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.

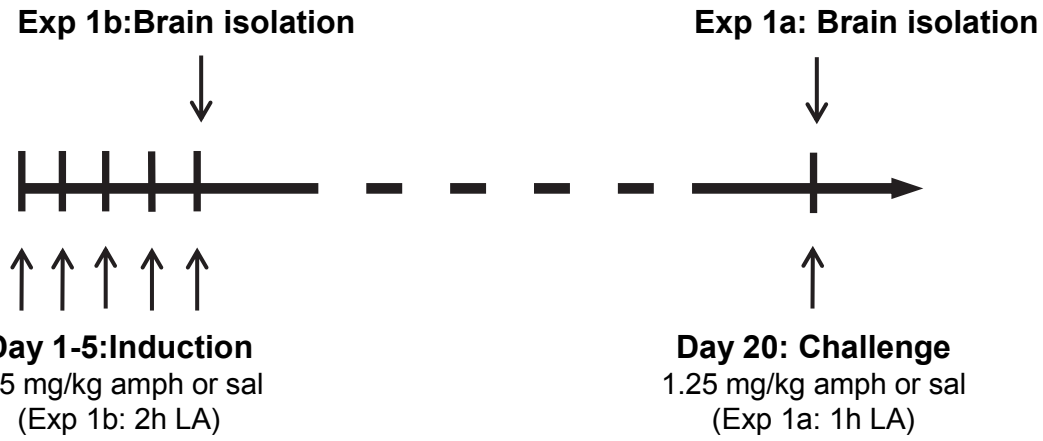

Figure 1| Amphetamine sensitization procedure in experiment 1. DBA/2J mice received daily injections of amphetamine (2.5 mg/kg) or saline for 5 consecutive days. After a withdrawal period, animals received a challenge injection of amphetamine (1.25 mg/kg) or saline at day 20. In experiment 1a locomotor 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).

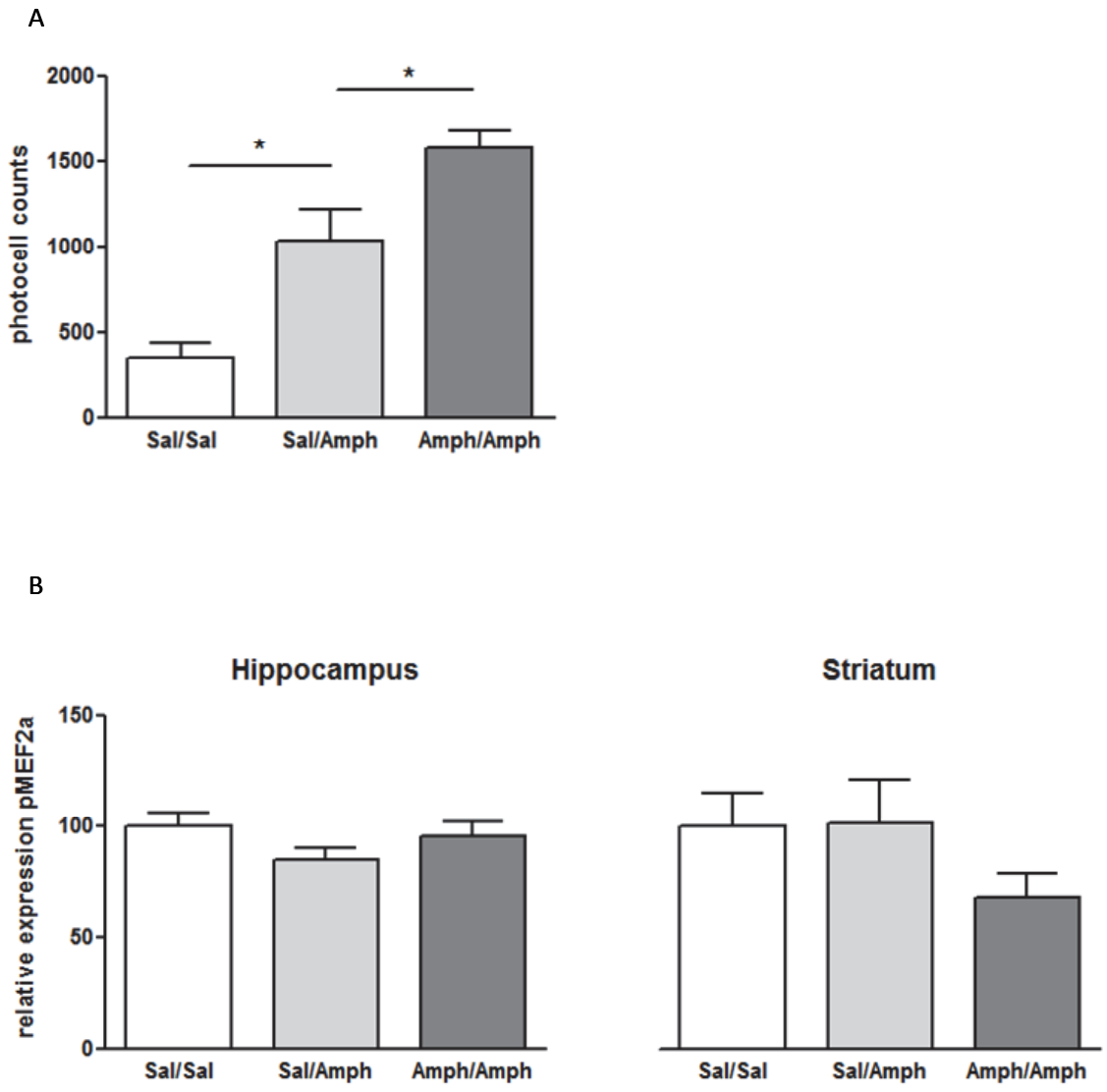

Figure 2 | Experiment 1a: Locomotor activity and MEF2a phosphorylation in hippocampus and striatum following a challenge dose at day 20 (A) Locomotor activity was measured for 1h after the challenge injection of amphetamine (Amph, 1.25 mg/kg) or saline (Sal). (B-C) Relative expression of MEF2 phosphorylation 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).

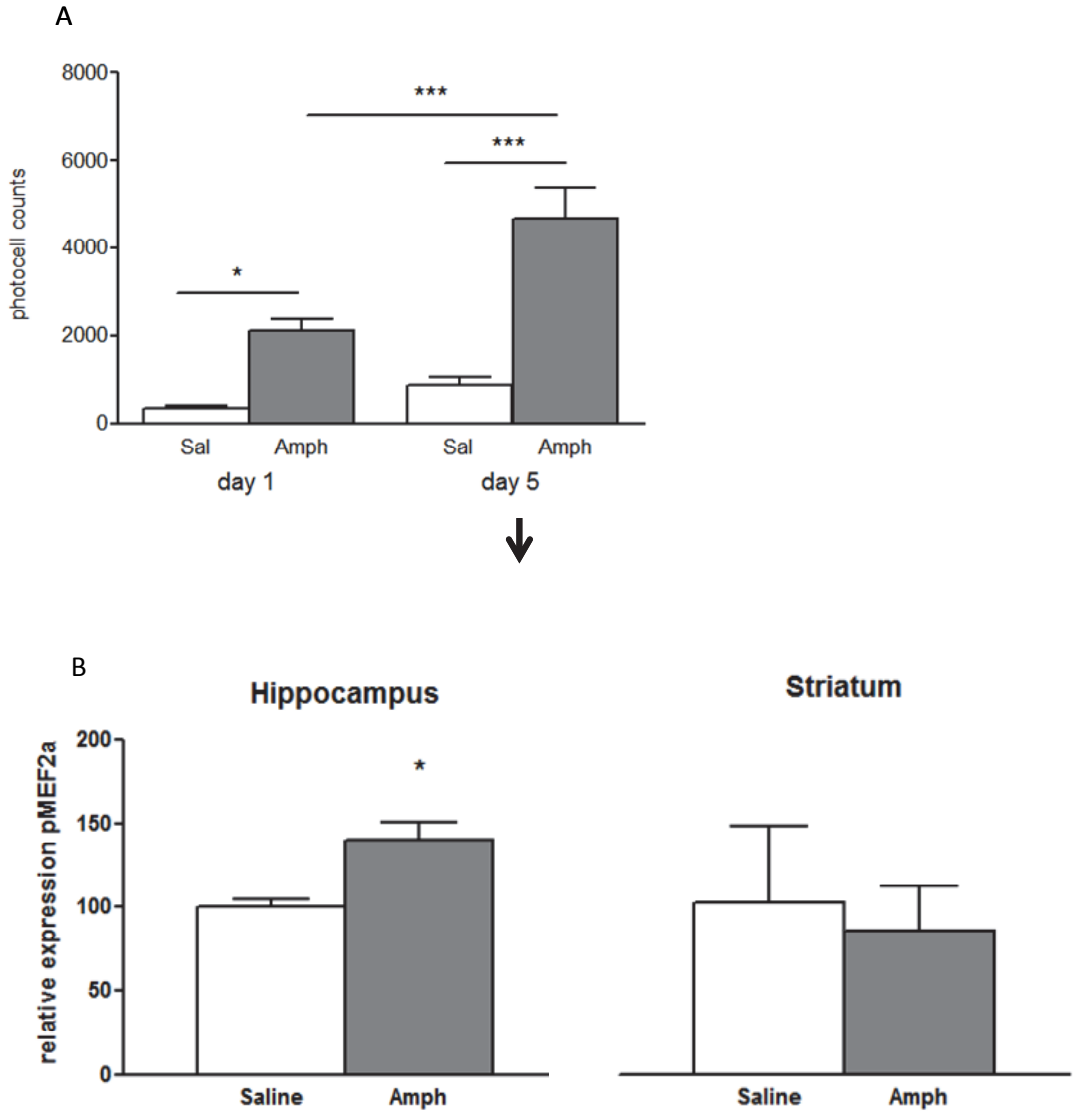

Figure 3 | Experiment 1b: Locomotor activity and MEF2a phosphorylation in hippocampus following daily injections. (A) Locomotor activity in response to amphetamine (2.5 mg/kg) or saline on day 1 and 5, total response over 2h. (B) Relative expression of MEF2 phosphorylation in hippocampus on day 5 of 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).

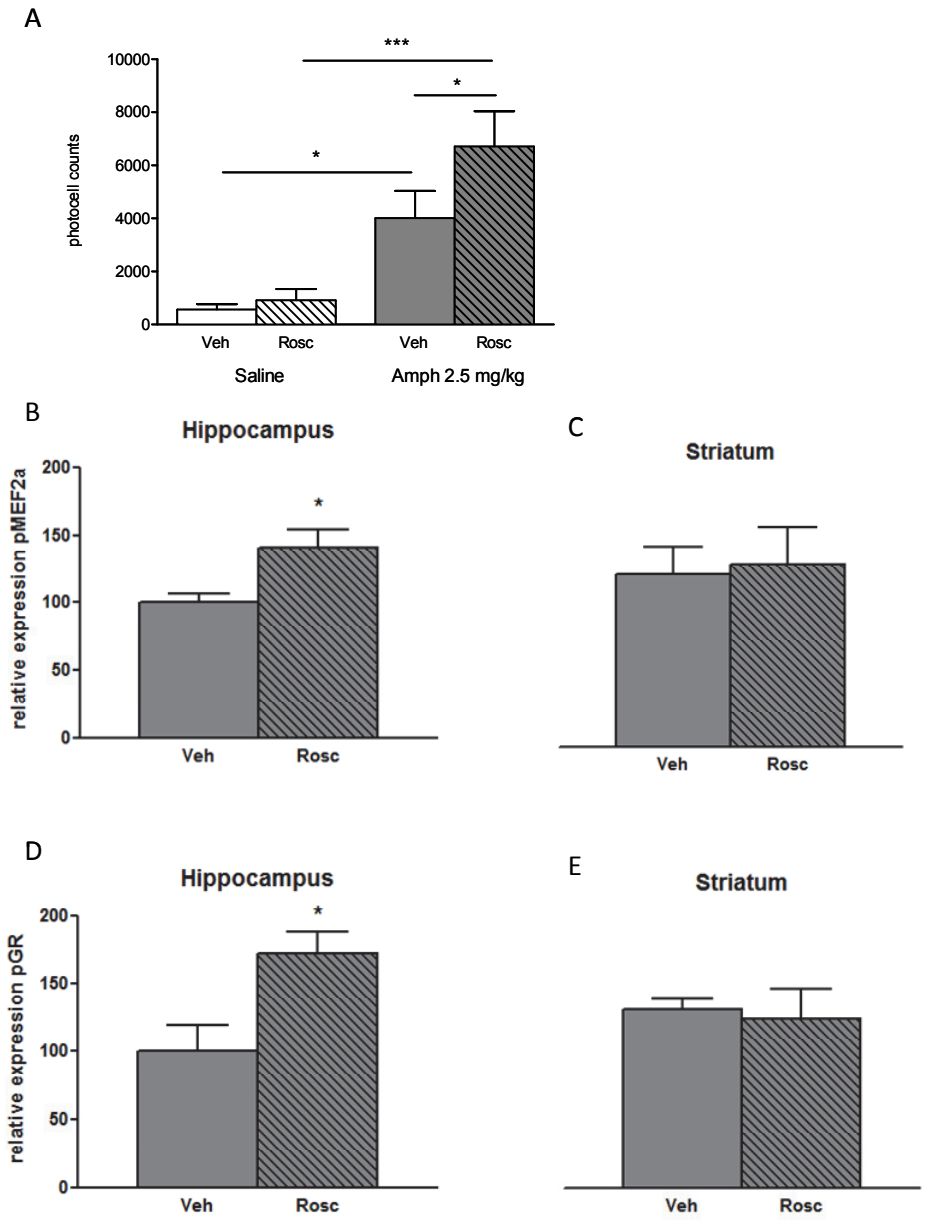

Figure 4 | Experiment 2: The effect of roscovitine on locomotor activity and MEF2a and GR phosphorylation in hippocampus and striatum following an amphetamine injection. (A) Locomotor activity in response to an amphetamine (2.5 mg/kg) or saline injection, total response over 2h. Animals were pretreated 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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