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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 pheochromocytoma (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 cm² plates (356478, BD Biosciences). Neuronal differentiation was achieved by giving 50 ng/ml NGF- β (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 184 μ g/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, 10 μ g/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, 80 μ g/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 μ M CaCl₂ and 120 μ M 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 μ M AraC (Cytosine Arabinoside). The cells were left for 14 days in vitro before assaying.

Lentiviral shRNA transduction, stimulation and RNA purification High titer batches ($>5 \times 10^8$ 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/ μ l 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 μ g per antibody) was incubated overnight (o/n) with 6 μ g 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 μ l 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 NaHCO₃ 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 Myoglobin 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% β -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.

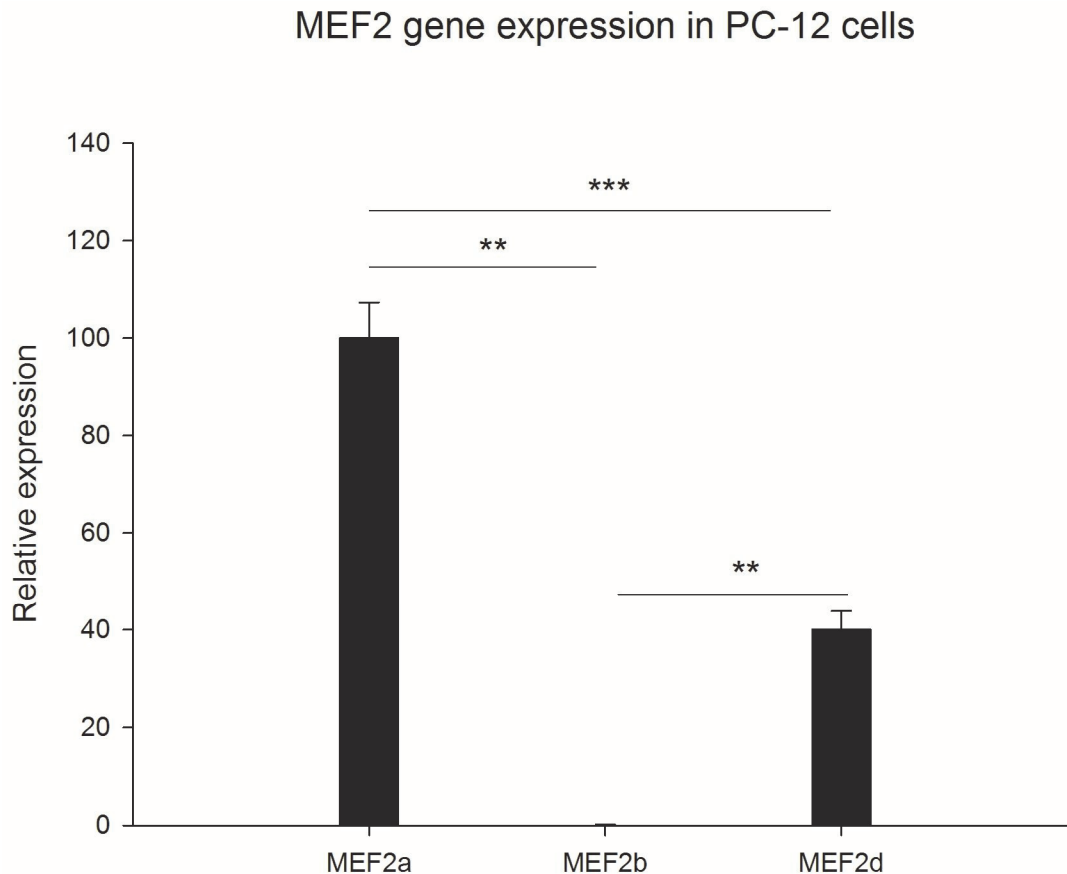


Fig. 1 |Relative expression levels of transcripts MEF2a, -b and -d in neuronally differentiated PC-12 cells under VEH conditions (n=6 per group). Expression is relative to MEF2a, which is set at 100%. Expression is normalized to TUBB2a. (** p<0.01; *** p <0.001 sign. between transcripts).

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).

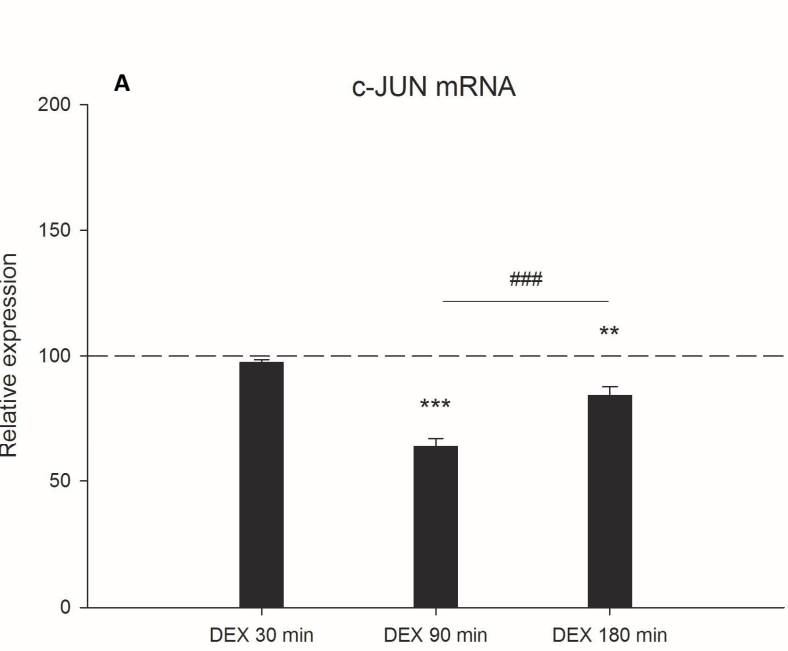


Fig. 2A |c-JUN mRNA expression at 30, 90 or 180 minutes of DEX treatment (n=6 per group). For each timepoint, expression level is relative to its VEH counterpart which is set at 100% and indicated by the dashed line. Expression is normalized to TUBB2a. (** $p < 0.01$; *** $p < 0.001$ sign. vs corresponding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

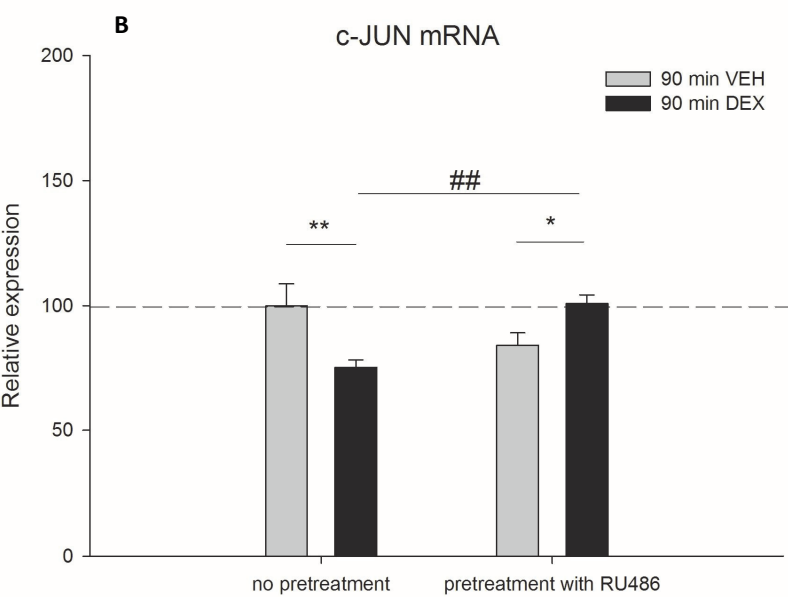


Fig. 2B |c-JUN mRNA expression at 90 minutes of DEX treatment with and without 60 minutes RU486 pretreatment (n=6 per group). Expression level is relative to VEH treated cells without pretreatment, set at 100%. Expression is normalized to TUBB2a. (* $p < 0.05$; ** $p < 0.01$ sign. vs VEH treatment) (##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

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.

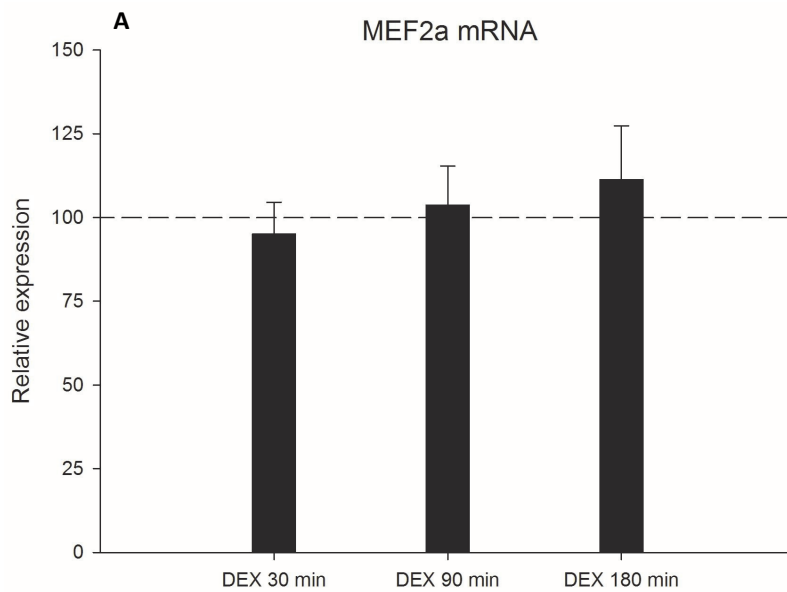


Fig. 3A | MEF2a mRNA expression in neuronal PC-12 cells at 30, 90 or 180 minutes of DEX treatment (n=6 per group). For each timepoint, expression level is relative to its VEH counterpart which is set at 100% and indicated by the dashed line. Expression is normalized to TUBB2a.

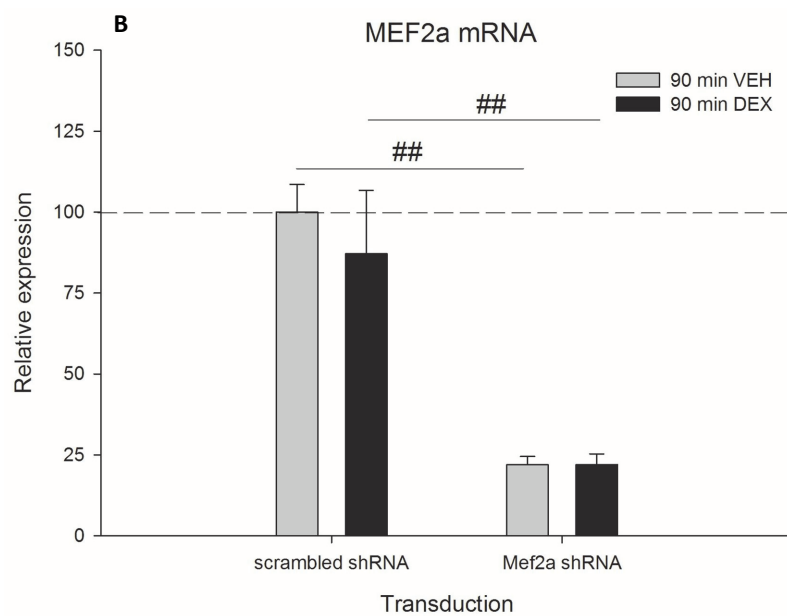


Fig 3B | MEF2a mRNA expression at 90 minutes of DEX treatment in lentiviral transduced primary hippocampal neurons expressing either scrambled shRNA or MEF2a shRNA (n=3 per group). Expression level is relative to scrambled shRNA transduced and VEH treated cells, set at 100%. Expression is normalized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

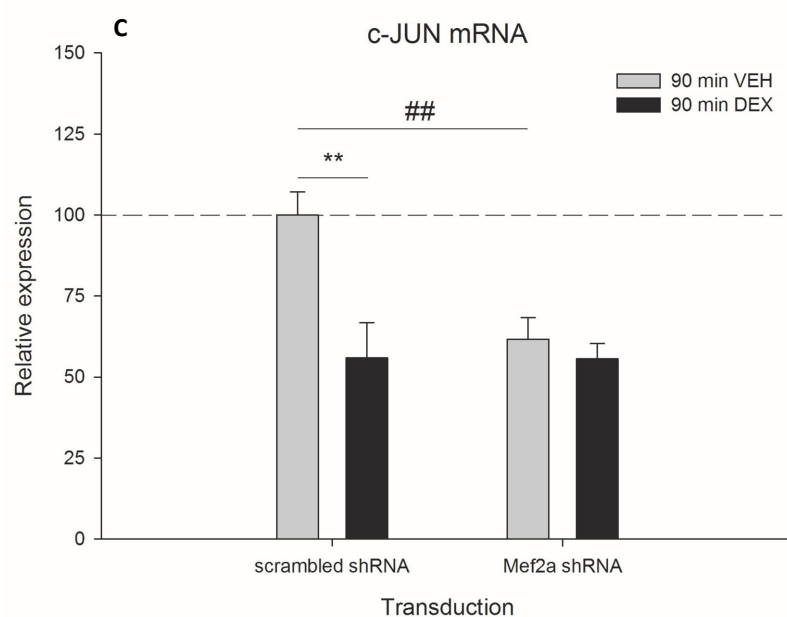


Fig 3C | c-JUN mRNA expression at 90 minutes of DEX treatment in lentiviral transduced primary hippocampal neurons expressing either scrambled shRNA or MEF2a shRNA (n=3 per group). Expression level is relative to scrambled shRNA transduced and VEH treated cells, set at 100%. Expression is normalized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

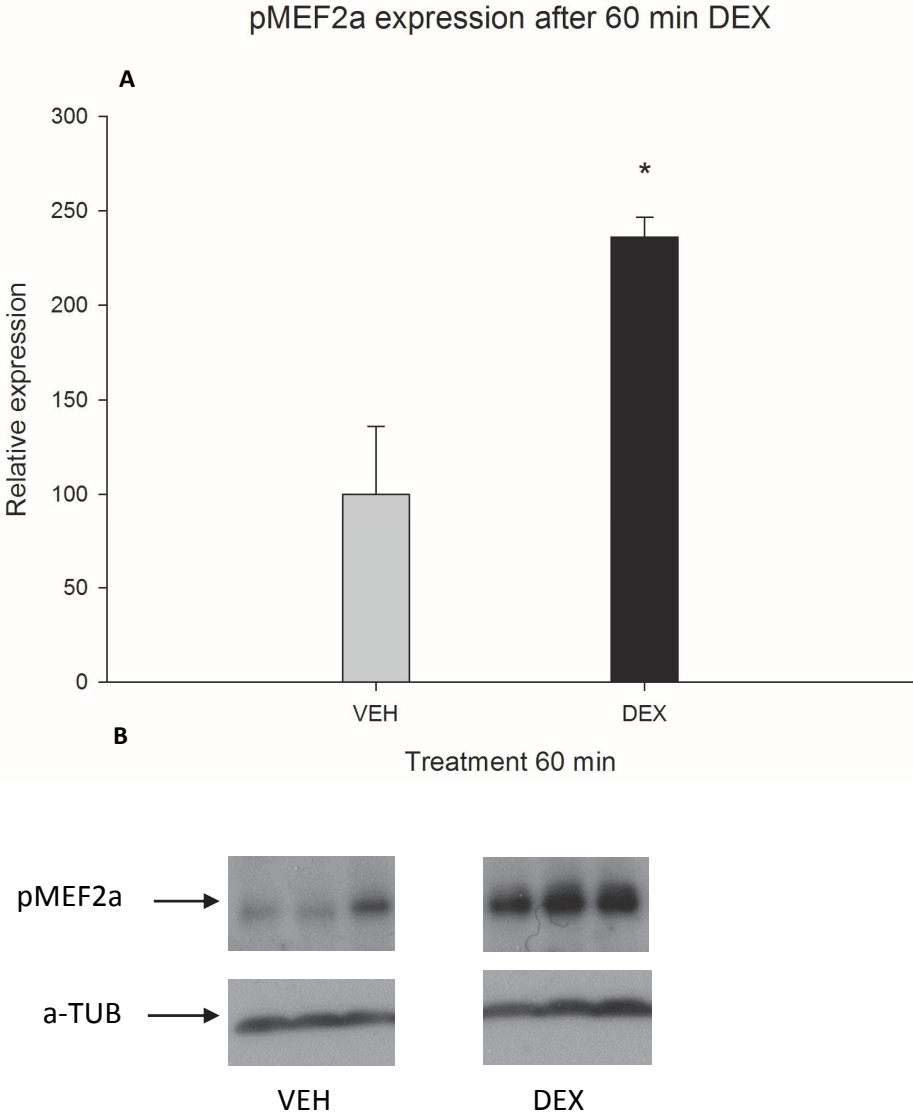


Fig. 4 | MEF2a S408 phosphorylation level after 60 minutes of DEX treatment (n=3 per group). A. Phosphorylation level is relative to 60 minutes VEH treatment, set at 100%. Expression is normalized to total MEF2a and alpha-tubulin. (* p<0.05 sign. vs VEH treatment). B. Representative example of protein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

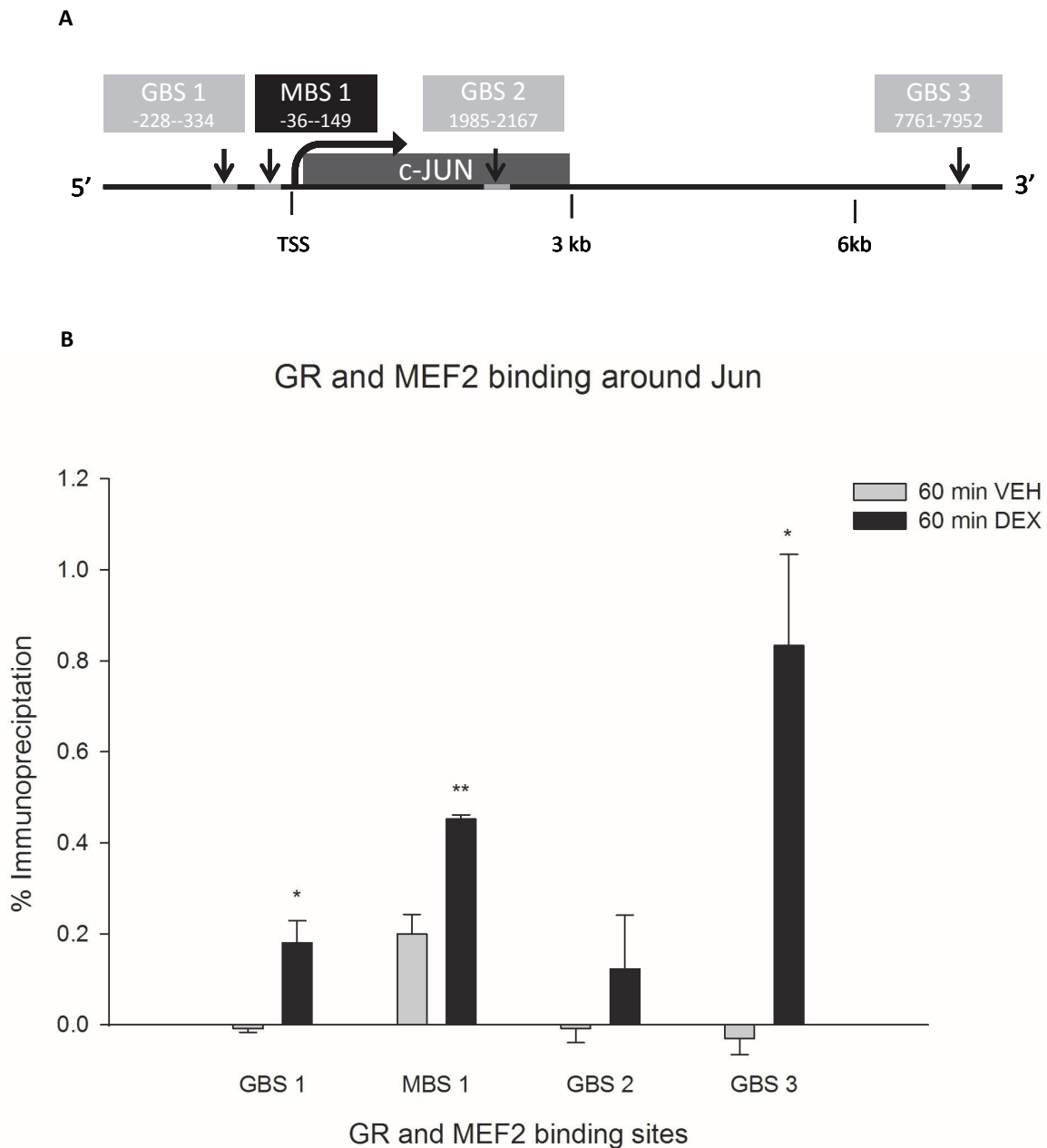


Fig. 5 | Binding levels of GR and MEF2a in the vicinity of the c-JUN gene (n=3 per group). A. Schematic overview of the c-JUN gene and surrounding sites. GR binding sites 1, 2 and 3 are depicted in red and the MBS1 is depicted in yellow. Numbers inside the boxes indicate distance from the beginning and 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 strengthened 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 modification 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 Phosphoinositide-3-kinase, catalytic, gamma polypeptide (PIK3CG) binding and expression, reveal a similar response pattern. Pulipparacharuvi *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 (Pulipparacharuvi *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 imply 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.

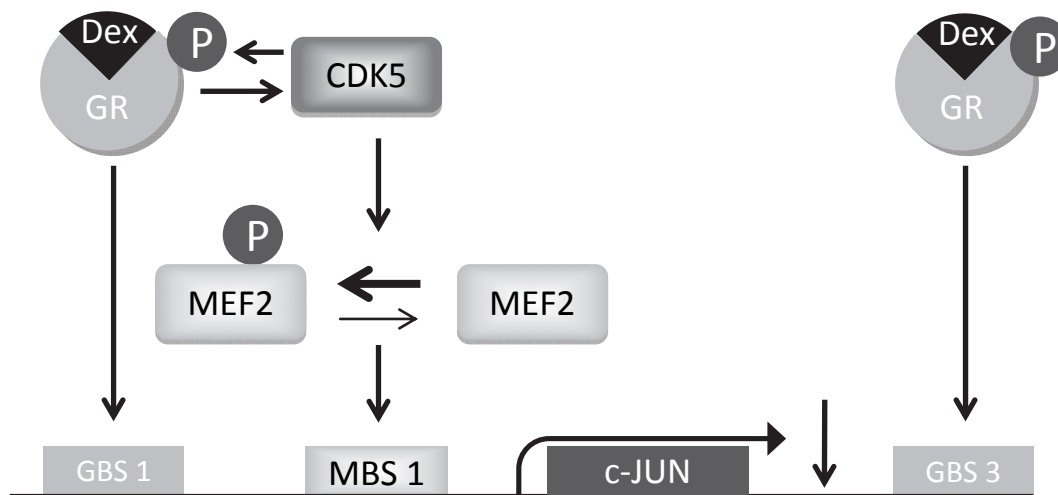


Fig. 6 | Schematic overview of the obtained results. Activation of GR by agonist DEX activates the kinase CDK5, responsible for phosphorylation of its target proteins, including GR and MEF2. MEF2, now phosphorylated, is increasingly bound to the DNA where it is suggested to act as a transcriptional repressor. 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	CGCGAAGGCTCAGGGATGA	CGGGAACACAAAGCCGGAGCA
	Jun oncogene	Rat	c-Jun	GR DNA-binding 2	2097	GGTCATGCAGTCTTGGTCA	TGGACTGGGTTGCGACCTGA
	Jun oncogene	Rat	c-Jun	GR DNA-binding 3	7871	TCTGATAACCCAAATTTCTGAAGCA	TGCCATGTCTCAGCAGTGT
	Jun oncogene	Rat	c-Jun	Mef2 DNA-binding	-36	AGTCTCTGCCACACTCAGTGCAA	TGGGAAGGCCCTGGGGTGACA
NC_005106.2	Mvoglobin	Rat	Mb	Negative control GR and MEF2 DNA-binding	3504	TAGTGTGCATCCAGCAGAGG	ACACTGTGGCCTTTTGTCC
NM_021835.3	Jun oncogene	Rat	c-Jun	Expression	NA	GCTGGAAGAAGAGGGTGTGG	CACAGGCCATGCTACTTTGAT
NM_010591.2	Jun oncogene	Mouse	c-Jun	Expression	NA	GGTGGAGGGGTTACAAACT	GGGGAGTTCATCTGCAGTCT
NM_001014035.1	Myocyte enhancer factor 2a	Rat	Mef2a	Expression	NA	TCAAGCCACACAACTCTTTG	GTGTTGTAGGCTGTGGGCAT
NM_001017507.1	Myocyte enhancer factor 2b	Rat	Mef2b	Expression	NA	GAACAGCCACTTGCACAAC	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	GACAGGCAAACTGAGCACCAT
NM_009450.2	Tubulin beta 2A	Mouse	Tubb2a	Expression	NA	TCCTTAGCCCTCTGTCCACGCA	ACCTCCAAAACCTTAGCGCCGATCT

