridge, UK) or anti-α-Tubulin DM1A mouse monoclonal antibody (T6199, Sigma). Blots were incubated for 1 hour at RT with the appropriate secondary antibody: goat-anti rabbit IgG HRP secondary antibody (sc-2054, Santa Cruz) or goat-anti mouse IgG HRP secondary antibody (sc-2055, Santa Cruz). Signals were quantified using ImageJ (v1.42; National Institute of Health, USA).

RESULTS

MEF2 is dephosphorylated under depolarizing conditions Neuronal PC-12 cells were treated with 55 mM KCl and proteins were harvested after 60 minutes to measure MEF2 s408 phosphorylation, the transcriptionally inactive form of MEF2. In agreement with the literature on other cell lines and *in vivo*, we observed that MEF2 phosphorylation at serine 408 is significantly decreased by KCl treatment (Fig. 1), thus enhancing the transcriptional potential of MEF2.

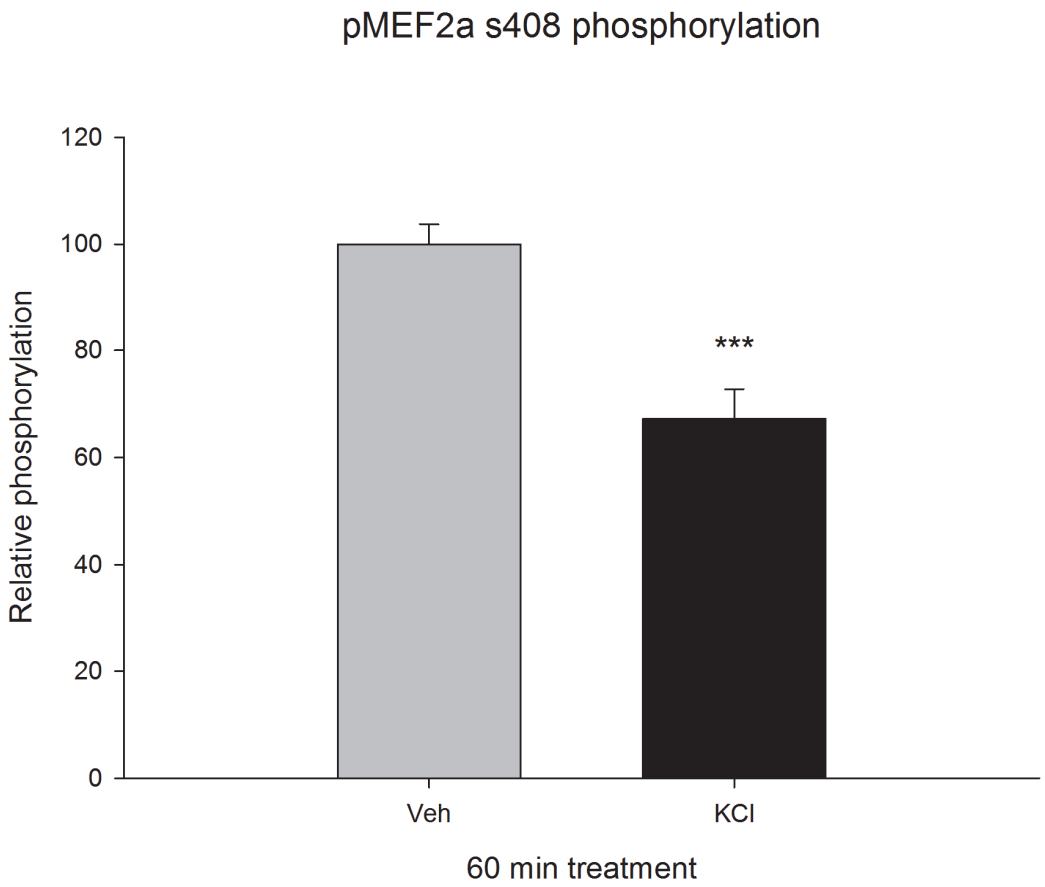

Figure 1 | MEF2a phosphorylation level at serine 408 after 60 minutes of KCl treatment (n=6 per group). A) Phosphorylation level is relative to 60 minutes VEH treatment, set at 100%. Expression is normalized to total MEF2a and alpha-tubulin. (* p<0.001 sign. vs VEH treatment).**

NR4A1 mRNA induction by KCl is attenuated by GR activation To study the effect of GR on mRNA expression of the MEF2 target gene NR4A1 under depolarizing conditions, neuronal PC-12 cells were treated with KCl, dexamethasone (DEX), or a combination of KCl and DEX for 90 minutes. This time point, 30 minutes after measuring MEF2 phosphorylation, was chosen since it typically takes 30 minutes to detect changes in gene expression of immediate-early genes (IEG). DEX treatment alone did not affect NR4A1 expression (Fig. 2), also not after 180 minutes of treatment (data not shown). Ninety minutes of a depolarizing concentration of KCl induced a large increase in NR4A1 expression

(~200-fold p<0.001) compared to the VEH condition. Surprisingly, DEX significantly attenuated this induction by 2-fold to a ~100-fold increase compared to the VEH condition (p<0.001 vs all other conditions) (Fig. 2).

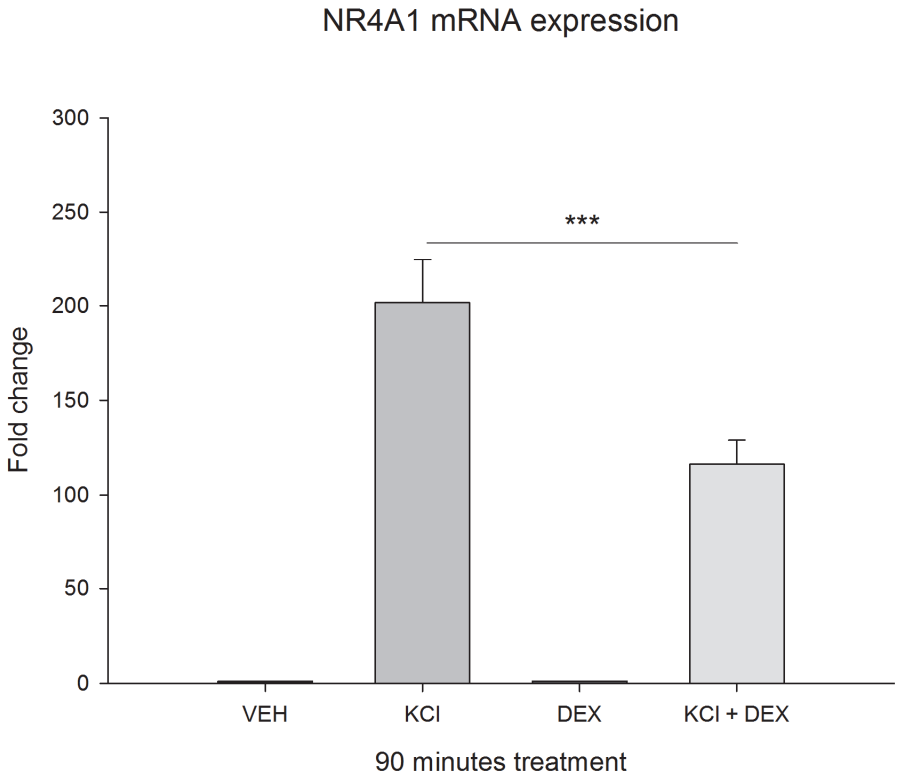

Figure 2 | NR4A1 mRNA expression at 90 minutes of KCl, DEX or KCl+DEX treatment (n=6 per group). Expression level is depicted as fold change relative to the VEH condition which is set at 1. Expression is normalized to TUBB2a. (ANOVA p<0.001; Tukey's post hoc test *** p<0.001).

MEF2 binding upstream and GR binding downstream NR4A1 is prevented by GR activation under depolarizing conditions Since KCl-induced depolarization resulted in both an increase in activity of MEF2 as well as a large induction of NR4A1, we next studied whether binding of MEF2 in the vicinity of NR4A1 was increased using chromatin immunoprecipitation of MEF2-bound genomic regions. Two MEF2 binding sites were identified in literature at -309 and -275 bp upstream of the transcription start site (TSS) of the NR4A1 gene (Lam *et al.* 2010). We did not differentiate between both sites, since they lie within 34 bp of each other and sonication of the DNA does not result in adequate discrimination between both sites. Therefore a DNA sequence harboring both sites was amplified, designated MBS1 (Fig. 3A) (Pulipparacharuvil *et al.* 2008). Following KCl-induced depolarisation, MEF2 binding to MBS1 was significantly enhanced (Fig. 3B). Although DEX treatment on its own did not result in an expression change of NR4A1, significant binding to MBS1 was observed (Fig. 3B). When both treatments were combined, MEF2-DNA binding was reduced by 3-fold compared to KCl treatment alone and was similar to VEH levels after 60 minutes of treatment (Fig. 3B).

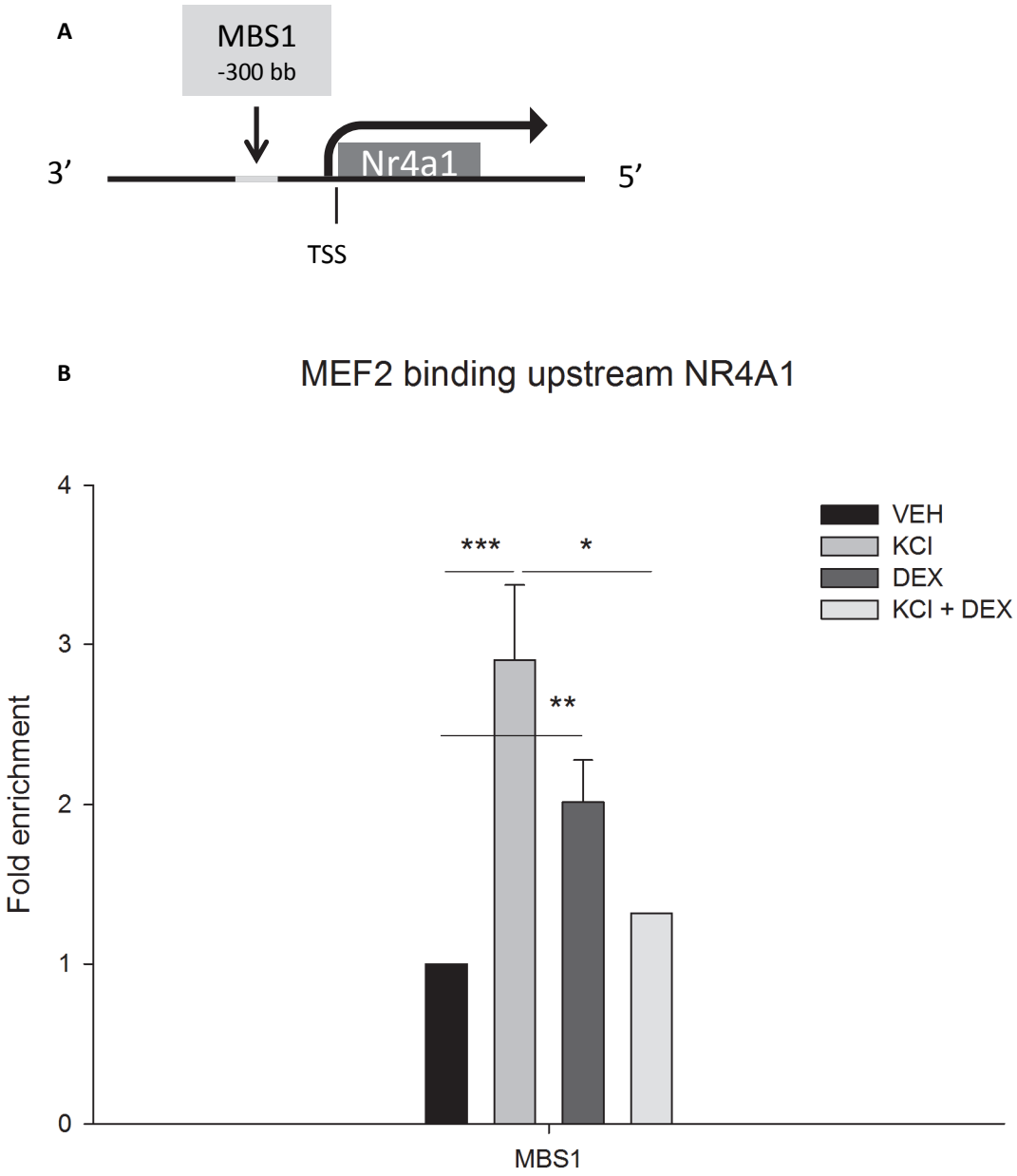

Figure 3 | Binding of MEF2 upstream NR4A1. A) Scheme representing the site of MEF2 binding upstream NR4A1, actually consisting of two neighboring MEF2 response elements. The number inside the box indicates the distance of the binding site to the TSS. B) Result of ChIP experiments representing DNA-binding of MEF2 upstream NR4A1 after VEH, KCl, DEX and KCl+DEX treatment (ANOVA p<0.01; Tukey's post hoc test * p<0.05, ** p<0.01, *** p<0.001). Results are fold changes in binding vs VEH treatment and normalized against IgG immunprecipitated fractions.

MEF2 target gene expression is modulated by GR activation in depolarized neurons Many IEGs are known to be regulated by MEF2, including c-JUN, c-FOS, activity-regulated transcript (ARC), and immediate-early response 2 (IER2) (Flavell et al. 2008, Flavell et al. 2006, Han & Prywes 1995, Lam et al. 2010). Since we observed a significant DEX-effect on KCl-mediated expression of NR4A1, we hypothesized that DEX may also attenuate the neuronal activity-dependent expression of several other MEF2 target genes. First we identified IER2 and c-FOS as genes that are significantly upregulated by KCl treatment in neuronally differentiated PC-12 cells (Fig. 4). Similar to NR4A1, GR activation by DEX also significantly attenuated the KCl-induced expression of both these genes (Fig. 4).

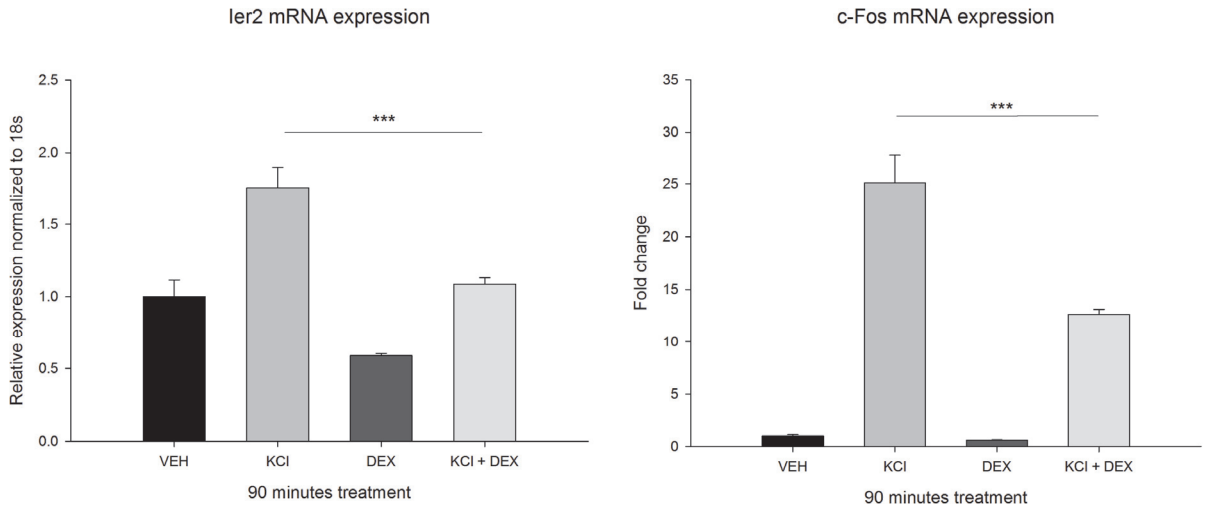

Figure 4 | Immediate-early genes Ier2 and c-FOS expression after 90 minutes of KCl, DEX and KCl+DEX treatment (n=6 per group). Expression levels are depicted as fold change relative to the VEH condition which is set at 1. Expression is normalized to TUBB2a. (ANOVAs p<0.05; Tukey's post-hoc *** p<0.001).

DISCUSSION

The MEF2 transcription factor is an important factor in synaptic plasticity and one of the target proteins of neuronal activation. We previously found that MEF2 and GR cooperate in the control of target gene expression in neuronally differentiated PC-12 cells under basal conditions. However, since MEF2 is activated by neuronal depolarization, we subsequently studied what the effect of GR is on MEF2 phosphorylation and DNA binding under depolarizing conditions.

NR4A1 mRNA is induced by KCl treatment We chose to investigate the effect of dexamethasone-activated GR on MEF2 in neuronally differentiated PC-12 cells, a cell model often used to study the effects of (neuronal) depolarization (Lam et al. 2010, Sheng *et al.* 1988, Ahn *et al.* 1998, Hansen *et al.* 2003). We show, in agreement with earlier reports, that MEF2 is dephosphorylated by KCl treatment. KCl-induced depolarization leads to calcium influx, which activates Calcineurin (CaN), resulting in dephosphorylation of MEF2 and a concomitant increase in its transcriptional potential. A well-known MEF2 target gene is NR4A1 and dephosphorylation of MEF2 is known to enhance NR4A1 expression. Conversely, blocking CaN, which prevents dephosphorylation of MEF2 was found to interfere with depolarization-induced NR4A1 expression (Lam et al. 2010, Ahn et al. 1998, Enslen & Soderling 1994, Tian *et al.* 2010, Blaeser et al. 2000). In agreement with the literature we show here that NR4A1 mRNA expression is induced by KCl. However, the magnitude of induction (~200-fold) is far greater compared to other studies in PC-12 cells. One study showed a 20-fold increase in NR4A1 expression using Northern blot analysis (Machado *et al.* 2008). Other studies using Northern blot analysis of KCl-induced induction of NR4A1 did not quantify the fold changes (Yoon & Lau 1993, Yoon & Lau 1994), or did not focus on direct mRNA expression of NR4A1 but instead used luciferase reporter assays to test activity of reporter constructs containing parts of the promoter region of NR4A1 (Lam et al. 2010)

Activation of GR in depolarizing neurons prevents MEF2-DNA binding to NR4A1 DEX treatment alone had no effect on NR4A1 expression, however, a clear attenuating effect of GR on depolarization-induced NR4A1 mRNA expression was observed. DNA binding of MEF2 is a prerequisite for MEF2-regulation of NR4A1. Two MEF2 binding sites were previously shown to reside just upstream of the TSS of NR4A1. MEF2 binding is enhanced by neuronal depolarization, which is consistent with a recent report by Lam et al, showing that constitutively active MEF2 attenuates KCl-induced expression of NR4A1 (Lam et al. 2010). Moreover, in unstimulated PC-12 cells, MEF2 actively represses NR4A1 expression, which is relieved by depolarization due to dephosphorylation of MEF2. In addition, DEX enhances binding of MEF2 to the DNA, which we previously also found to be the case for c-JUN (Speksnijder et al. 2012). Interestingly, we observed that MEF2-binding was

diminished to VEH levels when GR activation by DEX was combined with KCl-induced neuronal depolarization. Since we showed that treatment resulting in either GR activation or depolarization enhances binding of MEF2, this result was surprising.

Since MEF2-GR cooperation is a newly observed phenomenon, not much is known about this (Speksnijder et al. 2012). In the pituitary, it was observed that the MEF2 target gene NR4A1 and GR can antagonize each other. NR4A1 expression is enhanced by stress through induction of corticotrophin-releasing hormone (CRH). NR4A1 then binds to a negative glucocorticoid response element (nGRE) in the pro-opiomelanocortin (POMC) gene, preventing GR to block its expression and exert negative feedback of the hypothalamus-pituitary-adrenal (HPA)-axis (Okabe *et al.* 1998). In vitro, in AtT-20 cells, this antagonistic property was even found to result in decreased physical DNA binding of both GR and NR4A1 to a minimal POMC promoter. So, NR4A1 diminished GR-DNA binding while GR prevented NR4A1-DNA binding (Philips *et al.* 1997). This antagonistic mechanism might also play a role when both MEF2 and GR are activated and would explain why both MEF2-DNA and GR-DNA binding are diminished when they are activated at the same time by DEX and neuronal depolarization. Another possible explanation comes from the observation that the GR is able to

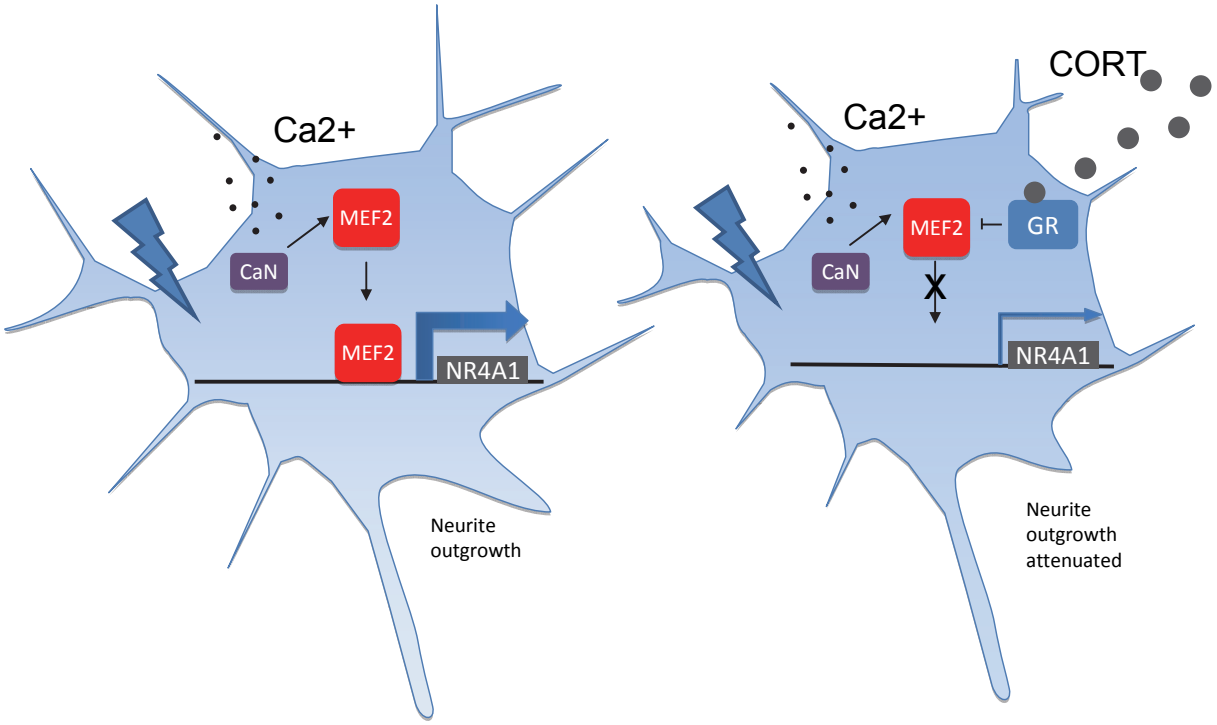

Figure 5 | Schematic overview of the obtained results. Depolarization by KCl leads to Ca^{2+}-ion influx (black dots) and activation of CaN. This would lead to dephosphorylation and transcriptional activation of MEF2 resulting in enhanced expression of NR4A1. GR activation by DEX leads to is known to enhance phosphorylation of MEF2, leading to transcriptional repression g. As depicted in this figure, combined depolarization and activation of GR leads to reduced binding of MEF2 to its binding site and attenuated NR4A1 expression compared to depolarization alone.

inhibit the action of nuclear factor of activated T cells (NFAT), a protein which is an activator of MEF2. NFAT is known to travel to the nucleus upon Ca^{2+} influx and bind to MEF2 to enhance transcription (Youn et al. 2000, Vacca *et al.* 1992, Blaeser et al. 2000). Simultaneous activation of GR by DEX and subsequent binding of GR to NFAT might prevent enhanced activation of MEF2 (Fig 5).

Following this hypothesis, another transcription factor might be responsible for the expression of NR4A1. Indeed, neuronal depolarization is also known to enhance the activity of cAMP response element binding protein (CREB) and NR4A1 is target gene of this transcription factor as well (Fass *et al.* 2003, Flavell & Greenberg 2008). This might indicate that expression of NR4A1 under the specific condition of both activated GR and depolarization is regulated solely by CREB. Since depolarization leads to dephosphorylation of MEF2, resulting in recruitment of transcriptional enhancers, the inability of MEF2 to bind upstream NR4A1 may lead to attenuated expression of NR4A1. NR4A1 expression gives rise to neurite outgrowth in PC-12 cells, one of the prerequisites for synaptic plasticity (Maruoka *et al.* 2010). Inhibition of NR4A1 expression by GR through preventing MEF2-DNA binding might pose a new mechanism of regulation by GR and might be an interesting new avenue of research on GR influencing plasticity.

Attenuating effect of GR on MEF2-regulated IEG expression In this study we mainly focused on IEGs since we previously identified these genes to be common target genes of GR and MEF2 (Datson *et al.* 2011). MEF2 is a neuronal activity-induced transcription factor and regulates transcription within a very short time span. Multiple IEGs apart from NR4A1 are known to contain binding sites for MEF2 such as c-JUN, ARC, IER2, c-FOS etc (Kawashima *et al.* 2009, Lam et al. 2010, Knoll & Nordheim 2009, Han *et al.* 1992, Han & Prywes 1995, Flavell et al. 2008). GR binding sites in the vicinity of these IEGs were found by GR ChIP-sequencing in PC-12 cells and rat hippocampus (unpublished data).

In PC-12 cells we found that KCl also induced the expression of IER2 and c-FOS but not of ARC or c-JUN. In both IER2 and c-FOS we also observed the attenuating effect of DEX on KCl-induced gene expression, comparable to NR4A1. Both IEGs showed lower mRNA expression after DEX + KCl treatment compared to KCl treatment alone. However, for IER2, GR activation by DEX by itself decreased expression of IER2. c-FOS expression was not changed by GR activation alone, but it significantly attenuated c-FOS expression induced by KCl. These results, are in line with in vivo observations where absence of endogenous glucocorticoids by adrenalectomy significantly enhanced IEG expression induced by kainate, a NMDA receptor agonist, resulting in Ca^{2+}-influx and hence neuronal activation (Li *et al.* 1992). Whether the obtained results can be extrapolated to other genes apart from IEGs remains to be studied.

CONCLUSION

The results clearly show an attenuating effect of GR activity on depolarization-induced NR4A1 expression. More importantly we show that under this condition MEF2-DNA binding upstream of NR4A1 is reduced to VEH levels. Depolarization-induced NR4A1 expression is an important mediator of synaptic plasticity, by facilitating neurite outgrowth. One of the known ways in which GR modulates synaptic plasticity is to balance calcium influx. Based on the presented results we propose another, more downstream, mechanism of modulation where GR attenuates NR4A1 expression by preventing MEF2-DNA binding, possibly leading to inhibited neurite outgrowth.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Table S1 Primer sequences used for gene expression measurements or immuno-precipitated DNA fragments bound by GR or MEF2.

NCBI accession #	Gene nam e	Gene Symbol	purpose	BP from TSS	Forward primer	Reverse primers
NC_005106.3	Nuclear Receptor, family 4, group A, member 1	NR4A1	MEF2 DNA binding	-271	GTGCAGCGGGCGAGAGGAAA	CGCGGGGTTCCATTGACGCA
NC_005106.2	Myoglobin	Mb	Negative control GR and MEF2 DNA-binding	3504	TAGTGTGCATCCAGCAGAGG	ACACTGTGGCCTTTTTGTCC
NM_024388.2	Nuclear Receptor, family 4, group A, member 1	NR4A1	Expression	NA	GCTTGGGTGTTGATGTTCCT	ACAGCTAGCAATGCGGTTCT
NM_022197.2	FBJ osteosarcoma oncogene	c-FOS	Expression	NA	GGGACAGCCTTTCCTACTACC	TGGCACTAGAGACGGACAGA
NM_001009541.1	Immediate-early response 2	IER2	Expression	NA	AACGTGCTGGTGCGAACCGT	CTTCGCCTCGGGTGTGCGTT
NM_001109119.1	Tubulin beta 2A	Tubb2a	Expression	NA	GAGGAGGGCGAGGATGAGGCTT	GACAGAGGCAAACTGAGCACCAT

Chapter 5 | Hippocampal MEF2 phosphorylation is enhanced during induction of sensitization

ABSTRACT

Inbred DBA/2J mice show profound individual differences in amphetamine-induced locomotor sensitization. We have previously shown differences in hippocampal gene expression patternsin these animals, in particular of target genes of the myocyte enhancer factor 2 (MEF2) and glucocorticoid receptor (GR). Interestingly, striatal phosphorylation of MEF2 has been suggested to be a key regulator of the psychomotor response to amphetamine. The present study was designed to investigate if and to what extent phosphorylation of hippocampal MEF2 might be related to individual differences in the induction and/or expression of amphetamine sensitization. In a first experiment, hippocampal MEF2 phosphorylation was measured at two distinct time points during amphetamine sensitization: a) after a challenge injection of amphetamine at day 20 during the expression of sensitization, and b) after the 5^{th} injection, on the last day of the induction phase. In a second experiment, MEF2 phosphorylation was manipulated by intracerebroventricular (ICV) injection with the CDK5 inhibitor roscovitine. While at day 20, after a challenge injection of amphetamine, phosphorylation of hippocampal MEF2 was not changed, a clear increase in phosphorylation was seen after 5 consecutive days of amphetamine injections. Roscovitine significantly enhanced the locomotor response to amphetamine, and was found to enhance the phosphorylation status of MEF2 and of glucocorticoid receptors (GR) in hippocampus, but not in striatum. Changes in hippocampal MEF2 target gene expression following a challenge dose of amphetamine are more likely to originate from changes induced in hippocampal MEF2 phosphorylation during the induction, rather than the expression phase of amphetamine sensitization.

INTRODUCTION

Dopamine sensitivity is thought to be an important hallmark of psychosis susceptibility (Seeman *et al.* 2005). Individuals at risk for psychosis have an hypersensitive dopamine system as demonstrated by enhanced psychostimulant sensitivity (Janowsky & Risch 1979). This can be modeled in rodents by the amphetamine sensitization paradigm, which can be monitored by locomotor acitivity (LA) (Featherstone *et al.* 2007, Peleg-Raibstein *et al.* 2008, Segal *et al.* 1981). The sensitization paradigm consists of an induction period, during which the animal is injected for several days with amphetamine, either consecutively or intermittently. This period is typically followed by a withdrawal period and a subsequent challenge with a lower dose of amphetamine to monitor the expression of sensitization. (Featherstone et al. 2007).

Previously, we observed large differences in amphetamine sensitivity between individual inbred DBA/2J mice, that correlate with small but consistent changes in hippocampal gene expression particularly in target gene networks affected by Glucocorticoid Receptor (GR) and Myocyte Enhancer Factor 2 (MEF2) activation (Datson *et al.* 2011). Both transcription factors are implicated in the regulation of neuronal plasticity and behavioral sensitization to psychostimulants (de Jong & de Kloet 2004, Deroche *et al.* 1992, Shalizi *et al.* 2006, Pulipparacharuvil *et al.* 2008).

MEF2 has shown to be involved in the dendritic remodeling after cocaine treatment (Zhang *et al.* 2012). Moreover, MEF2 activity has been shown to influence the sensitized behavioral response to repeated cocaine administration (Pulipparacharuvil *et al.* 2008). It was found that cocaine treatment reduces MEF2 transcriptional activity in striatal neurons as a result of enhanced phosphorylation of MEF2a at serine 408(Pulipparacharuvil et al. 2008, Zhang *et al.* 2012). Phosphorylation at this serine site is mediated by CDK5, a highly-expressed kinase in neurons (Gong *et al.* 2003, Gregoire *et al.* 2006), that can be inhibited by roscovitine (Knockaert *et al.* 2002, Meijer *et al.* 1997). CDK5 inhibition by roscovitine was reported to potentiate the cocaine induced locomotor activity during a 5-day sensitization protocol when infused in the nucleus accumbens, prior to each cocaine injection (Chen & Chen 2005, Bibb *et al.* 2001). However, when infused in the nucleus accumbens prior to a challenge dose of methamphetamine (1 mg/kg), after a 14-day pre-treatment of methamphetamine (4 mg/kg) and a 7-day withdrawal period, roscovitine decreased the methamphetamine-induced locomotor response(Chen & Chen 2005).

Although previous studies have been focusing on the striatal region for the effects of cocaine on MEF2 activity and MEF2 effects on psychostimulant sensitization, we have found hippocampal MEF2 related gene expression to be changed correlated to amphetamine sensitization. MEF2 activity has shown to regulate synapse density in cultured hippocampal neurons (Flavell et al. 2006). The present

study was designed to investigate if and to what extent phosphorylation of MEF2 in hippocampus might be related to the induction and/or expression of amphetamine sensitization.

METHOD

Animals Male DBA/2J mice (Charles River Laboratories, Arbresle, France) were obtained at 7 weeks of age. Mice were housed in groups of four in Perspex cages (35x19x14 cm) with food and water available ad libitum. They were kept in a temperature (21°C) and humidity (55%) controlled room with a 12h light-dark cycle (lights on: 7:30 am). Cages were changed weekly, body weight was measured and general health status was checked. After arrival in our animal facilities, the mice were given an acclimatization period of at least two weeks. All experiments were conducted during the light phase. Experiments were approved by the local committee for Animal Health, Ethics and Research of Leiden University. Animal care was conducted in accordance with the EC Council Directive of November 1986 (86/609/EEC).

DrugsAmphetamine (OPG Groothandel, Oss, The Netherlands) was dissolved in 0.9% NaCl. Injections were administered subcutaneously in the neck region using a 30-gauge needle (BD Breda, The Netherlands). Animals were weighed and injection volumes were adjusted according to bodyweight with 0.1 ml/10 g bodyweight. Control animals received the same amount of vehicle solution. Roscovitine (Sigma, R7772) was dissolved in DMSO and 0.9% NaCl 1:1 v/v. Injections were given ICV using A 27-gauge needle (BD, Breda, The Netherlands) connected via polythene tubing (ID 0.4mm, OD 0.8mm, Smiths Medical International Ltd, Kent, Uk) and tubing adapters (CMA, Stockholm, Sweden) to a 100 µl Hamilton 710 RN syringe (Hamilton, Bonaduz, Switzerland). The syringe was placed in a CMA 400 microsyringe pump (CMA, Stockholm, Sweden)**.** Mice were firmly restrained with immobilization of the head and the needle was inserted into the lateral ventricle (2 mm lateral from midline, 3 mm deep). Roscovitine or vehicle was injected, with a volume of 4 µl per 30 seconds. After the injection the mice returned to their home cage and a recovery period of 1.5h followed.

Procedures In experiment 1hippocampal MEF2 phosphorylation was measured at two distinct time points during amphetamine sensitization: a) after a challenge injection of amphetamine at day 20 during the expression of sensitization (see also (Datson et al. 2011), and b) after the 5^{th} injection, on the last day of the induction phase (Fig. 1). Animals were injected for 5 consecutive days with 2.5 mg/kg amphetamine s.c. In experiment 1a, animals were injected again on day 20 with 1.25 mg/kg s.c. after a withdrawal period of 14 days. Following this amphetamine challenge, LA of the animals was measured for 1 hour after which the animals were sacrificed. In experiment 1b after each daily amphetamine injection, locomotor activity was measured for 2h and animals were sacrificed 2 hours after the last injection on day 5.

In experiment 2 we studied whether the amphetamine induced LA response could be manipulated by treating the animals with the CDK5 inhibitor roscovitine. Roscovitine or vehicle was administered ICV

2.5h before a single 2.5 mg/kg amphetamine injection. Locomotor activity was measured for 2h following the amphetamine injection, after which animals were sacrificed. In all experiments, animals were habituated to the test cages for 1h before amphetamine injection.

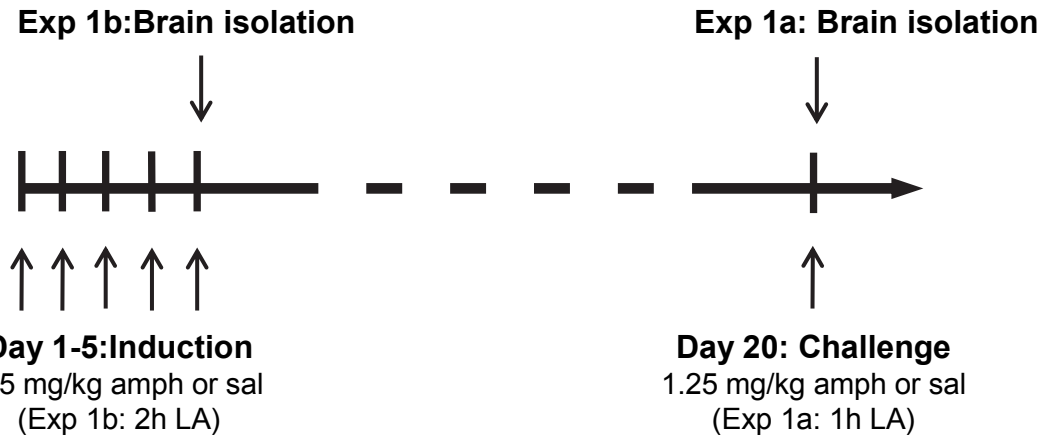

Figure 1| Amphetamine sensitization procedure in experiment 1. DBA/2J mice received daily injections of amphetamine (2.5 mg/kg) or saline for 5 consecutive days. After a withdrawal period, animals received a challenge injection of amphetamine (1.25 mg/kg) or saline at day 20. In experiment 1a locomotor activity was measured for 1h following the challenge injection and animals were sacrificed immediately thereafter. In experiment 1b, locomotor activity was measured for 2h following daily injections and animals were sacrificed 2h following the injection on day 5.

Analysis of locomotor activity Animals were placed in a test cage of the same type and size as the home cage (35x19x14 cm), covered with a Perspex lid. The cage was placed in a Photobeam Activity System (SD Instruments, San Diego, CA, USA). This system is equipped with photoelectric cells to measure horizontal and vertical activity (4x8 photobeam configuration and an 8 photobeam rearing frame). Locomotor activity is represented as the amount of photocell counts measured.

Tissue dissection and protein isolation Directly after decapitation, brains were isolated and hippocampus and striatum were dissected. The tissue was minced using a razorblade and dissolved in ice-cold, fresh RIPA buffer containing Protease Inhibitors (#04693124001, Roche) and phosphatase inhibitors ($NaVO_3$ and B-glycerophosphate). The tissue was then further ruptured using a homogenizer and incubated in RIPA for another 30 minutes. The cell lysate was then centrifuged and the supernatant transferred to a new tube.

Western blot Protein concentration was measured using the Pierce BCA Protein Assay kit (23225, Thermo Scientific, Rockford, IL, USA), according to the manufacturers protocol. Diluted samples were supplemented with 1:2 v/v of sample buffer (including 2.5% ß-mercaptoethanol and BromoPhenol Blue). Twenty µg of each sample was loaded on a 10% polyacrylamide gel. After sufficient separation

of the proteins, they were transferred o/n at 4°C to a PVDF (Polyvinylidene fluoride) membrane. The membrane was subsequently blocked in 5% low fat milk for 1 hour at RT or 5 hours at 4°C for phospho-proteins. Primary antibodies were added in the blocking buffer and incubated for 1 hour at RT or at 4°C o/n for phospho-proteins with either one of the following primary antibodies: Anti-phospho S408 MEF2 rabbit monoclonal (ab51151, Abcam, Cambridge, UK), anti-MEF2a rabbit polyclonal (sc-313X, Santa Cruz) or anti-α-Tubulin DM1A mouse monoclonal antibody (T6199, Sigma). Blots were incubated for 1 hour at RT with the appropriate secondary antibody: goat-anti rabbit IgG HRP secondary antibody (sc-2054, Santa Cruz) or goat-anti mouse IgG HRP secondary antibody (sc-2055, Santa Cruz). Signals were quantified using ImageJ (v1.42; National Institute of Health, USA). .

Statistical analysis Statistical analysis was performed using Statistica (StatSoft). Locomotor response to a challenge dose of amphetamine (1.25 mg/kg) and phosphorylation of MEF2a in the hippocampus and striatum 1h following challenge, was analyzed using a one-way ANOVA with treatment (sal/sal, sal/amph or amph/amph) as between subjects factor (experiment 1a). Locomotor response at the start (day 1) and end (day 5) of the repeated amphetamine injections (2.5 mg/kg) was analyzed with a repeated measures analysis of variance (ANOVA) with day (day 1 and 5) as within-subjects and dose (amphetamine or saline) as between-subjects factor (experiment 1b). The locomotor response to a single injection of amphetamine (2.5 mg/kg) was analyzed using a factorial ANOVA, with dose (amph or saline) and treatment (roscovitine or vehicle) as between-subjects factors (experiment 2). When statistical significance was found, post hoc testing was performed using Duncan's test. MEF2 phosphorylation in hippocampus and striatum at day 5 of repeated injections and after a single injection was analyzed using student's t-tests (experiment 1b and 2).

RESULTS

Animals sensitize to amphetamine, but show no changes in hippocampal and striatal MEF2a phosphorylation

After a withdrawal period of 2 weeks, animals that had received a pretreatment with amphetamine clearly showed a sensitized response to a challenge dose of amphetamine (1.25 mg/kg), (challenge dose effect $F(2,29)=12.1$, $p<0.001$, with sal/sal < sal/amph < amph/amp, $p<0.05$) (Fig. 2a). Western blot measurements did not reveal a difference in the amount of phosphorylated MEF2a in either hippocampus or striatum 60 minutes after the amphetamine challenge (Fig. 2b and 2c).

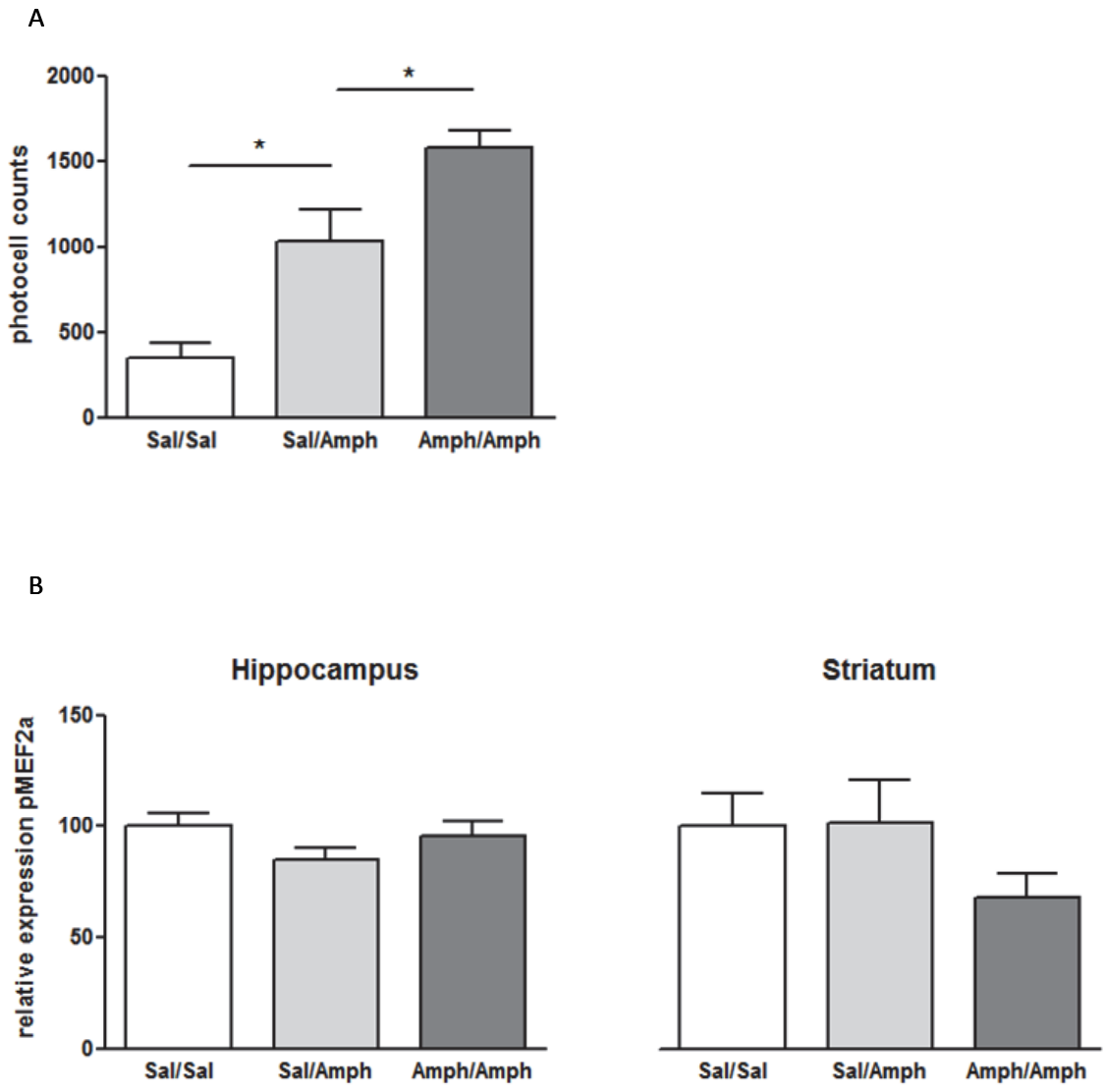

Figure 2 | Experiment 1a: Locomotor activity and MEF2a phosphorylation in hippocampus and striatum following a challenge dose at day 20 (A) Locomotor activity was measured for 1h after the challenge injection of amphetamine (Amph, 1.25 mg/kg) or saline (Sal). (B-C) Relative expression of MEF2 phosphorylation in hippocampus (B) and striatum (C), normalized against alpha-tubulin expression. * p<0.05, Sal/Sal n = 8, Sal/Amph n = 8, Amph/Amph n = 16 . Graphs represent mean ± SEM

Phosphorylation of MEF2a is enhanced after 5 consecutive days of amphetamine treatment

We next studied whether MEF2 phosphorylation was modified during the induction phase of sensitization. To this end DBA/2J mice were injected for 5 consecutive days with 2.5 mg/kg amphetamine and sacrificed 2 hours after the last injection. Animals clearly heighten their locomotor activity in response to the first amphetamine dose of 2.5 mg/kg and a sensitized response to the drug is apparent at day 5 (dose*day interaction $F(1,14)=14.3$, $p<0.01$, with sal ≠ amph on day 1 ($p<0.05$) and day 5 ($p<0.001$) and day1 ≠ day5 for amph ($p<0.001$), but not for saline) (Fig. 3a). Interestingly, phosphorylation of MEF2a was found to be significantly enhanced ($p <0.05$)at the fifth day of amphetamine injection in the hippocampus (Fig. 3b) but not the striatum (Fig. 3c).

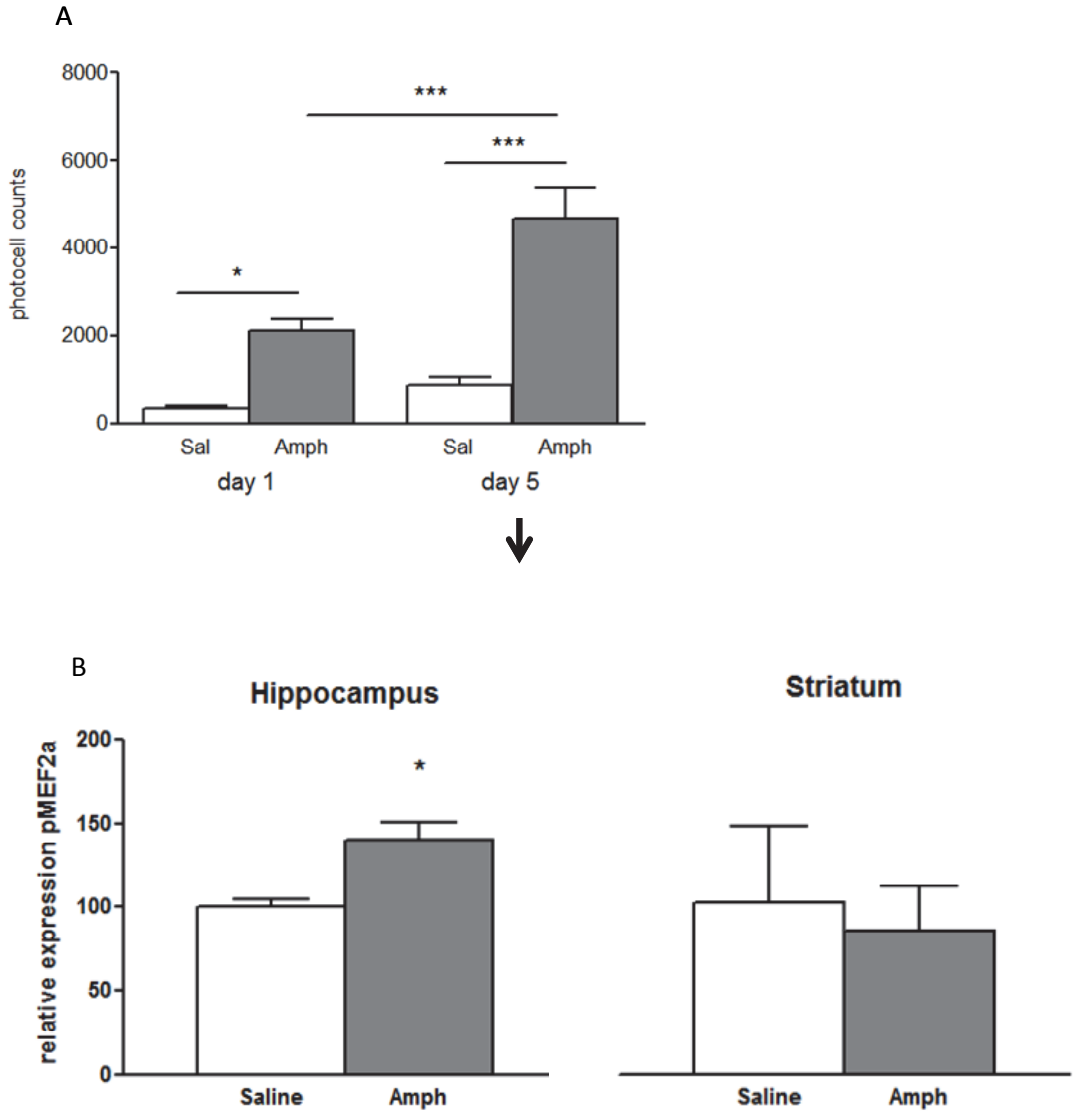

Figure 3 | Experiment 1b: Locomotor activity and MEF2a phosphorylation in hippocampus following daily injections. (A) Locomotor activity in response to amphetamine (2.5 mg/kg) or saline on day 1 and 5, total response over 2h. (B) Relative expression of MEF2 phosphorylation in hippocampus on day 5 of amphetamine treatment, normalized against alpha-tubulin expression. * p<0.05, *** p<0.001. Sal n = 8, Amph n = 8. Graphs represent mean ± SEM

Roscovitine is associated with enhanced LA in response to a single amphetamine injection and increased phosphorylation of MEF2a

We next studied whether MEF2a phosphorylation could be modified using the CDK5 inhibitor roscovitine. CDK5 is responsible for phosphorylation of MEF2a at serine 408 and inhibition of CDK5 by roscovitine icv was therefore expected to lead to decreased levels of MEF2 phosphorylation. Amphetamine led to an increase in locomotor activity in both treatment groups, with a trend for roscovitine to enhance this increase (dose effect $F(1,28)=28.1$, $p<0.001$, and treatment effect $F(1,28)=3.1$, $p=0.09$, with a dose*treatment $F(1,28)=1.8$, $p=0.19$). Post-hoc testing revealed an amphetamine effect that was much stronger in roscovitine treated animals (sal≠amph Rosc $p<0.001$) compared to Vehicle treated animals (sal≠amph Veh $p<0.05$). Moreover, roscovitine did not affect the locomotor response to saline (Rosc≠Veh for the amph dose ($p<0.05$), but not for the saline dose) (Fig. 4a). Roscovitine did not change phosphorylation of MEF2a in the hippocampus nor in the striatum when the animals were subsequently injected

with saline sc (graphs not shown). Surprisingly, hippocampal MEF2a phosphorylation was significantly enhanced after amphetamine injection in animals pretreated with roscovitine ($p <0.05$) (Fig. 4b). No changes were observed in striatum (Fig. 4c). GR phosphorylation at serine 211, a site also known to be phosphorylated by CDK5, was also significantly enhanced following amphetamine in animals pretreated with roscovitine ($p <0.05$) (Fig. 4d). As for pMEF2a, in striatum no effect of roscovitine on pGR was observed (Fig. 4e).

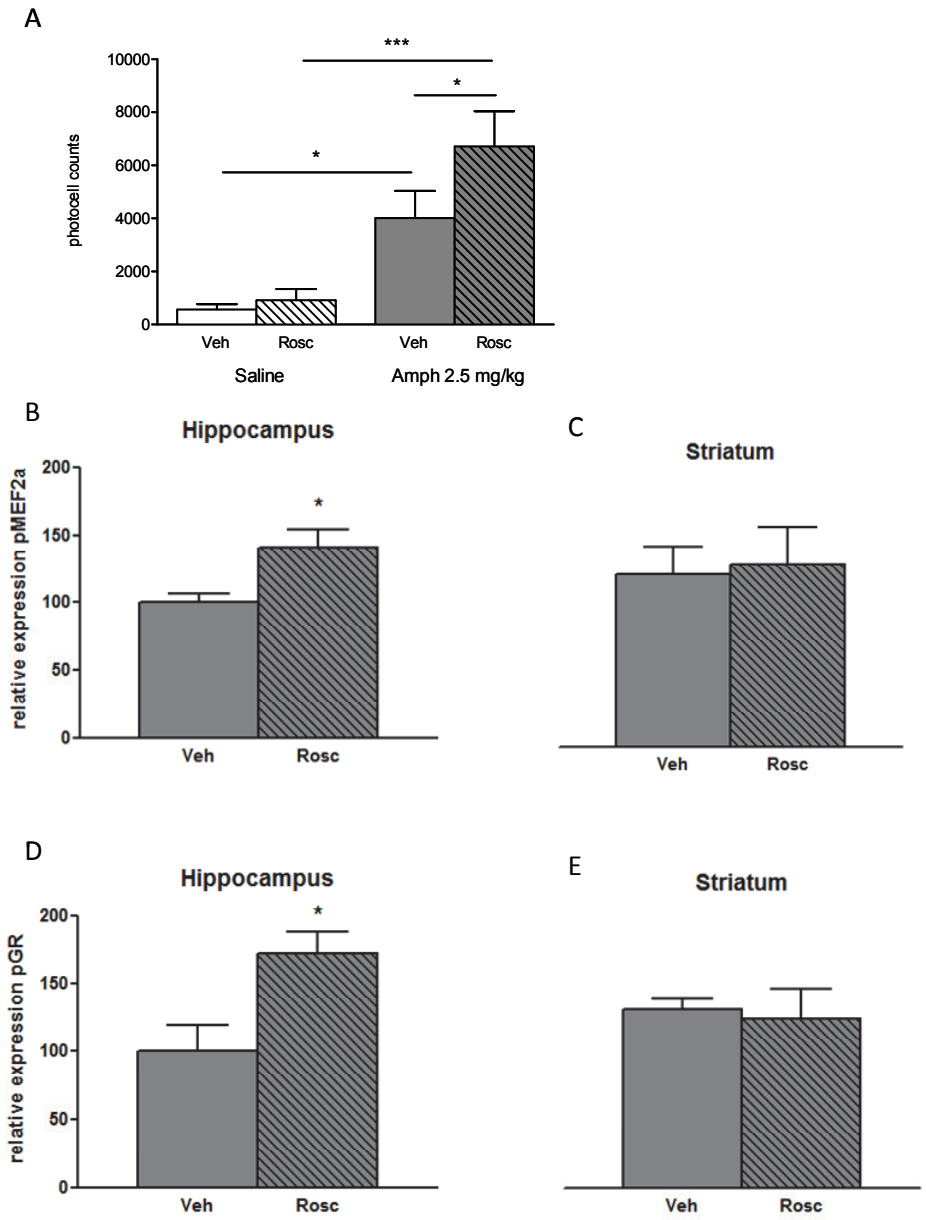

Figure 4 | Experiment 2: The effect of roscovitine on locomotor activity and MEF2a and GR phosphorylation in hippocampus and striatum following an amphetamine injection. (A) Locomotor activity in response to an amphetamine (2.5 mg/kg) or saline injection, total response over 2h. Animals were pretreated with roscovitine (Rosc) or vehicle (Veh) icv. (B-E) Relative expression of MEF2 (B-C) and GR (D-E) phosphorylation in hippocampus and striatum following a 2.5 mg/kg amphetamine injection, normalized against alpha-tubulin. * p<0.05, *** p<0.001. Veh/Sal *n* = 8, Veh/Amph *n* = 8, Rosc/Sal *n* = 8. Rosc/Amph *n* = 8. Graphs represent mean ± SEM

DISCUSSION

The present study demonstrated that MEF2a phosphorylation seems implicated in the induction rather than in the expression of amphetamine sensitization as measured by locomotor activity. Although the expression of psychomotor sensitization to the repeated amphetamine injection was clear at a challenge dose after a 2 week withdrawal period, no differences were observed in MEF2 phosporylation at this timepoint in either hippocampus or striatum. When observing in an earlier stage of sensitization however, we did find differences and at the last day of a 5-day injection protocol, MEF2 phosphorylation was enhanced in hippocampus. Differences in the expression of MEF2 target genes in hippocampus at the expression of sensitization, might thus find their basis at an earlier stage, during the initiation of sensitization.

Previously, phosphorylation of MEF2a was found enhanced in the striatum(Pulipparacharuvil et al. 2008) and nucleus accumbens (Zhang et al. 2012)by chronic psychostimulant treatment. In contrast to these reports we did not observe a change in MEF2a phosphorylation in the striatum after chronic treatment. However, several other reports showed genomic differences in psychostimulant action, that may well explain the difference in MEF2a phosphorylation after psychostimulant treatment in different brain areas per animal strain (Ventura *et al.* 2004, van der Veen *et al.* 2007, de Jong & de Kloet 2004).

After ICV roscovitine infusion, the DBA/2J mice showed an enhanced locomotor response to an amphetamine injection. This is in line with a study showing potentiation of cocaine induced locomotor response after roscovitine infusion in the nucleus accumbens during a 5 day injection protocol in Sprague-Dawley rats (Chen & Chen 2005, Bibb et al. 2001). However, in a different setting, a decrease in locomotor activity was found in this same strain of rats; Roscovitine infused in the nucleus accumbens prior to either a single dose (4 mg/kg) or a challenge dose of methamphetamine (1 mg/kg), after a 14-day pre-treatment of methamphetamine (4 mg/kg) and a 7-day withdrawal period, decreased the methamphetamine-induced locomotor response (Chen & Chen 2005, Bibb et al. 2001)). It might be that Cdk5 has a differential role during the initiation (stimulatory) versus the expression (inhibitory) stage of sensitization, although this cannot explain the repressing effect of roscovitine also observed after a single dose of amphetamine in the latter study. In our study, we provide further evidence for a stimulatory role of Cd5 during the initial stages of sensitization to a psychostimulant.

We found that the increased psychomotor response to amphetamine after roscovitine treatment was paralleled by an enhanced phosphorylation of both MEF2a and GR. For this effect a combined treatment of roscovitine with amphetamine was required, since roscovitine alone affected neither

phosphorylation nor locomotor activity. This stimulatory effect of roscovitine icv on phosphorylation, if combined with amphetamine, came as a surprise, since it is an inhibitor of CDK5 (Meijer et al. 1997, Garrofe-Ochoa *et al.* 2011). Several*in vitro* studies showed that roscovitine treatment decreased phosphorylation of MEF2 as well as GR (Kino *et al.* 2007, Gong et al. 2003), a finding that is therefore in line with its reported function of CDK5 inhibition (Meijer et al. 1997, Kino *et al.* 2007). In contrast, we showed that roscovitine treatment enhances MEF2a phosphorylation, but only if given prior to amphetamine administration and not in saline treated animals.

Furthermore, roscovitine is also known for other effects. For instance, roscovitine decreases dopamine transporter function independently of CDK5, it blocks elongation of transcription via CDK9 and it induces intracellular Calcium release in neuronally differentiated PC-12 cells (Price *et al.* 2009, Choi & Chung 2010, Garrofe-Ochoa et al. 2011). Moreover, a recent study by Zhang et al. showed that cocaine can enhance phosphorylation of MEF2 in the nucleus accumbens and striatum when pretreated with a dopamine D1 receptor antagonist, while phosphorylation is decreased when pretreated with a dopamine D3 receptor antagonist (Zhang et al. 2012). It was also shown that CDK5 has a direct effect on the activity of D3 receptors (Chen *et al.* 2009), which have been suggested to play an important role in psychostimulant sensitization (Zhu *et al.* 2012, Newman *et al.* 2012). Accordingly, these findings suggest that dopaminergic signaling is implicated in control of MEF2 activity which can be modulated by roscovitine. The precise involvement of CDK5 and roscovitine therefore should be studied in greater detail, since it may provide an interesting novel approach to modulate psychostimulant sensitivity.

The expression of the different MEF2 genes (Mef2a-d) shows large developmental changes. In the adult mouse brain, the regional expression pattern is uneven for each gene with especially high expression of MEF2b and MEF2c in the forebrain, while MEF2a is highly expressed in the hippocampus. MEF2d is ubiquitously expressed in the brain (Lyons *et al.* 1995). The CA1 area of the hippocampus mainly expresses MEF2a and MEF2d and both gene products have a somewhat overlapping characteristics. For example, psychostimulant treatment leads to phosphorylation of both MEF2a and MEF2d. Hippocampal neurons have comparable expression profiles of both proteins and both MEF2a and MEF2d can downregulate synapse number in hippocampal neurons (Flavell *et al.* 2006, Pulipparacharuvil et al. 2008). MEF2a in particular was shown to control dendritic arborization and synaptic plasticity (Shalizi et al. 2006).

MEF2a is a member of a family of four proteins of which three, MEF2a, MEF2c and MEF2d are highly overlapping in structure and expression pattern. Hence it is not surprising that some studies showed additive effects of these MEF2 members. Brain specific MEF2a knockout mice for example showed

normal behavior, while combined knockout of MEF2a and MEF2d significantly affected motor coordination. Knocking out MEF2a, MEF2c and MEF2d in the brain even resulted in significant decreased survival rate compared to wild-type animals (Akhtar et al. 2012). In vitro it was shown in cerebellar granule neurons, that overexpression of dominant-active MEF2d prevents apoptosis while cotransfection with increasing amounts of dominant-inactive MEF2a nullifies this effect (Li *et al.* 2001). The effect of amphetamine on MEF2a phosphorylation might be an underestimation of the overall, and possibly additive, effect of amphetamine on MEF2. It would therefore be of interest to study the effect of amphetamine on MEF2c and MEF2d as well. Next to MEF2, GR phosphorylation was also found enhanced following the combined roscovitine – amphetamine treatment. The available literature suggests however, that GR phosphorylation is decreased after *in vitro* roscovitine treatment alone (Kino et al. 2007), but this effect of roscovitine has not been studied *in vivo* to our knowledge. Given the important role of stress in psychostimulant sensitization, our finding raises the question whether stress effects on sensitization might implicate GR and MEF2 changes in phosphorylation. For this possibility, we recently found indirect evidence in an *in vitro* study, showing that glucocorticoids can indeed enhance the phosphorylation of MEF2 (Speksnijder *et al.* 2012). An interesting avenue for follow-up research would be then to study the effect of GR on manipulation of MEF2 activity and its consequence for psychostimulant sensitization.

Overall, our results suggest that changes in hippocampal MEF2 phosphorylation upon amphetamine treatment develop transiently after repeated administration and can be manipulated by roscovitine icv. These findings suggest that the function of MEF2a- and GR-responsive gene networks in hippocampus warrants further study in relation to amphetamine sensitization.

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Chapter 6 | General Discussion

Contents of General Discussion

6.1 Objective

The overall objective of this thesis was to gain insight in the genes and pathways involved in psychosis susceptibility. For this purpose we first generated transcriptional profiles of the dopaminergic Prefrontal Cortex (PFC), Nucleus Accumbens (NAc) and hippocampal CA1 areas of low and high responders in an amphetamine sensitization paradigm. The hippocampal CA1 area displayed the most prominent differences in gene expression between low and high responders. Among the differentially expressed genes we identified profound changes in the expression of target genes of the transcription factors GR and MEF2. Pathways driven by both these transcription factors are known to play an important role in structural and functional neuroplasticity underlying sensitization to amphetamine effects (Crombag *et al.* 2005)

Because GR and MEF2 have several common target genes, we next studied whether GR and MEF2 signalling pathways cooperate in the regulation of genes involved in neuroplasticity underlying amphetamine sensitization. For this purpose we focused on the genomic control exerted via GR and MEF2 in an *in vitro* cell model: neuronally differentiated PC12 cells (Chapters 3 and 4). In Chapter 3 we demonstrated that GR-activation increases MEF2 phosphorylation, which is the inhibitory form of MEF2, thereby decreasing transcription of MEF2-target genes such as c-jun. Since MEF2 is predominantly activated by neuronal depolarization, we extended our *in vitro* studies by treating the PC12 cells with KCl, thus creating a depolarized environment (Chapter 4). We showed that the enhanced expression of the MEF2 target gene NR4A1 in response to neuronal depolarization, was attenuated by GR activation. Since NR4A1 plays a role in synaptic plasticity, by decreasing the number of dendritic spines, we propose that this attenuation by GR is aimed at balancing the depolarization-induced changes (Shalizi *et al.* 2006). In Chapter 5 we returned to the *in vivo* amphetamine sensitization paradigm, and we studied whether MEF2 phosphorylation changes during the sensitization process. Moreover, we tested the possibility that manipulation of MEF2 phosphorylation may influence sensitization to amphetamine. We indeed found a relationship between amphetamine-induced locomotor activity and MEF2 phosphorylation, but this relation is complex and requires further study.

6.2 Amphetamine sensitization as a model for psychosis

Schizophrenia is a multi-faceted disorder comprising positive, negative and cognitive symptoms. Because of this complexity, most animal models are designed to 'test specific causative or mechanistic hypotheses regarding schizophrenia' (Marcotte *et al.* 2001). Although some models, such as sensitization to phencyclidine (PCP) and maternal separation (Javitt & Zukin 1991), do indeed show some of the positive and negative symptoms,it is extremely challenging to design an appropriate model. The main reason for this challenge is probably that schizophrenia affects mainly higher brain functions that are less developed or non-existent in other species.

The amphetamine sensitization model, as used in **Chapters 2 and 5**, has received both negative and positive criticism on whether it appropriately mimics specific aspects of schizophrenia. The most important point of critique concerns the fact that in schizophrenia dopaminergic changes are probably preceded by glutamatergic changes in the brain. According to this reasoning dopamine is therefore not considered to be causal for the disorder (Coyle 2006). A large body of research showed that glutamate release is enhanced in the NAc and ventral tegmental area (VTA) by repeated amphetamine treatment (Reid *et al.* 1997, Wolf & Xue 1999). Moreover, glutamatergic neurons are known to regulate the activity of dopaminergic neurons in the VTA and NAc (Gorelova & Yang 1997, Taber *et al.* 1995). Therefore, it has been argued that amphetamine sensitization might not be the best model to study the causative neurodevelopmental changes underlying schizophrenia (Stone *et al.* 2007, Coyle 2006).

Nonetheless, one of the most important advantages of the amphetamine sensitization model is that in spite of the primary changes in glutamatergic input the underlying dopaminergic changes are comparable to the human situation **(Chapter 1)**. Repeated amphetamine treatment enhances sensitization to subsequent psychostimulant challenges, particularly under stressful conditions, a phenomenon that is also observed in schizophrenia patients (**Figure 1**) (Pierce & Kalivas 1997, Ujike & Sato 2004). Moreover, dopamine antagonists, the prevalent medication in schizophrenia patients, block the expression of amphetamine sensitivity. Therefore, we consider the genomic differences in the dopaminergic PFC, NAc and hippocampal CA1 area of animals that show either low or high sensitivity to amphetamine, to represent a valuable first step in understanding the pathogenic mechanism of schizophrenia.

6.3 Individual differences in amphetamine sensitization

Several studies have been conducted towards understanding the cause of individual differences in amphetamine sensitivity (Shilling *et al.* 2006, Conversi *et al.* 2006, Scholl *et al.* 2009, Segal & Kuczenski 1987). However, these studies either used outbred strains, or compared amphetamine treatment under different environmental circumstances. In order to study which genes and pathways are involved in susceptibility differences we used inbred animals with a comparable genetic background. In **Chapter 2** a large divergence in locomotor activity after the amphetamine challenge in DBA/2j mice was reported. This phenomenon was previously observed by de Jong *et al*. using the psychostimulant cocaine (de Jong *et al.* 2007). Sensitization of DBA/2j mice is dependent on stress hormones and since stress is an important factor in the onset of psychosis, this makes DBA/2j mice a good model to study the role of environmental factors on the development and expression of individual differences in amphetamine sensitivity (de Jong *et al*. 2007, Ventura *et al.* 2004).

One of the questions we wanted to answer was how this large divergence in amphetamine sensitivity could arise. Since all animals were treated identically, it seems plausible that the differences in amphetamine sensitivity occurred prior to or during the amphetamine sensitization paradigm. Amphetamine-induced locomotor activity in DBA/2j mice is known to be enhanced by stress (Badiani *et al.* 1992). Since stress hormones are a key to the development of sensitivity, one of the factors that could play a role is an unstable social hierarchy in the home cage, which is known to be extremely stressful. Studies by Avitsur *et al*. pointed out that a social disruption stressor (SDR), caused by introducing an aggressive intruder into the home cage of another singly-housed mouse, has divergent effects even in genetically identical mice (Avitsur *et al.* 2001, Avitsur *et al.* 2003, Avitsur *et al.* 2007). SDR also differentially affected group-housed mice depending on their dominant or submissive status (Avitsur et al. 2003). These differential effects that seem related to degree of subordination could be explained by the fact that submissive behavior was correlated with glucocorticoid resistance (Avitsur et al. 2001).

These reports suggest that, although individual mice share their genetic background and even their environment (home cage), their response to a stressor can be entirely different. In humans it is known from many studies that social stress is an important factor in the development of schizophrenia. Several studies have shown that schizophrenia prevalence is increased in urban environments (Johns *et al.* 2004, van Os *et al.* 2003). Recently it was shown that especially the processing of social stress is different between persons raised in urban versus rural areas (Lederbogen *et al.* 2011). Although many factors are hypothesized to attribute to the overall

increased risk, social 'defeat' stress or discrimination are considered to be important factors. This view is backed up by animal studies showing activation of the dopaminergic system under such adverse conditions (Akhtar *et al.* 2012, Tidey & Miczek 1996).

Another possible explanation for the differential amphetamine sensitivity of the adult animals may be variation in the extent of maternal care they received as pups. Maternal care in rats, expressed as the percentage of time the pups were exposed to maternal licking and grooming (LG), is correlated with long-lasting effects on stress responsiveness, emotional arousal and cognitive performance (Champagne *et al.* 2003). Offspring of low LG mothers had an enhanced stress-induced release of corticosterone and ACTH, low hippocampal GR expression (Liu *et al.* 1997), and also reduced dendritic arborization and lower spine density in the hippocampal CA1 area. Moreover, fear conditioned behaviour and also long term potentiation in hippocampal slices of these animals was disturbed (Champagne *et al.* 2008). Importantly, within a single litter not all pups receive the same amount of maternal care; within litters the individual low LG pups showed enhanced stress-induced corticosterone and ACTH release compared to their high LG littermates (van Hasselt *et al.* 2012, Claessens *et al.* 2011)

Interestingly, during the amphetamine sensitization paradigm, the animals showed no difference in LA after their first amphetamine injection and there was no correlation between LA at the first or fifth injection and the challenge. Nevertheless, the individual differences to amphetamine that emerged after the challenge were a stable trait and likely developed during the withdrawal phase on days 6-14 **(Chapter 2, Figure 3b)**.

6.4 Differential gene expression patterns

From human studies it is evident that genetic vulnerability does not necessarily lead to schizophrenia development (van Erp *et al.* 2004, Tsujita *et al.* 1998, Petronis *et al.* 2003, Onstad *et al.* 1991, Goldberg *et al.* 1993). The concordance rate for schizophrenia in monozygotic twins is 'only' 50%, while a decreasing genetic homogeneity with the schizophrenic individual reduces the risk of developing schizophrenia concomitantly to approximately 1% in the general population (Cardno & Gottesman 2000). This means that an identical genetic background and childhood environment, considered to be the most important contributors to schizophrenia, does still not fully predict who will develop schizophrenia. In this thesis we aimed to investigate which molecular mechanism may underlie the variability in LA following a psychostimulant sensitization paradigm by generating transcriptional profiles of low and high responders to amphetamine **(Chapter 2)**.

To this end microarrays were used harboring probes compatible to all known mRNA's in the mouse genome (~31,000). An advantage of this unbiased hypothesis-generating approach, is that it allows for the identification of novel genes that correlated with amphetamine sensitivity relevant for the pathogenesis of schizophrenia. A limitation, however, of the type of microarray we applied was that it did not allow the measurement of expression levels of non-coding RNAs, that are increasingly appreciated as factors playing an important role in (psycho)pathology. At present RNA-sequencing is considered to be an even better approach since it allows assessment of all expressed RNAs.

Chapter 2 presents a list of genes that were found to be differentially expressed between LR and HR (Table S2). The number of differentially expressed genes was disappointingly low in the PFC and the NAc. In the hippocampal CA1 area the relative differences in expression were mild and validation of expression differences was mainly successful for the genes with the largest fold changes combined with the smallest p-values. Nevertheless, gene expression changes could be validated quite well in the CA1 area, especially considering the fact that both LR and HR received an equal treatment and were of the same genetic background. Using a p-value of 0.01, 63 genes were found to be differentially expressed in the CA1 area between LR and HR. Although this number is rather low, Ingenuity Pathway Analysis revealed that CREB, MEF2 and GR target genes were significantly overrepresented when compared to a random set of 2,000 genes.

The strength of our initial search for gene targets was that the microarray approach was combined with laser-capture microdissection, which enabled us to specifically target the desired structures (**Chapter 2; Figure 2**). A limitation of laser-capture microdissection, however, is that the procedure is labor intensive and time-consuming with a risk of reduced mRNA integrity, if compared to mRNA extraction directly from fresh tissue (unpublished results).

6.5 The hippocampus as key region in sensitivity to amphetamine

One of the intriguing findings in this thesis is that gene expression in the hippocampus, or more specifically in the CA1 area of the hippocampus, seems to be more robustly affected by the amphetamine sensitization paradigm than in the striatum, NAc and PFC. Proof that the hippocampus plays a major role in amphetamine sensitization comes from studies focusing on projection of neurons from the hippocampus to the NAc. Bilateral lesions of the hippocampus resulted in enhanced amphetamine-induced LA, suggesting that the hippocampus is involved in controlling NAc neuronal activity (Wilkinson *et al.* 1993). Furthermore, pharmacological blockade with lidocaine of the dorsal hippocampus excitatory input to the NAc significantly blocked the expression of behavioural sensitization (Degoulet *et al.* 2008). Within the hippocampus, the CA1 area has been proposed to modulate activity of the ventral subiculum of the hippocampus which affects the firing of dopamine neurons in the VTA. Valenti et al reported that inhibition of the excitatory output of the ventral hippocampus blocked the activity of dopaminergic neurons in the VTA as well as the behavioural effects induced by amphetamine (Valenti *et al.* 2011).

Stress can affect the functioning of this hippocampal – VTA dopaminergic circuit in various ways with consequences for amphetamine sensitization. First, stress can directly modulate the hippocampal - dopaminergic circuit (Valenti et al. 2011, Tidey & Miczek 1996). Second, the stress-induced rise in corticosterone levels activates glucocorticoid receptors (GR) which are widely expressed in this circuit. These GR-mediated effects display an enormous diversity, which is collectively aimed to facilitate the coordination exerted by the corticosterone. Thus, D1 receptors are down regulated by corticosterone in hippocampus (Mizoguchi *et al.* 2004, Mizoguchi *et al.* 2002, Mizoguchi *et al.* 2008). Furthermore, acute amphetamine treatment induces GR mRNA expression in the CA1 area of the hippocampus, while GR is downregulated after chronic amphetamine (Shilling *et al.* 1996). Moreover, GR is expressed in dopamine neurons controlling the activity of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis (Cintra et al. 1994): GR expressed in the dopaminoceptive neurons in frontal cortex and NAc sustains the corticosterone-induced activity of a positive feedback loop to the VTA (Barik *et al.* 2010).

Accordingly, it cannot be excluded that a difference in the level of circulating corticosterone participates in the differential gene expression between LR and HR. Of interest is therefore that post-mortem GR expression is lower in the CA1 area of schizophrenia patients, but not of depressed or bipolar patients, making this a quite specific finding for schizophrenia (Webster et al. 2002).

6.6 Crosstalk of GR-MEF2 pathways: functional implications

A striking observation reported in this thesis is that a large percentage of the genes differentially expressed in the CA1 region of the hippocampus are target genes of MEF2, GR and CREB. Since the role of CREB and GR in psychostimulant sensitization already has been documented (Alboni *et al.* 2011, Datson *et al.* 2012, Levine *et al.* 2005, McClung & Nestler 2003, Shaw-Lutchman *et al.* 2003), we decided to explore the possible interaction between MEF2 and GR. that had not been previously reported (**Chapter 2**). We first studied this interaction *in vitro* in neuronally differentiated PC-12 cells (**Chapter 3**) in which GR and MEF2 are also both expressed (Polman et al., 2012: Morsink et al., 2006 (**Chapter 3**). The possibility to differentiate the PC-12 cells to a neuronal phenotype makes this cell line a suitable system to study the interaction between both transcription factors (Kim et al. 2011, Lam et al. 2010, Morsink et al. 2006). The results described in **Chapter 3** show that GR readily modulates the transcriptional activity of MEF2 by enhancing its phosphorylation and DNA-binding capacity.

Amphetamine treatment has been observed to affect neuronal plasticity and connectivity as a reaction to behavioral and environmental processes. This effect on neuronal plasticity has been shown in different brain areas, such as the VTA as well as the NAc (Jones *et al.* 2000, Thomas & Malenka 2003). These neuroplastic changes are very stable and hence have long-term consequences. In rodents, a single exposure to a psychostimulant is known to cause an enhanced response to the same psychostimulant one year later (Vanderschuren *et al.* 1999). The same results have been observed in humans leading to the conclusion that this enhanced sensitivity might last a life-time (Nestler 2001). Amphetamine treatment was shown to increase the number of dendritic branches and synaptic spines of medium spiny neurons in the NAc as well as on pyramidal neurons in the prefrontal cortex, both of which are long-lasting phenomena (Robinson & Kolb 1999, Robinson & Kolb 1997).

This increase in spine density is abrogated by MEF2 activity, as shown when constitutively active MEF2 was injected directly into the NAc (Pulipparacharuvil *et al.* 2008). This MEF2 activity not only modified spine density, but appeared necessary for the loss of dendritic spines (Tian *et al.* 2010, Shalizi *et al.* 2006). Results in **Chapter 4** showed that neuronal depolarization, inducing neuronal plasticity, led to dephosphorylation of MEF2 and enhanced DNA binding of MEF2, suggesting an enhanced transcriptional potential of MEF2. This might have direct effects on neuronal plasticity *in vivo* with consequences for behavioral sensitization.

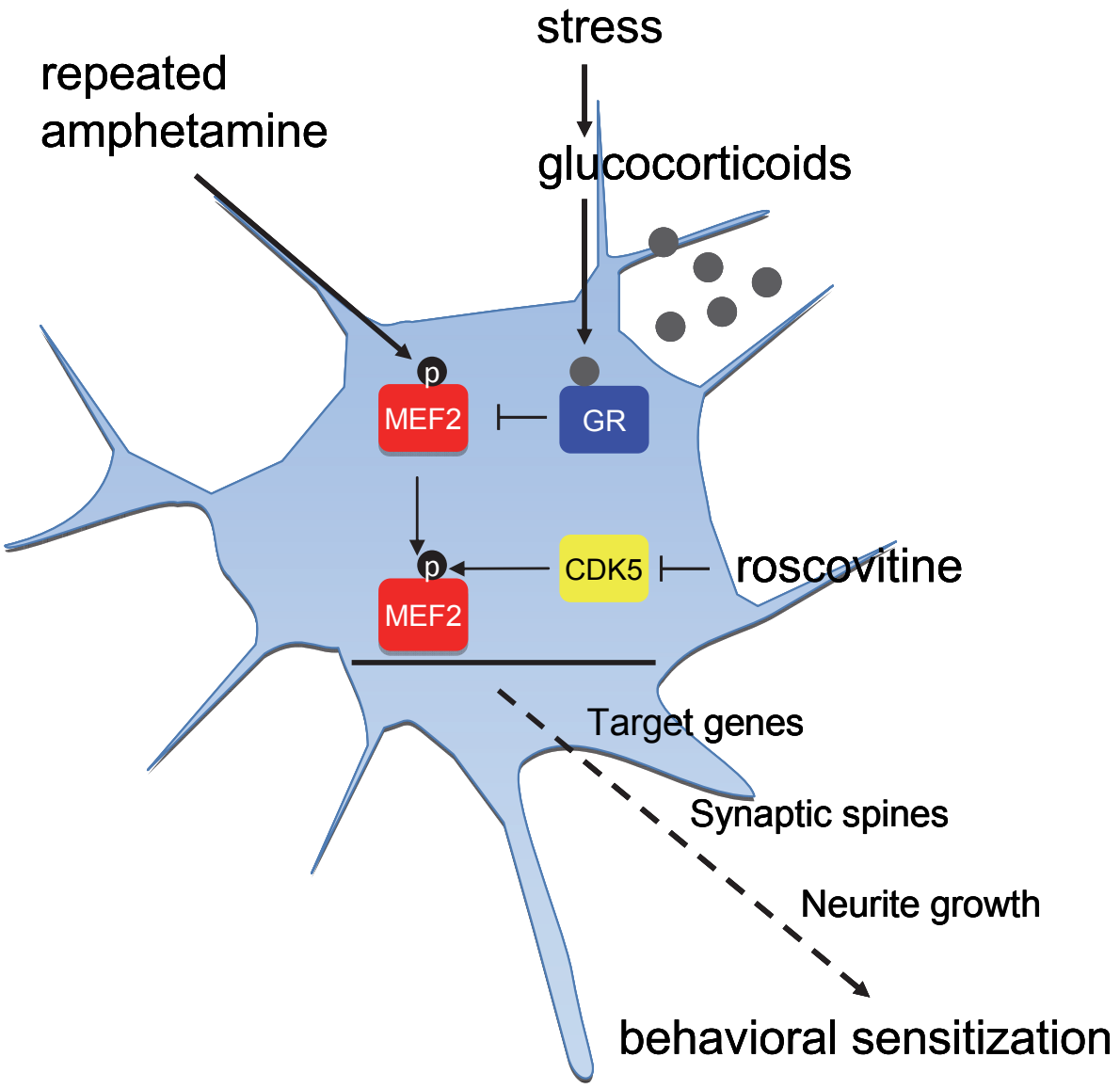

Figure 1 | Phosphorylation of MEF2 can be manipulated in several ways. Chronic treatment with psychostimulants such as cocaine and amphetamine results in enhanced phosphorylation of MEF2 at serine 408. This modification is known for its inhibitory effects on the transcriptional potential of MEF2. The synthetic glucocorticoid dexamethasone and its endogenous counterpart corticosterone can bind to GR, leading to phosphorylation of GR. In this thesis we show that activation of GR by glucocorticoids causes MEF2 to be phosphorylated, resulting in differential expression of its target genes. MEF2 target genes are known for their effects on synaptic spines and neurite growth, possibly affecting behavioral sensitization itself. The discovery that glucocorticoids via GR, either directly or indirectly, influence MEF2 transcriptional activity may lead to new insights in how stress can play a role in psychostimulant sensitization and hence psychosis susceptibility.

6.7 Hippocampal MEF2 phosphorylation and amphetamine sensitization

Several reports have shown that MEF2 transcriptional activity is altered by cocaine in the NAc (Zhang *et al.* 2012, Pulipparacharuvil et al. 2008). An upregulation of MEF2C was found in the striatum of cocaine-treated rats (Dietrich *et al.* 2012). Cellular studies on psychostimulant treatment have shown that amphetamine and other psychostimulants such as cocaine, induce calcium influx in the neuron. This is even more enhanced after repeated psychostimulant treatment (Pierce & Kalivas 1997). In agreement with this observation, caveolin-deficient (CaV1.3-/-) mice, that miss an essential part of L-type calcium channels, do not show expression of sensitization to amphetamine, suggesting that calcium influx after amphetamine treatment and subsequent changes in synaptic plasticity are a crucial hallmark of sensitization (Giordano *et al.* 2006).

Results in **Chapter 2** revealed that after repeated amphetamine administration LR and HR differed in their expression of GR and MEF2 target genes. Since transcriptional activity of MEF2is mainly mediated by posttranslational modifications and we observed that MEF2 phosphorylation is enhanced by glucocorticoid treatment *in vitro*, it is of interest to know whether MEF2 phosphorylation is different between LR and HR to amphetamine. Two studies demonstrated that this could indeed be the case. It was found that MEF2 phosphorylation is enhanced in the striatum after cocaine treatment (Pulipparacharuvil et al. 2008), Another study showed enhanced CDK5 activity in the striatum of methamphetamine treated animals (Chen & Chen 2005). CDK5 is the kinase responsible for phosphorylation of MEF2 and enhanced activity of CDK5 would indicate a higher amount of phosphorylated MEF2. It was therefore a logical follow-up to study modification of CDK5 activity on MEF2 activity *in vivo* after repeated amphetamine administration.

Using a comparable paradigm as described in **Chapter 2,** DBA/2j mice were treated with amphetamine for 5 consecutive days and challenged at day 20 in order to measure MEF2 phosphorylation in the hippocampus and striatum (**Chapter 5**). Previously, MEF2 phosphorylation in the striatum was found significantly enhanced in C57Bl/6 mice both after acute and chronic cocaine treatment (Pulipparacharuvil et al. 2008). Our results in DBA/2 mice showed that MEF2 phosphorylation was not enhanced by amphetamine in either the hippocampus or the striatum after application of the full sensitization paradigm (**Chapter 5**). These results suggest strain differences between DBA/2 and C57Bl/6 mice regarding the role of MEF2 phosphorylation in sensitization to amphetamine, as was found previously for cocaine (de Jong et al. 2007, van der Veen *et al.* 2007). However, we observed that MEF2 phosphorylation was enhanced at the end of the 5 day induction period of amphetamine sensitization. Interestingly this was only found in the hippocampus and not in the striatum. These results strengthen our conclusion from the microarray experiment that the

hippocampus plays an important role in amphetamine sensitization, although direct proof still has to be provided. Possibly, because of the relatively large amount of tissue that was needed for the Western blots, the measurement of phosphorylation of MEF2 directly in the CA1 might have been masked in our experiments. Based on the work by Datson *et al* focusing on transcriptional divergence between hippocampal subregions it could very well be that phosphorylation differences might exist between the subregions as well (Datson *et al.* 2004). Future studies might benefit from studying the posttranslational modifications of MEF2 in more neuro-anatomical detail.

Roscovitine treatment was used to manipulate MEF2 phosphorylation since it is known to be a CDK5 inhibitor, the kinase responsible for phosphorylation of MEF2 (**Figure 1**). However, our results suggest the opposite. Roscovitine treatment significantly enhanced phosphorylation in the hippocampus, an observation that to our knowledge has not been previously described. Possible explanations for this result have been discussed in **Chapter 5**.Future studies should focus on direct manipulation of MEF2 by RNA interference or siRNA targeting MEF2 mRNAs. A more specific inhibitor of CDK5 could also avoid 'off-target' effects. Nevertheless, the fact that roscovitine influenced both MEF2 phosphorylation as well as amphetamine sensitivity points towards a role for MEF2 in psychostimulant sensitization.

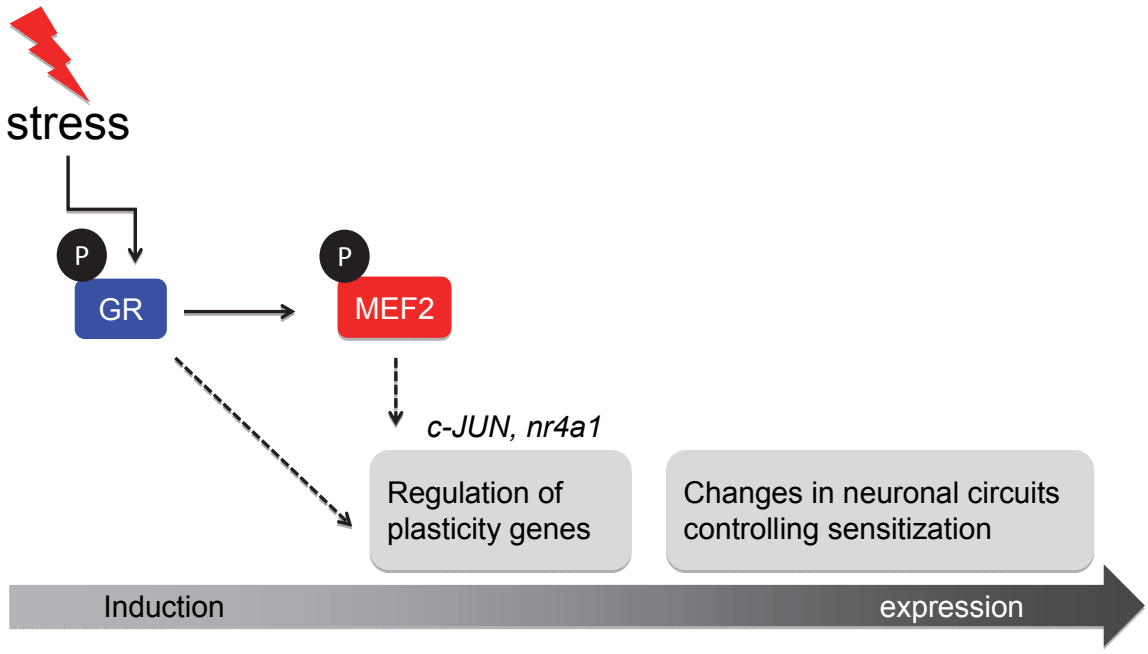

Figure 2 | A model for the interplay between stress, GR and MEF2 on the induction and expression of sensitization to psychostimulants. In short, stress causes a rise in endogenous glucocorticoid levels, resulting in activation of GR and phosphorylation of MEF2. MEF2 transcriptional activity is now affected leading to a differential regulation of genes such as c-JUN and NR4A1, involved in neuronal plasticity and changes in neuronal circuits controlling sensitization. Depending on the amount of stress perceived, this might influence the sensitivity for psychostimulants. This reasoning may help to understand how stress affects the susceptibility for psychosis.

6.8 Future perspectives

The experiments described in this thesis have provided evidence for the influence of GR on MEF2 activity, as well as for the role of MEF2 and GR interaction in amphetamine sensitization. However, many aspects of this MEF2-GR interplay have yet to be determined. From a molecular point of view it would be interesting to test whether upon GR activation GR and MEF2 engage in physical interaction, either directly or indirectly via other proteins. This would give more insight in how GR changes the transcriptional activity of MEF2. Moreover, it would also be important to know the proportion of phosphorylated and unphosphorylated MEF2 bound to the DNA upstream of genes involved in neuroplasticity.

In this thesis we did not discriminate between both modifications of MEF2 and we could therefore not tell with certainty whether expression of target genes would be enhanced or repressed. One of the problems we encountered was the lack of specific inhibitors of MEF2. Since lentiviral and/or siRNA mediated knockdown was unsuccessful in PC-12 cells, we could not directly manipulate MEF2. Alternatively, pharmacological inhibition of MEF2, with compounds that, to our knowledge, have yet to be discovered, could be a means to directly study the effect on the target genes of MEF2 with and without GR activation by DEX as well as in depolarizing and non-depolarizing conditions.

In vivo experiments would also benefit from direct MEF2 inhibition, although the effect on animals is not known at the moment. The most crucial follow-up experiment with respect to the work described in this thesis would be to subject DBA/2j mice to the amphetamine sensitization paradigm with and without treatment with MEF2 inhibitors and study its effect on the different stages of amphetamine sensitization.

6.9 Conclusion

In the present study we have used the sensitization of the DBA/2j mouse to repeated amphetamine as a model for psychosis susceptibility. Our data showed that a number of common GR- and MEF2 target genes are differentially expressed in the CA1 region of the mouse hippocampus of high and low responders to amphetamine sensitization. This finding suggested a role for both transcription factors in generating the divergent phenotypes.

In vitro in PC12 cells we found that GR and MEF2 signaling pathways converge at multiple levels in the control of their shared target gene c-JUN and we showed that the glucocorticoid-induced downregulation of c-JUN is mediated by MEF2. Using another MEF2 target gene, NR4A1, we observed that under depolarizing conditions the activation of GR can attenuate MEF2 signaling among others by affecting MEF2-DNA binding. The *in vivo* study using roscovitine, a potent CDK5 inhibitor, revealed that changes induced in hippocampal MEF2 phosphorylation are rather related to the induction than the expression phase of amphetamine sensitization.

Taking our data together, the findings suggest that in hippocampus the effect of stress, via glucocorticoid activation of GR, can modulate the role of MEF2 target genes in behavioural sensitization. This finding points to the hippocampus as an exciting target for further studies on the role of MEF2 and GR in the precipitation of psychosis susceptibility.

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Addendum | Summary

SUMMARY

Schizophrenia is a serious developmental psychiatric disorder affecting approximately 1% of the general population. It is characterized by a wide variety of symptoms, divided in positive, negative and cognitive aspects. Some factors that may contribute to the development are obstetric events, chronic social adversity and drug abuse. It is recognized that these adverse environmental effects will only result in the development of schizophrenia if they are combined with genetic predisposition. Genetic make-up accounts for 50% of the risk of schizophrenia development.

Numerous animal models have been developed in order to mimic aspects of schizophrenia. One of these models is the amphetamine sensitization paradigm. When animals are treated with amphetamine for several days and are injected again after a withdrawal period with a so-called challenge injection of the stimulant, this results in a sensitized state of the animal. This sensitized state has some similarities to the human situation of schizophrenia, reflected in a hyperactive dopamine system and the ability of dopamine antagonists to block this expression of sensitization.

Important brain areas playing a role in this sensitized state are the nucleus accumbens (NAc), the prefrontal cortex (PFC) and the hippocampus that all receive direct dopaminergic input from the ventral tegmental area. In **Chapter 2** we studied gene expression differences in these three dopaminergic brain areas in genetically identical DBA2/j mice that differ in their response to the amphetamine sensitization paradigm. The goal was to study the genes that might be responsible for the differences in sensitivity in order to identify potential psychosis susceptibility genes. We showed that of three brain areas, the CA1 area of the hippocampus harbored the most robust differences in gene expression between low or high responders to the amphetamine sensitization paradigm. Moreover, we found that the differentially expressed genes were common target genes of the Glucocorticoid Receptor (GR) and Mycocyte Enhancer Factor 2 (MEF2), suggesting a role for both transcription factors in generating the divergent phenotypes. The hippocampus is directly involved in inhibiting amphetamine-induced sensitivity, probably by controlling NAc neuronal activity and interfering with the firing of dopaminergic neurons form the ventral tegmental area. Furthermore, GR is highly expressed in the hippocampus and glucocorticoids are known to modulate amphetamine sensitivity.

At the time no reports existed on a possible interaction between GR and MEF2. **Chapter 3** therefore focused on studying whether such an interaction exists *in vitro* in neuronally differentiated PC-12 cells. Treating cells with the GR agonist dexamethasone (DEX), resulted in phosphorylation of MEF2

at serine 408, a modification known to convert MEF2 into a transcriptional repressor. Moreover, MEF2-DNA binding upstream of the MEF2 target gene c-JUN, was significantly enhanced by DEX. This suggests a state of active transcriptional repression which correlated with the downregulation of c-JUN. Finally, we showed that downregulation of MEF2 by means of lentiviral knockdown, resulted in inhibition of c-JUN expression to a similar extent as normally seen after DEX treatment, which could not be further reduced by DEX. These results suggest that GR-regulated downregulation of c-JUN is mediated by MEF2.

GR and MEF2 transcription factors are known to play an important role in synaptic plasticity, which is a crucial hallmark of psychostimulant sensitization. Neuronal depolarization is an important prerequisite to demonstrate synaptic plasticity and MEF2 is known to be activated in a depolarizing environment. In **Chapter 4** a study is described in which we examined how GR regulates MEF2 activity when neuronally differentiated PC-12 cells are depolarized.The results showed that expression of the MEF2 target gene NR4A1, which is known to be affected by depolarization, was attenuated when depolarization was combined with GR activation. Surprisingly, while GR activation or depolarization alone resulted in enhanced MEF2-DNA binding upstream of NR4A1, MEF2-DNA binding was completely reduced to vehicle levels when DEX was administered within a depolarized environment. The results demonstrate that glucocorticoids acting via GR can maintain the balance in depolarization-induced synaptic plasticity.

In Chapter 5 the previous results were used to design a study *in vivo* to ask the question whether phosphorylation of MEF2 in the hippocampus and striatum is modified by amphetamine sensitization and at what time point in the sensitization process this may happen. The results showed that, although locomotor activity is enhanced by amphetamine at all-time points measured, MEF2 phosphorylation is increased only after 5 consecutive days of amphetamine treatment. This effect was observed in the hippocampus but not in the striatum, where amphetamine treatment did not have any effect on MEF2 phosphorylation. We subsequently tried to manipulate MEF2 phosphorylation *in vivo* in order to study its effect on locomotor activity. For this purpose we used roscovitine, which is an inhibitor of the kinase CDK5, that is known for its effect on MEF2 phosphorylation as well as on amphetamine sensitization. Roscovitine administered intracerebroventricularly enhanced amphetamine-induced locomotor activity while it increased phosphorylation in the hippocampus. The results suggest that MEF2 phosphorylation in the hippocampus is transiently modulated by amphetamine treatment and that roscovitine-induced changes in locomotor activity correlate with enhanced MEF2 phosphorylation. We therefore propose

that MEF2 activity may play an important role in the process of psychostimulant sensitivity and psychosis susceptibility.

The results that were obtained in this thesis precipitated a model (Figure 7.2) in which stress and the subsequent rise in circulating glucocorticoid concentration activate GR and modulate the activity of MEF2. This altered MEF2 activity causes a change in the expression of its target genes that play a role in synaptic plasticity. Regulation of MEF2 is proposed as a new mechanism via which glucocorticoids via GR regulate synaptic plasticity. We suggest that this mechanism is implicated in behavioral sensitization. Interestingly, the *in* vivo results are only observed in the hippocampus making this brain area an exciting target for further studies to understand its involvement in behavioral sensitization, in particular with respect to the role MEF2 plays in this process. In conclusion, we have identified a GR-mediated pathway influencing MEF2 activity, that might give new insight in the mechanism of psychostimulant sensitization and psychosis susceptibility.

Addendum | Samenvatting

SAMENVATTING

Schizofrenie is een ernstige psychiatrische ontwikkelingsstoornis die voorkomt bij ongeveer 1% van de bevolking. Het wordt gekenmerkt door een grote variëteit aan symptomen waarbij, positieve, negatieve en cognitieve aspecten worden onderscheiden. Factoren die kunnen bijdragen aan de ontwikkeling van de ziekte zijn complicaties bij de zwangerschap, chronische sociale problemen en drugsgebruik. Het is bekend dat deze negatieve omgevingsinvloeden het risico op schizofrenie met name verhogen in combinatie met genetische kwetsbaarheid. Het genetische profiel draagt voor ongeveer 50% bij aan het risico op schizofrenie.

Verschillende diermodellen zijn ontwikkeld met als doel bepaalde aspecten van schizofrenie na te bootsen. Eén van deze modellen is het “amfetamine sensitisatie“ model. Wanneer dieren meerdere dagen behandeld worden met amfetamine resulteert dit in een overgevoelige (of gesensitiseerde) staat van het dier. Deze sensitisatie kan aangetoond worden wanneer na een onthoudingsperiode van enkele weken, de dieren opnieuw amfetamine toegediend krijgen als zogenaamde “challenge”-injectie en daar sterker dan voorheen op reageren. Deze voor amfetamine overgevoelige toestand vertoont gelijkenissen met schizofrenie bij de mens, zoals onder andere een bij schizofrenie vergelijkbare sensitisatie van het dopamine systeem en het vermogen de expressie van sensitisatie te blokkeren met behulp van dopamine-antagonisten.

Hersengebieden die een belangrijke rol spelen in het sensitisatie proces zijn de nucleus accumbens, de prefrontale cortex en de hippocampus. Deze hersengebieden ontvangen allen directe dopaminerge input van de ventrale tegmentale regio. In **Hoofdstuk 2** is in deze drie hersenregio’s de expressie van genpatronen beschreven van genetisch identieke DBA2/j muizen die extreem verschilden in het verloop van amfetamine sensitisatie. Het doel van dit onderzoek was om genen te identificeren die een rol spelen bij het ontstaan van deze sensitisatie en daardoor mogelijk een rol spelen bij schizofrenie en gevoeligheid voor psychoses. In de CA1 regio van de hippocampus vonden we de meest robuuste verschillen in genexpressie tussen laag en hoog responsieve dieren in het amfetamine sensitisatie experiment. De resultaten lieten bovendien zien dat de genen die differentieel tot expressie kwamen, target genen zijn van de glucocorticoïd receptor (GR) en myocyte enhancer factor 2 (MEF2). Dit suggereert dat beide transcriptiefactoren een rol spelen in het tot stand komen van amfetamine sensitisatie. De hippocampus is betrokken bij het onderdrukken van amfetamine-geïnduceerde sensitisatie. Dit gebeurt waarschijnlijk door het controleren van de neuronale activiteit van de nucleus accumbens en het reguleren van het vuren van dopaminerge neuronen uit de ventrale tegmentale regio. Bovendien komt de GR hoog tot expressie in de hippocampus en moduleren glucocorticoiden de amfetamine gevoeligheid.

Bij de start van dit onderzoek was het nog onbekend dat GR en MEF2 invloed op elkaar kunnen uitoefenen. **Hoofdstuk 3** beschrijft onderzoek naar een mogelijke interactie tussen beide transcriptiefactoren *in vitro* in neuronaal gedifferentieerde PC-12 cellen. Na behandeling van de cellen met de GR agonist dexamethason, bleek MEF2 gefosforyleerd te worden op serine 408. Dit is een modificatie waarvan bekend is dat het de werking van MEF2 verandert van stimulatie naar inhibitie van transcriptie. Verder werd de DNA-binding van MEF2 vóór het c-JUN gen significant verhoogd. Dit wees op een actieve transcriptionele repressie van c-JUN die correleerde met de downregulatie van c-JUN op mRNA niveau. Tot slot lieten we zien dat downregulatie van MEF2, met behulp van lentivirale knockdown, resulteerde in verminderde expressie van c-JUN, zoals dat eerder was waargenomen na DEX behandeling. Bovendien had DEX behandeling na knockdown van MEF2 geen effect meer op de expressie van c-JUN. Deze resultaten suggereren dat de c-JUN downregulatie door glucocorticoïden via GR en MEF2 tot stand komt.

Aangezien de transcriptiefactoren GR en MEF2 een belangrijke rol spelen bij de regulatie van synaptische plasticiteit, hetgeen een belangrijk aspect is van sensitisatie met psychostimulantia, is vervolgens onderzocht of GR de MEF2 activiteit reguleert in een omgeving van neuronale depolarisatie. Neuronale depolarisatie is een belangrijke voorwaarde om synaptische plasticiteit te bewerkstelligen en van MEF2 is bekend dat het wordt geactiveerd in een depolariserende omgeving. De resultaten beschreven in **Hoofdstuk 4** lieten zien dat de door depolarisatie geïnduceerde expressie van het MEF2 target gen NR4A1, significant wordt onderdrukt indien tevens GR geactiveerd wordt door dexamethason. Verrassend genoeg bleek, dat terwijl GR activatie of depolarisatie op zichzelf leidde tot verhoogde MEF2-DNA binding vóór NR4A1, deze verhoogde DNA binding weer verdween bij combineren van depolarisatie en GR activatie. Deze resultaten tonen aan dat glucocortioïden via GR de door depolarisatie-geïnduceerde synaptische plasticiteit in balans kunnen houden.

In **Hoofdstuk 5** is gebruik gemaakt van de eerdere resultaten om te onderzoeken of *in vivo* de fosforylatie van MEF2 in de hippocampus en striatum verandert bij amfetamine sensitisatie. Ook is vastgesteld op welke punt tijdens het sensitisatieproces deze fosforylatie plaatsvindt. De resultaten lieten zien dat de locomotor activiteit op elk gemeten tijdpunt na amfetamine behandeling verhoogd is. MEF2 fosforylatie echter was alleen verhoogd na 5 dagelijkse behandelingen met amfetamine. De verhoogde fosforylatie van MEF2 werd tot onze verrassing waargenomen in de hippocampus en niet in het striatum. Vervolgens is het effect van het manipuleren van MEF2 fosforylatie op locomotor activiteit gemeten. Hiertoe kregen de dieren intracerebroventriculair een injectie met roscovitine, dat een remmer is van het kinase CDK5 verantwoordelijk voor de fosforylatie van MEF2. Roscovitine behandeling versterkte het effect van amfetamine op de locomotor activiteit en resulteerde in meer

fosforylatie van MEF2. Dit suggereert dat de door roscovitine geïnduceerde verandering in locomotor activiteit correleert met verhoogde MEF2 fosforylatie. De resultaten geven derhalve een aanwijzing dat MEF2 activiteit betrokken is bij amfetamine sensitisatie en psychose gevoeligheid

De resultaten beschreven in dit proefschrift leiden tot een model (Figuur 7.2) waarin stress en glucocorticoïden via GR de activiteit van MEF2 kunnen veranderen. Dit heeft gevolgen voor de regulatie van de target genen van MEF2, die een rol spelen bij de synaptische plasticiteit. De regulatie van MEF2 in plasticiteit kan derhalve mede ten grondslag liggen aan de glucocorticoïd-afhankelijke mate van amfetamine sensitisatie. Verrassend genoeg laten de *in vivo* resultaten zien dat de effecten op MEF2 voorkomen in de CA1 regio van de hippocampus. Deze bevinding benadrukt nog eens de belangrijke rol van de hippocampus wat betreft de werking van MEF2 en GR in amfetamine sensitisatie. Samengevat, in dit promotieonderzoek is een nieuw mechanisme geïdentificeerd waarin glucocorticoïden via GR de activiteit van MEF2 beïnvloedt. Dit mechanisme kan een nieuw aanknopingspunt geven in het mechanisme van amfetamine sensitisatie en gevoeligheid voor psychose.

Addendum | Dankwoord

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Addendum | Curriculum Vitae

CURRICULUM VITAE

Niels Speksnijder werd geboren op 10 januari 1982 in Sliedrecht. Na het voltooien van de HAVO in 1999 aan de Guido de Bres te Rotterdam startte hij met de Hogere Laboratorium Opleiding aan de Hogeschool West-Brabant in Etten-Leur. Na het behalen van zijn propedeuse in 2000 is hij in Utrecht aan de faculteit Farmaceutische Wetenschappen gestart met de opleiding Farmacie. Het bachelorexamen behaalde hij in 2004 waarna hij de master Drug Innovation heeft gevolgd, eveneens aan dezelfde faculteit. Tijdens de masteropleiding heeft Niels zich bij de afdeling Farmacoepidemiologie en Farmacotherapie en de afdeling Psychofarmacologie verdiept in de effecten van stress. Niels behaalde zijn masterexamen in 2006 en begon in februari 2007 aan zijn promotietraject aan het LACDR/Universiteit Leiden/LUMC in Leiden onder begeleiding van Prof. dr. E.R. de Kloet en Dr. N.A. Datson. In augustus 2012 is Niels als projectmanagement trainee gaan werken bij de afdeling Z-Index van de KNMP in Den Haag. Sinds augustus 2013 is Niels werkzaam als beleidsmedewerker ICT bij de afdeling Zorg Onderzoek & Innovatie van de KNMP in Den Haag.

Addendum | Publication list

PUBLICATION LIST

Speksnijder N, Van der Veen R, Datson N, de Kloet ER; Hippocampal MEF2 phosphorylation is enhanced during induction of sensitization; submitted

Speksnijder N, de Kloet ER, Datons NA; Depolarization-induced binding of MEF2 to the promoter region of NR4A1 is prevented by GR activation; submitted

Speksnijder N, Vielsted Christensen K, Didriksen M, de Kloet ER, Datson NA; Glucocorticoid Receptor and Myocyte Enhancer Factor 2 cooperate to regulate the expression of c-JUN in a neuronal context; Journal of Molecular Neuroscience 2012 Sep; 48(1): 209-18.

Polman JA, Hunter RG, Speksnijder N, van den Oever JM, Korobko OB, McEwen BS, de Kloet ER, Datson NA; Glucocorticoids modulate the mTOR pathway in the hippocampus: differential effects depending on stress history; Endocrinology 2012 Sep; 153(9): 4317-27.

Datson NA/Speksnijder N, De Jong IE, Steenbergen PJ, Vielsted Christensen K, Potempa K, Torleif Pedersen J, Egebjerg J, Kallunki P, Nielsen EB, de Kloet ER, Didriksen M; Hippocampal CA1 region shows differential regulation of gene expression in mice displaying extremes in behavioral sensitization to amphetamine: relevance for psychosis susceptibility?; Psychopharmacology 2011 Oct; 217(4): 525-38.

Datson NA, Speksnijder N, Mayer JL, Steenbergen PJ, Korobko O, Goeman J, de Kloet ER, Joëls M, Lucassen PJ. The transcriptional response to chronic stress and glucocorticoid receptor blockade in the hippocampal dentate gyrus; Hippocampus 2010 Feb; 22(2): 359-71.