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Stable immediate early gene expression patterns in medial prefrontal cortex and striatum after long-term cocaine self-administration

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ABSTRACT

The transition from casual to compulsive drug use is thought to occur as a consequence of repeated drug taking leading to neuroadaptive changes in brain circuitry involved in emotion and cognition. At the basis of such neuroadaptations lie changes in the expression of immediate early genes (IEGs) implicated in transcriptional regulation, synaptic plasticity and intracellular signalling. However, little is known about how IEG expression patterns change during long-term drug self-administration. The present study, therefore, compares the effects of 10 and 60-day self-administration of cocaine and sucrose on the expression of 17 IEGs in brain regions implicated in addictive behaviour, i.e. dorsal striatum, ventral striatum and medial prefrontal cortex (mPFC). Increased expression after cocaine self-administration was found for 6 IEGs in dorsal and ventral striatum (*c-fos*, *Mkp1*, *Fosb*/Δ*Fosb*, *Egr2*, *Egr4*, and *Arc*) and 10 IEGs in mPFC (same 6 IEGs as in striatum, plus *Bdnf*, *Homer1*, *Sgk1* and *Rgs2*). Five of these 10 IEGs (*Egr2*, *Fosb*/Δ*Fosb*, *Bdnf*, *Homer1* and *Jun*) and *Trkb* in mPFC were responsive to long-term sucrose self-administration. Importantly, no major differences were found between IEG expression patterns after 10 or 60 days of cocaine self-administration, except *Fosb*/Δ*Fosb* in dorsal striatum and *Egr2* in mPFC, whereas the amount of cocaine obtained per session was comparable for short-term and long-term self-administration. These steady changes in IEG expression are, therefore, associated with stable self-administration behaviour rather than the total amount of cocaine consumed. Thus, sustained impulses to IEG regulation during prolonged cocaine self-administration may evoke neuroplastic changes underlying compulsive drug use.

Keywords Cocaine self-administration, dorsal striatum, immediate early genes, prefrontal cortex, ventral striatum.

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INTRODUCTION

The transition from casual to compulsive drug use is thought to be initiated by pathological neuroadaptations in neural circuits that are involved in reward processing, habit formation and cognitive control over behaviour, including the ventral striatum (VS), dorsal striatum (DS) and prefrontal cortex (PFC) (Jentsch & Taylor 1999; Koob & Volkow 2010; Pierce & Vanderschuren 2010; Everitt 2014). That is, the reinforcing properties of psychostimulants such as cocaine are widely assumed to be mediated by the mesolimbic dopaminergic system that targets the VS (Pierce & Kumaresan 2006). The DS has been implicated in habit formation (Yin & Knowlton 2006) and drug-induced maladaptive changes in this process might play a major role in the development of inflexible cocaine use (Vanderschuren, Di Ciano, & Everitt 2005; Zapata, Minney, & Shippenberg 2010; Jonkman, Pelloux, & Everitt 2012a). Evidence suggests that a gradual shift in striatal control over drug use takes place from VS to DS with a prolonged drug taking history, as drug use devolves from casual to compulsive (Porrino *et al.* 2004b; Everitt & Robbins 2005; Pierce & Vanderschuren 2010). Concomitantly, drug-induced changes leading to malfunction of mPFC are thought to cause failure of cognitive control over drug

seeking (Jentsch & Taylor 1999; Goldstein & Volkow 2011; Chen *et al.* 2013).

Cocaine-induced neuroadaptations affecting striatal and PFC function have been shown to involve complex molecular changes in transcriptional regulation and cellular signalling pathways (Lüscher & Malenka 2011; Robison & Nestler 2011), in which immediate early genes (IEGs) are key components. IEG expression profiles have been widely studied to establish the effects of cocaine exposure, but few studies have looked at IEG changes as a consequence of cocaine self-administration (Perrotti *et al.* 2008; Larson *et al.* 2010; Zahm *et al.* 2010; Besson *et al.* 2013; Fumagalli *et al.* 2013). Importantly, studies in rats and primates have shown that cocaine exposure-evoked changes in striatal metabolic activity and dopamine function spread in extent and intensity as a function of self-administration experience (Letchworth *et al.* 2001; Porrino *et al.* 2004a; Besson *et al.* 2013). Moreover, prolonged cocaine self-administration has been demonstrated to engender addiction-like behaviour, for example insensitivity to punishment (Deroche-Gamonet, Belin, & Piazza 2004; Vanderschuren & Everitt 2004; Pelloux, Murray, & Everitt 2013; Vanderschuren & Ahmed 2013; Limpens *et al.* 2014b). In the present study, we, therefore, measured the expression of a panel of IEGs in VS, DS and mPFC of animals that had been self-administering cocaine for either a limited (10 days) or a prolonged (60 days) time period, using quantitative real-time PCR (RT-PCR). The panel of IEGs encompassed genes responsive to cocaine or dopaminomimetic drugs that are involved in a broad range of functions ranging from transcriptional regulation (*Fos* and *Egr* family) to synaptic plasticity (*Arc*, *Bdnf*, *Homer1*, *Dclk1*, *Ccnl1*), intracellular signalling (*Bdnf*, BDNF receptor *Trkb* and *Rgs2*) and cellular stress or phosphorylation (*Sgk1* and *Mkp1*) (Hope *et al.* 1994; Berke *et al.* 1998; Burchett, Bannon, & Granneman 1999; Thiriet, Aunis, & Zwiller 2000; Berke *et al.* 2001; Takaki *et al.* 2001; Courtin *et al.* 2006; Corominas *et al.* 2007; McGinty *et al.* 2008; Fumagalli *et al.* 2009; Ghasemzadeh *et al.* 2009; Larson *et al.* 2010; Zahm *et al.* 2010; Guez-Barber *et al.* 2011; McCoy *et al.* 2011; Fumagalli *et al.* 2013; Jia *et al.* 2013; Otis, Fitzgerald, & Mueller 2014; Heller *et al.* 2015). IEG expression levels in the cocaine-exposed animals were compared with those after self-administration of the natural reinforcer sucrose and a self-administration control.

Our hypothesis was that after short-term cocaine exposure VS would display an increase in the expression levels of particular IEGs, which would abate after extended access to cocaine. For DS, we expected to find enhanced expression mainly after long-term exposure to cocaine. With respect to mPFC, we expected to see increased IEGs expression after short-term cocaine self-administration, followed by either further increases or decreases after long-term cocaine exposure.

METHODS

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 320–380 g were housed individually in Macrolon cages (L = 40, W = 25 and H = 18 cm) under controlled conditions (temperature = 20 – 21°C, 55 ± 15 percent relative humidity) and a reversed 12 hour light dark cycle (lights on at 19:00). Each subject received 20 g laboratory chow (SDS Ltd, UK) per day and free access to water, which was sufficient to maintain body weight and growth. Self-administration sessions were carried out between 9 AM and 6 PM, for 5 days a week. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86 / 609 / EEC).

Apparatus

Subjects were trained and tested in operant conditioning chambers (L = 29.5 cm, W = 32.5cm, H = 23.5cm; Med Associates, Georgia, VT, USA). The chambers were placed in light and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8 cm wide retractable levers, placed 11.7 cm apart and 6.0 cm from the grid floor. A cue light (28 V, 100 mA) was present above each active lever and a white house light (28 V, 100 mA) was located on the opposite wall. Sucrose pellets (45 mg, formula F, Research Diets, New Brunswick, NJ, USA) were delivered at the wall opposite to the levers via a dispenser. Cocaine infusions were controlled by an infusion pump placed on top of the cubicles. During the cocaine self-administration sessions, polyethylene tubing ran from the syringe placed in the infusion pump via a swivel to the cannula on the animals' back. In the operant chamber, tubing was shielded with a metal spring. Experimental events and data recording were controlled by procedures written in MedState Notation using MED-PC for Windows.

Surgery

Rats allocated to the cocaine self-administration group were anaesthetized with ketamine hydrochloride (0.075 mg/kg, i.m) and medetomidine (0.40 mg/kg, s.c.) and supplemented with ketamine as needed. A single catheter was implanted in the right jugular vein aimed at the left vena cava. Catheters (Camcaths, Cambridge, UK) consisted of a 22 g cannula attached to silastic tubing (0.012 ID) and fixed to nylon mesh. The mesh end of the catheter was sutured subcutaneously (s.c.) on the dorsum. Carprofen (50 mg/kg, s.c.) was administered once before and twice after surgery. Gentamycin (50 mg/kg, s.c.) was

administered before surgery and for 5-day post-surgery. Animals were allowed 10 days to recover from surgery.

Cocaine and sucrose self-administration procedures

Rats were trained to self-administer cocaine under a fixed ratio-1 (FR-1) schedule of reinforcement. During the self-administration sessions, two levers were present, an active lever and an inactive lever. The left or right position of the active and inactive levers was counterbalanced for individual animals. Pressing the active lever resulted in the infusion of 0.25 mg cocaine in 0.1 ml saline over 5.6 seconds, retraction of the levers and switching off of the house light. During the infusion, a cue light above the lever was switched on, followed by a 20 seconds time-out period after which the levers were reintroduced and the house light illuminated. The time-out period was changed to 3 minutes after five training sessions in order to increase the session length. The session ended after 2 hours or if animals had obtained 40 cocaine infusions, whichever occurred first. Responding on the inactive lever had no programmed consequences but was recorded to assess general levels of activity. After each self-administration session, intravenous catheters were flushed with a gentamycin–heparin–saline solution to maintain the patency of the catheters.

The training procedure for the rats in the sucrose group was similar to that for cocaine self-administration, with the exception that each response on the active lever resulted in delivery of a sucrose pellet. Subjects in the control group were also exposed to the self-administration box. Each response on the active lever resulted in illumination of the cue-light for 5.6 seconds.

Rats were trained to self-administer cocaine or sucrose for either 10 or 60 days, and thereafter used for the quantitative RT-PCR experiments. In the 10-day experiment, 20 rats were used (control: $n = 6$, sucrose: $n = 6$, cocaine: $n = 8$), and in the 60-day experiment the number of animals was 24 (control: $n = 8$, sucrose: $n = 8$, cocaine: $n=8$). To validate our findings in the RT-PCR experiments for selected genes (i.e. *c-fos* and *Bdnf*) with a different technique, separate rats were trained in the same procedures and used for *in situ* hybridization (ISH). In the 10-day experiment, control: $n = 8$, sucrose: $n = 8$, cocaine: $n = 8$ and in the 60-day experiment, control: $n = 8$, sucrose: $n = 8$, cocaine: $n = 8$.

Tissue dissection

After the last self-administration session, rats were moved back to their home cages, and decapitated after 30 minutes. Brains were quickly removed and immediately frozen in cold isopentane, then stored at −80°C. For RT-PCR experiments, 200 µm coronal sections were cut at −15°C in a Leica CM 1950 cryostat. Three different brain regions including mPFC, DS and VS were collected and stored separately. For ISH experiments, 14-µm thick coronal sections were cut at −25°C in the cryostat and mounted on SuperFrost® Plus glass slides (Menzel-Gläser, Braunschweig, Germany)

RNA extraction and quantitative real-time PCR

Total RNA was extracted by Guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987) and stored in RNase-free water (Ambion Inc., Austin, TX, USA). A reverse transcription reaction was performed using TaqMan® reverse transcription reagents (Applied Biosystems, Branchburg, NJ, USA) with the total reaction volume of 20 µl (1 µg RNA template, 4 µl 5× iScript reaction Mix, 1 µl iScript reverse transcriptase and nuclease-free water) at the following conditions: 25°C for 10 minutes, 37°C for 1 hour and stopped by heating at 85°C for 5 minutes.

Primers for 17 IEGs were designed using Primer 3 software. Details of all primers are given in Table S1. A total of 10 µl reaction volume contained 1 µl of cDNA, 5 µl of 2× SYBR Green mix (Applied Biolosystems, USA), 1 µl RNase-free H_2O and 3 µl primer pair mix (1 pmol/µl each). The temperature of the cycling protocol was 95°C for 2 minutes, followed by 40 reaction cycles of 95°C for 15 seconds and 60°C for 60 seconds, using the Applied Biosystems® StepOnePlus™ real-time PCR system. For correcting results, target IEGs transcripts were normalized to internal endogenous reference genes. IEGs *Dclk1*, *Ccnl1* and *Homer1* were normalized using the mean of two reference genes (*Gapdh* and *Actb*), and the other IEGs were normalized using the mean of three reference genes (*Gapdh*, *Actb* and *Eno2*). Analysis was performed according to the $2_{\mathrm{T}}^{-\Delta\Delta C}$ method (Livak & Schmittgen 2001). Data from the cocaine and sucrose groups are presented as the n-fold change in gene expression relative to the self-administration control group.

In situ hybridization

ISH for *c-fos* and *Bdnf* in striatum and mPFC after cocaine or sucrose self-administration was performed as previously published (van Kerkhof *et al.* 2014). In brief, DNA fragments for generating RNA probes were produced using Phusion® High-Fidelity PCR Kit (Thermo, New England Biolabs, Germany) with PCR conditions 98°C/2 minutes, followed by 30 cycles of 98°C for 20 seconds, 66°C for 40 seconds, 72°C for 60 seconds and 72°C for 10 minutes. Primers containing either a T7 or T3 RNA polymerase promoter sequence were:

c-fos: sense 5′-GTAATACGACTCACTATAGGGTCACCC TGCCTCTTCTCAAT-3′, and antisense 5′-AATTAACCCT CACTAAAGGGCACAGCCTGGTGTGTTTCAC-3′.

Bdnf: sense 5′-AATTAACCCTCACTAAAGGGGCGGATATTGCAAAGGGTTA-3′, and antisense 5′-GTAATACGACTCACTATAGGGCGGCATCCAGGTAATTTTTG-3′.

Probes were labelled by incorporating digoxigenin-labelled UTP (Roche Diagnostics GmbH, Mannheim, Germany). Sections were fixated, acetylated, dehydrated and delipidated as described previously and hybridized with digoxigenin- labelled antisense RNA probe (5 ng/section). Post-hybridization stringency washings were in 1× SSC at 60°C. Digoxigenin was visualized using immunocytochemistry with alkaline phosphatase as reporter molecule using NBT/BCIP as a substrate (van Kerkhof *et al.* 2014).

Quantification of hybridized probe was carried out on a MCID Elite image system (Interfocus Imaging Ltd., Linton, UK) by assessing the surface area of individual immunopositive cells multiplied by the individual cellular staining intensity (optical density) (van Kerkhof *et al.* 2014).

Statistical analysis

Data were analyzed using SPSS software 20 (IBM, New York, NY, USA). A repeated measures ANOVA was used for analyzing cocaine/sucrose self-administration in the 10- and 60-day experiments, with treatment as a between-subject factor and self-administration session as a within-subject factor. A Greenhouse–Geisser correction was applied when Mauchly's test of sphericity indicated that the assumption of sphericity had been violated. To determine whether the effects of short-term cocaine self-administration on IEG expression differed from those after long-term administration, the quantitative RT-PCR data were analyzed using two-way ANOVA with treatment and number of self-administration sessions (10 or 60) as factors, followed by Tukey's *post hoc* test if appropriate. ISH signal of *c-fos* in three brain regions (VS, DS and mPFC) and *Bdnf* in mPFC were also analyzed with two-way ANOVA followed by Tukey's *post hoc* test.

RESULTS

Cocaine and sucrose self-administration

Rats were trained to self-administer cocaine or sucrose for either 10 or 60 days (Fig. 1 A and 1B). The animals self-administered under a FR-1 schedule of reinforcement, so that the number of active lever presses is equal to the number of rewards obtained. Repeated measures ANOVA showed a main effect of treatment on the active lever presses in the 10 days [$F(2, 17) = 21.172$, $p < 0.001$] and 60-days experiments [$F(2, 21) = 181.993$, $p < 0.001$]. *Post hoc* analysis showed that the sucrose group differed significantly from the control and cocaine groups in both experiments (both $p < 0.001$). A significant difference between the cocaine and control groups was found in the 60-day experiment ($p < 0.001$). In both experiments, there was a main effect of session on active lever presses [10 days: $F(3.645, 61.97) = 3.026$, $p = 0.028$; 60 days: $F(3.388, 71.15) = 6.312$, $p < 0.001$], and there was a significant interaction between treatment and session [10 days: $F(7.29, 61.97) = 7.462$, $p < 0.001$; 60 days: $F(6.522, 61.96) = 2.946$, $p = 0.011$]. When the numbers of active lever presses between the first and the last session of the experiment were compared, significant increases were seen in the cocaine group (10 days: $t = -9.205$, $df = 14$, $p < 0.001$; 60 days: $t = -9.521$, $df = 14$, $p < 0.001$), but not in the sucrose group (10 days: $t = -2.131$, $df = 5.004$, $p = 0.086$; 60 days:

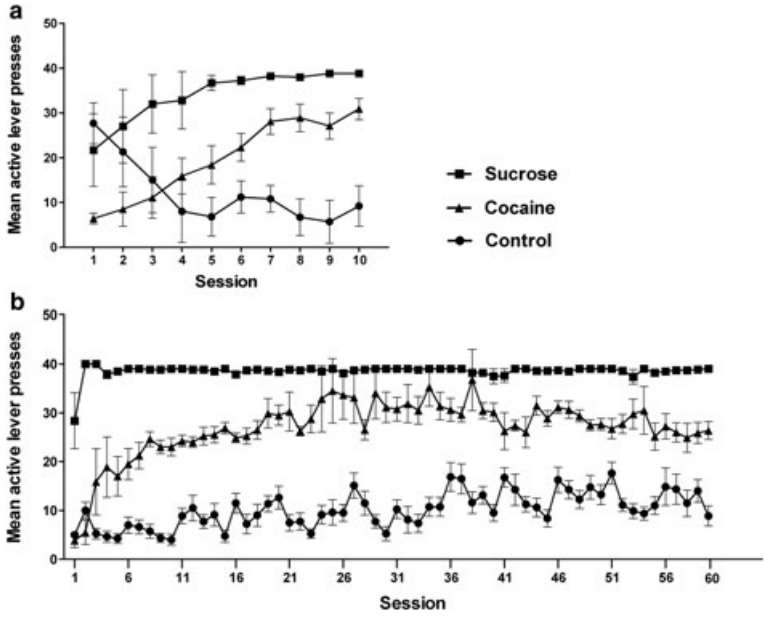

Figure 1 Short-term (10 days, A) versus long-term (60 days, B) self-administration of cocaine or sucrose. Data are presented as mean ± SEM number of active lever presses over animals per group

$t = -1.854$, $df = 7.00$, $p = 0.106$). In contrast, in the control group active lever presses showed a decrease between the first and the final session in the 10-day experiment (10 days: $t = 3.201$, $df = 10$, $p = 0.009$; 60 days: $t = -1.802$, $df = 14$, $p = 0.093$). Responses to the inactive lever in the cocaine and sucrose groups were below 7 per session from the third self-administration session onwards, in both experiments. The number of active lever presses was significantly higher than the response to the inactive lever in the cocaine [10 days: $F(1, 14) = 42.933$, $p < 0.001$; 60 days: $F(1, 14) = 407.68$, $p < 0.001$] and sucrose self-administration groups [10 days: $F(1, 10) = 123.739$, $p < 0.001$; 60 days: $F(1, 14) = 19305.395$, $p < 0.001$], but not in the control groups [10 days: $F(1, 10) = 3.564$, $p = 0.08$; 60 days: $F(1, 14) = 2.336$, $p = 0.149$].

Gene expression after cocaine or sucrose self-administration

Ventral striatum

In VS, a significant main effect of treatment was seen in the expression of 9 IEGs: *c-fos* [$F(2, 37) = 28.428$, $p < 0.001$], *Mkp1* [$F(2, 37) = 28.402$, $p < 0.001$], *Fosb/ΔFosb* [$F(2, 37) = 18.194$, $p < 0.001$], *Egr2* [$F(2, 37) = 23.405$, $p < 0.001$], *Arc* [$F(2, 37) = 7.086$, $p = 0.002$], *Egr4* [$F(2, 37) = 9.751$, $p < 0.001$], *Egr3* [$F(2, 37) = 7.882$, $p = 0.001$, *Jun* [$F(2, 37) = 3.457$, $p = 0.042$], and *Fosl1* [$F(2, 37] = 3.707$, $p = 0.034$ (Fig. 2A–2I) (for complete overview of all 17 IEGs refer to Table S2). Please note that the designation IEG for *Fosb/ΔFosb* and *Trkb* are used for ease of discussion; *Fosb/*Δ*Fosb* is a specific transcript, and *Trkb* was included because of its high affinity for BDNF). *Post hoc* tests showed that mRNA levels of five of these genes were significantly higher in the cocaine self-administration group (Con) than the sucrose self-administration (Suc) and control (Con) groups: *c-fos* (Coc versus Con/Suc, $p < 0.001$), *Mkp1* (Coc versus Con/Suc, $p < 0.001$), *Fosb/ΔFosb* (Coc versus Con/Suc, $p < 0.001$), *Egr2* (Coc versus Con/Suc, $p < 0.001$), *Arc* (Coc versus Con: $p = 0.001$, Coc versus Suc: $p = 0.006$) (Fig. 2A–2E). For *Egr4*, *Egr3*, *Jun, and Fosl1*, significant differences were only found between the cocaine self-administration and control groups (*Egr4*: $p = 0.001$, *Egr3*: $p = 0.001$, *Jun*: $p = 0.036$, *Fosl1*: $p = 0.034$) (Fig. 2F–2I).

A main effect of session (10 versus 60 days) was found for the expression of *Egr4* ($F(1, 37) = 6.383$, $p = 0.016$), *Ccnl1* ($F(1, 37) = 22.730$, $p < 0.001$), *Trkb* [$F(1, 37) = 8.469$, $p = 0.006$], *Egr1* [$F(1, 37) = 8.143$, $p = 0.007$], and *Rgs2* [$F(1, 37) = 7.099$, $p = 0.011$] (Figs 2F, 2J–2M).

For *Ccnl1* a significant interaction between session and treatment was found [$F(2, 37) = 5.929$, $p = 0.006$] (Fig. 2J). *Post hoc* tests showed that after 10 days of self-administration the *Ccnl1* mRNA levels in the cocaine group were significantly higher than in control ($p = 0.006$), but not higher than in the sucrose group ($p = 0.499$). The value in the sucrose group was not significantly different from control ($p = 0.08$). After 60 days, no significance was observed for any comparison among control, sucrose and cocaine groups (Coc versus Con: $p = 0.413$, Coc versus Suc: $p = 1$, Suc versus Con: $p = 0.413$) (Fig. 2J). In addition, for both cocaine and sucrose groups, significant differences were seen between 10 and 60 days of self-administration sessions (cocaine: $t = 6.16$, $df = 14$, $p < 0.001$; sucrose: $t = 4.918$, $df = 12$, $p < 0.001$) (Fig. 2J).

Dorsal striatum

In DS, increased expression of 6 IEGs was found after 10 or 60 days of cocaine self-administration. Two-way ANOVA revealed a significant main effect of treatment on the induction of *c-fos* [$F(2, 38) = 91.535$, $p < 0.001$], *Mkp1* [$F(2, 38) = 33.507$, $p < 0.001$], *Fosb/ΔFosb* [$F(2, 38) = 60.208$, $p < 0.001$], *Egr2* [$F(2, 38) = 42.690$, $p < 0.001$], *Arc* [$F(2, 38) = 14.260$, $p < 0.001$], and *Egr4* [$F(2, 38) = 18.323$, $p < 0.001$] (Fig. 3A–2F) (for complete overview of all 17 IEGs refer to Table S2). *Post hoc* tests showed that the mRNA levels of these 6 genes were significantly higher after cocaine self-administration than after sucrose self-administration and control (*c-fos*: Coc versus Con/Suc, $p < 0.001$, *Mkp1*: Coc versus Con/Suc, $p < 0.001$, *Fosb/ΔFosb*: Coc versus Con/Suc, $p < 0.001$, *Egr2*: Coc versus Con/Suc, $p < 0.001$, *Arc*: Coc versus Con/Suc, $p < 0.001$, *Egr4*: Coc versus Con/Suc, $p < 0.001$), whereas no differences were found between the sucrose and the control groups (*c-fos*: $p = 0.14$, *Mkp1*: $p = 0.466$, *Fosb/ΔFosb*: $p = 0.326$, *Egr2*: $p = 0.076$, *Arc*: $p = 0.75$, *Egr4*: $p = 0.234$) (Fig. 3A–3F).

For *Egr4*, a main effect of session (10 versus 60 days) was seen in DS [$F(1, 38) = 8.672$, $p = 0.006$] (Fig. 3F).

For *Fosb/*Δ*Fosb*, there was a significant interaction between session and treatment [$F(2, 38) = 3.651$, $p = 0.035$] (Fig. 3C). *Post hoc* tests showed that *Fosb/ΔFosb* mRNA levels in the cocaine group were significantly higher than those in the sucrose and control groups in both the 10 and 60 day experiments ($p < 0.001$ for all comparisons), but no differences were present between sucrose and control (10 days: $p = 0.951$, 60 days: $p = 0.489$) (Fig. 3C). In addition, significant differences were seen between 10- and 60-day self-administration in the cocaine groups ($t = 2.524$, $df = 14$, $p = 0.024$) but not in the sucrose groups ($t = -0.918$, $df = 12$, $p = 0.377$) (Fig. 3C).

Medial prefrontal cortex

In mPFC, two-way ANOVA revealed a main effect of treatment on the expression of 10 IEGs, i.e. *c-fos* [$F(2, 38) = 41.875$,

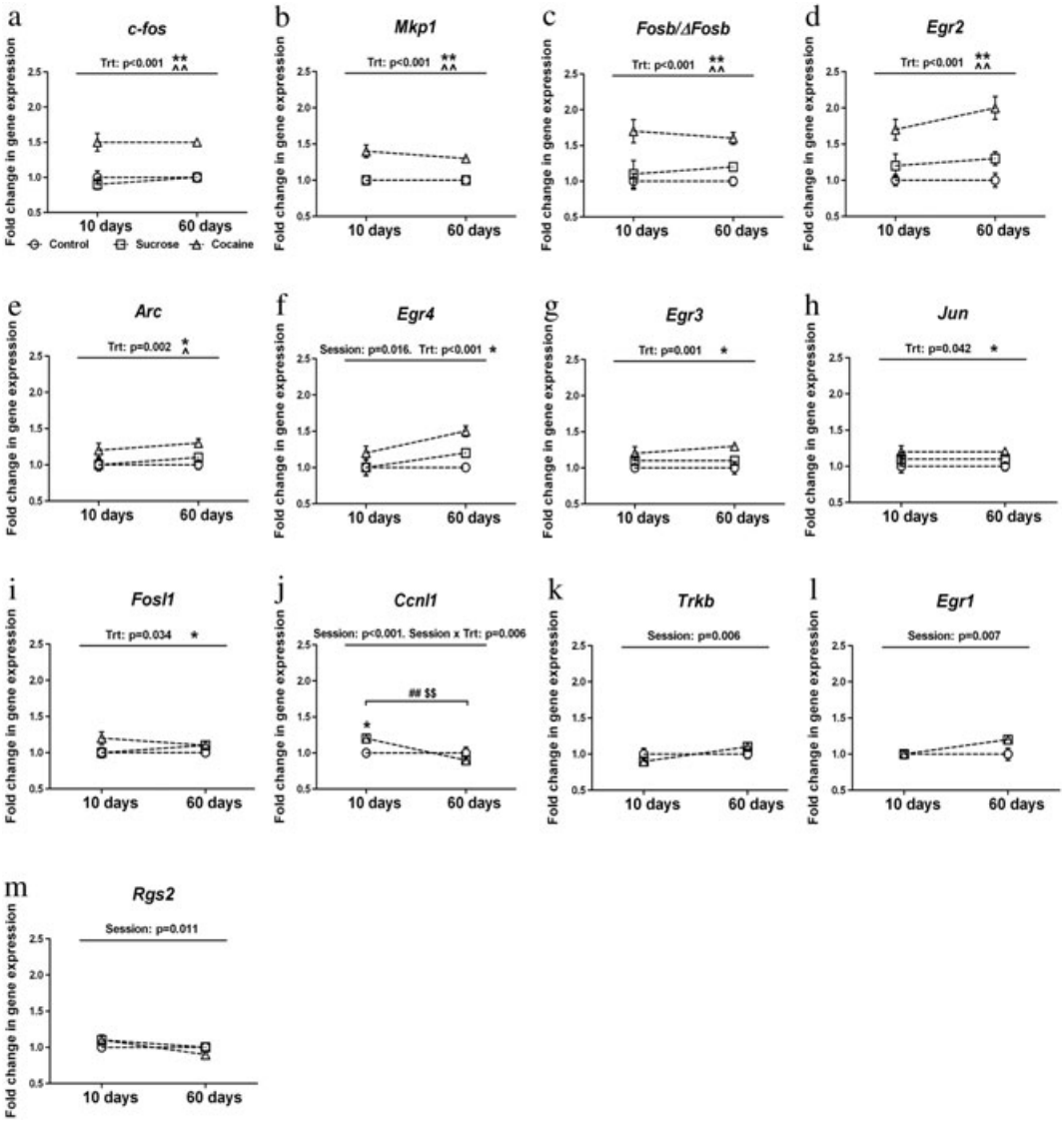

Figure 2 Effects of cocaine or sucrose self-administration on 13 IEGs in VS. Relative transcript expression levels of IEGs after 10- or 60-day self-administration as determined by quantitative RT-PCR. Data from the cocaine and sucrose groups were calculated as fold change in gene expression relative to the control group, and presented as mean ± SEM. *P* values are shown if the effects of treatment (Trt), session (Session) or the interaction (Session x Trt) were significant in two-way ANOVA. Symbols refer to significant differences between experimental groups after *post hoc* testing: *, cocaine and control groups, $*p < 0.05$, $**p < 0.001$; ^, cocaine and sucrose groups, ^ $p < 0.05$, ^^$p < 0.001$; $^{\#}$, 10 and 60 days cocaine groups, $^{\#}p < 0.05$, $^{\#\#}p < 0.001$; $^{\$}$, 10 and 60 days sucrose groups, $^{\$}p < 0.05$, $^{\$\$}p < 0.05$

$p < 0.001$], *Mkp1* [$F(2, 38) = 44.508$, $p < 0.001$], *Egr2* [$F(2, 38) = 39.807$, $p < 0.001$], *Fosb*/Δ*Fosb* [$F(2, 38) = 42.594$, $p < 0.001$], *Arc* [$F(2, 38) = 10.027$, $p < 0.001$], *Egr4* [$F(2, 38) = 8.144$ $p = 0.001$], *Bdnf* [$F(2, 38) = 6.102$, $p = 0.005$], *Homer1* [$F(2, 38) = 8.881$, $p < 0.001$], *Sgk1* [$F(2, 38) = 5.387$, $p = 0.009$], and *Rgs2* [$F(2, 38) = 5.385$, $p = 0.009$] (Fig. 4A–4J) (for complete overview of all 17 IEGs, refer to Table S2). *Post hoc* tests showed that cocaine self-administration, compared with sucrose self-administration and control, significantly enhanced the mRNA levels of *c-fos* (Coc versus Con/Suc, $p < 0.001$), *Mkp1* (Coc versus Con/Suc, $p < 0.001$), *Fosb*/Δ*Fosb* (Coc versus Con/Suc, $p < 0.001$), *Egr2* (Coc versus Con/Suc, $p < 0.001$), *Arc* (Coc versus Con, $p = 0.001$; Coc versus Suc, $p = 0.003$), and *Egr4* (Coc versus Con, $p = 0.005$; Coc versus Suc, $p = 0.002$) (Fig. 4A–4F). Both *Egr2* and *Fosb*/Δ*Fosb* were significantly up-regulated by sucrose self-administration in comparison to the control group (*Egr2*: $p = 0.018$, *Fosb*/Δ*Fosb*: $p = 0.012$) (Fig. 4C and 4D). For *Bdnf* and *Homer1*, cocaine and sucrose self-administration significantly increased mRNA levels compared with the control group (*Bdnf*: Coc versus Con, $p = 0.009$, Suc versus Con, $p = 0.008$; *Homer1*: Coc versus Con, $p < 0.001$, Suc versus Con, $p = 0.001$, Fig. 4G and 4H), but there was no significant difference between the cocaine and the sucrose self-administration

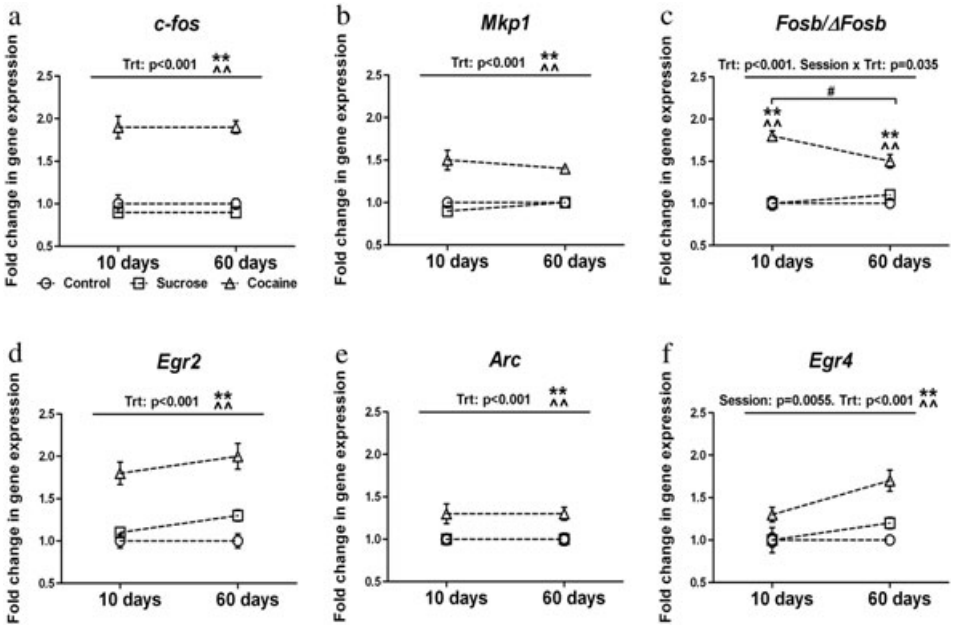

Figure 3 Effects of cocaine or sucrose self-administration on six IEGs in dorsal striatum. Relative transcript expression levels of IEGs after 10- or 60-day self-administration as determined by quantitative RT-PCR. Data from the cocaine and sucrose groups were calculated as fold change in gene expression relative to the control group, and presented as mean ± SEM. *P* values are shown if the effects of treatment (Trt), session (Session), or the interaction (Session x Trt) were significant in two-way ANOVA. Symbols refer to significant differences between experimental groups after *post hoc* testing: *,cocaine and control groups, *$p < 0.05$, **$p < 0.001$; ^, cocaine and sucrose groups, ^$p < 0.05$, ^^$p < 0.001$; $^{\#}$, 10 and 60 day cocaine groups, $^{\#}p < 0.05$

groups. For *Sgk1*, significant differences were seen only between the cocaine and control groups ($p = 0.007$) (Fig. 4I). Interestingly, ISH for *Sgk1* demonstrated labelled cells not only in cortical and striatal gray matter but also in corpus callosum. Small-sized cells in white and gray matter—probably oligodendrocytes—were more heavily labelled than larger cells in gray matter (Fig. S1). For *Rgs2*, significant differences were seen only between the cocaine and sucrose groups ($p = 0.01$) (Fig. 4J).

A significant main effect of session was seen on the expression of *Egr2* [$F(1, 38) = 22.088$, $p < 0.001$], *Fosb/ΔFosb* [$F(1, 38) = 7.725$, $p = 0.008$], *Arc* [$F(1, 38) = 5.445$, $p = 0.025$], *Bdnf* [$F(1, 38) = 4.154$, $p = 0.049$], *Homer1* [$F(1, 38) = 11.321$, $p = 0.002$], *Jun* [$F(1, 38) = 21.460$, $p < 0.001$], *Trkb* [$F(1, 38) = 21.989$, $p < 0.001$], *Egr3* [$F(1, 38) = 10.727$, $p = 0.002$], *Dclk1* [$F(1, 38) = 10.757$, $p = 0.002$], *Ccnl1* [$F(1, 38) = 5.822$, $p = 0.021$] and *Egr1* [$F(1, 38) = 4.717$, $p = 0.036$] (Fig. 4C–4E, 4G–4H, and 4K–4P).

A significant interaction between session and treatment was observed for the expression of *Egr2* [$F(2, 38) = 8.457$, $p = 0.001$], *Jun* [$F(2, 38) = 11.343$, $p < 0.001$] and *Trkb* [$F(2, 38) = 5.504$, $p = 0.008$] (Fig. 4C, 4K, and 4L). *Post hoc* tests showed that the *Egr2* mRNA levels in the cocaine self-administration group were significantly higher than in the sucrose self-administration ($p = 0.001$) and control groups ($p = 0.005$) in the 10-day experiment. After 60 days, the *Egr2* mRNA levels in the cocaine self-administration group still significantly differed from the other two groups (Coc versus Con/Suc, $p < 0.001$), but also a significant increase in *Egr2* expression was seen in the sucrose self-administration group compared with the control group ($p = 0.033$). Furthermore, the *Egr2* up-regulation after 60 days of cocaine or sucrose self-administration was significantly greater than that after 10 days (cocaine: $t = -4.568$, $df = 14$, $p < 0.001$; sucrose: $t = -2.619$, $df = 12$, $p = 0.022$) (Fig. 4C). For *Jun*, the mRNA levels in the cocaine self-administration group were significantly higher than in the sucrose self-administration group after 10 days (Coc versus Suc: $p = 0.022$; Coc versus Con: $p = 0.054$). In contrast, after 60 days, the expression of *Jun* in the cocaine or sucrose self-administration groups was significantly lower than in the control group (Coc versus Con: $p = 0.001$, Suc versus Con: $p = 0.039$). In addition, the induction of *Jun* mRNA after 60 days of cocaine and sucrose self-administration was significantly lower than after 10 days (cocaine: $t = 6.566$, $df = 14$, $p < 0.001$; sucrose: $t = 3.454$, $df = 12$, $p = 0.005$) (Fig. 4K). For *Trkb*, no significant differences were observed between groups after 10-day administration (Coc versus Con: $p = 0.858$, Con versus Suc: $p = 0.889$, Suc versus Con: $p = 0.630$), while after 60 days, the mRNA levels in both cocaine and sucrose groups were significantly higher than in control (Coc versus Con: $p = 0.001$, Suc versus Con: $p = 0.029$). No difference was seen between cocaine and sucrose

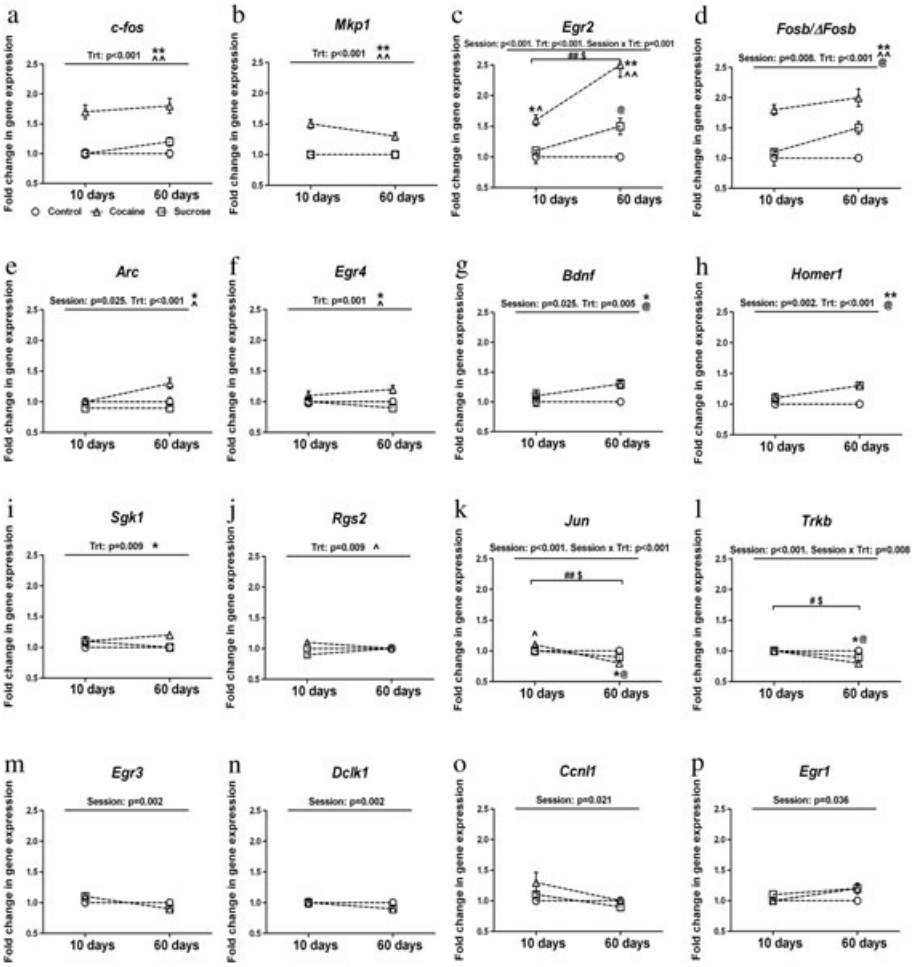

Figure 4 Effects of cocaine or sucrose self-administration on 16 IEGs in medial prefrontal cortex. Relative transcript expression levels of IEGs after 10- or 60-day self-administration as determined by quantitative RT-PCR. Data from the cocaine and the sucrose groups were calculated as fold change in gene expression relative to the control group, and presented as mean ± SEM. P values are shown if the effects of treatment (Trt), session (Session), or the interaction (Session x Trt) were significant in two-way ANOVA. Symbols refer to significant differences between experimental groups after *post hoc* testing: *, cocaine and control groups, $*p < 0.05$, $**p < 0.001$; ^, cocaine and sucrose groups, $^p < 0.05$, $^^p < 0.001$; $^{@}$, sucrose and control groups, $^{@}p < 0.05$; $^{\#}$, 10 and 60 day cocaine groups, $^{\#}p < 0.05$, $^{\#\#}p < 0.001$; $^{\$}$, 10 and 60 day sucrose groups, $^{\$}p < 0.05$

self-administration ($p = 0.339$). In addition, for both the cocaine and sucrose groups, *Trkb* mRNA levels after 60 days of self-administration were significantly different from those after 10 days (cocaine: $t = 3.558$, $df = 14$, $p = 0.003$; sucrose: $t = 3.991$, $df = 12$, $p = 0.002$) (Fig. 4L).

Histological validation for representative IEGs

ISH was performed in order to confirm the effects of cocaine self-administration that had been established with RT-PCR. *c-fos* and *Bdnf* were selected as two representative genes for ISH, because the RT-PCR data showed a strong increase of *c-fos* in both striatum and mPFC, whereas up-regulation of *Bdnf* was only observed in mPFC.

For *c-fos*, visual inspection showed a high signal in animals that had self-administered cocaine for either 10 or 60 days with a large amount of *c-fos* positive cells in striatum and a vast accumulation of cells particularly in the dorsomedial striatum compared with the control animals (compare Fig. 5A with 5B, and 5C with 5D). *c-fos* positive cells had a label-free nucleus and darkly labelled cytoplasm that clearly differed in labelling intensity between the cocaine self-administration (Fig. 5F) and control groups (Fig. 5E).

Two-way ANOVA indicated a significant main effect of treatment on the total amount of *c-fos* ISH signal in VS, DS, and mPFC [VS: $F(2, 41) = 24.876$, $p < 0.001$, DS: $F(2, 41) = 29.973$, $p < 0.001$, mPFC: $F(2, 41) = 25.482$, $p < 0.001$]. A main effect of session was seen in VS, DS, and mPFC [VS: $F(1, 41) = 10.065$, $p = 0.003$, DS: $F(1, 41) = 8.544$, $p = 0.006$, mPFC: $F(1, 41) = 22.383$, $p < 0.001$]. There was a significant interaction between session and treatment in all three regions [VS: $F(2, 41) = 10.486$, $p < 0.001$, DS: $F(2, 41) = 6.065$, $p = 0.005$,

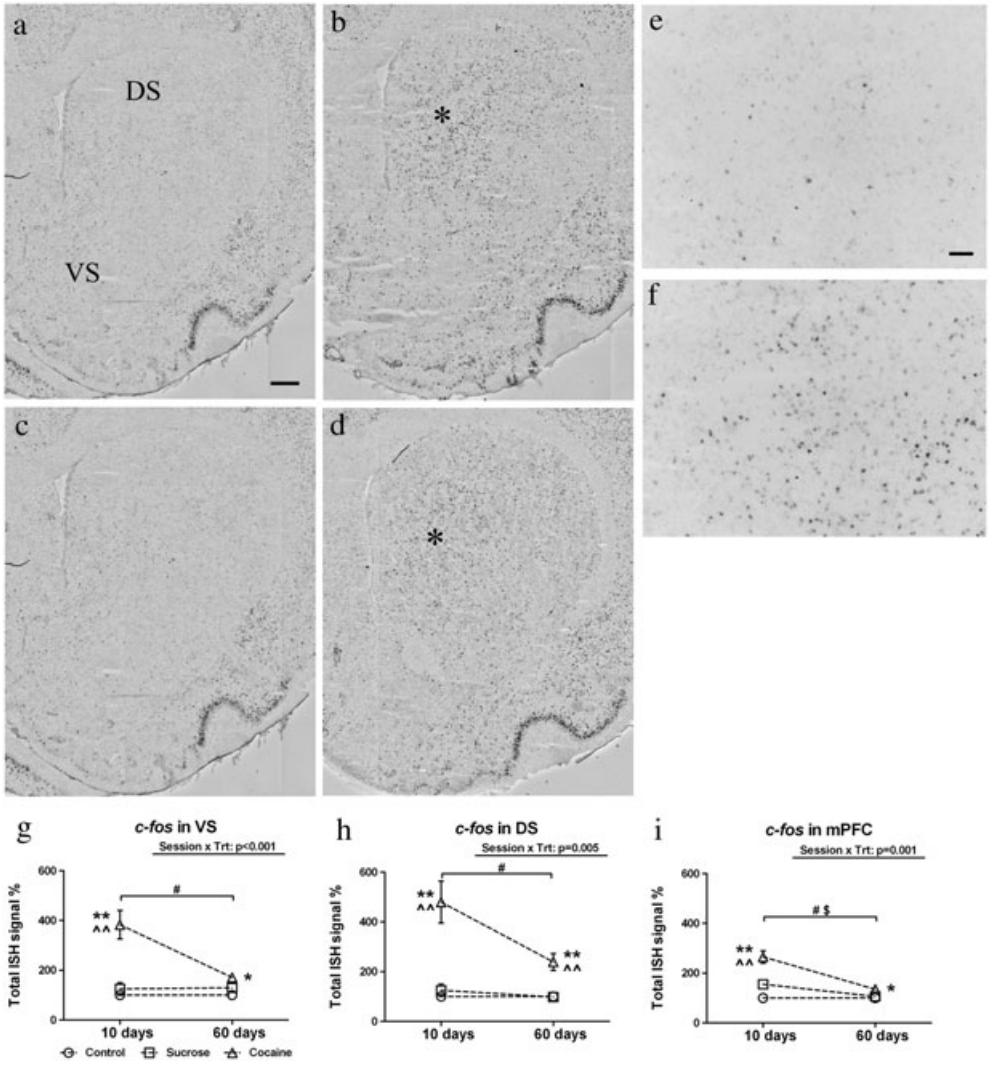

Figure 5 *C-fos* transcript in striatum and mPFC. Representative micrographs of hybridized coronal sections through striatum (Bregma +1.2) showing distribution of *c-fos* mRNA-positive cells after 10-day (A, control; B, cocaine) and 60-day self-administration (C, control; D, cocaine). Asterisks in B and D indicate areas with high cell density in dorsomedial striatum. At high magnification (E, control; F, cocaine, 10-day self-administration), differences in number and individual labelling intensity of the striatal neurons can be seen. Quantification of *c-fos* probe hybridization (total ISH signal) in VS (G), DS (H), and mPFC (I) is expressed as percentage of control. Values are presented as mean ± SEM after normalizing to the control group. *P* values refer to two-way ANOVA, symbols refer to significant differences between groups after *post hoc* testing: *, cocaine and control groups, $^{*}p < 0.05$, $^{**}p < 0.001$; ^, cocaine and sucrose groups, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.001$; $^{\#}$, 10 and 60 day cocaine groups, $^{\#}p < 0.05$; $^{\$}$, 10 and 60 day sucrose groups, $^{\$}p < 0.05$. Scale bar: 500 μm in A–D, 100 μm in E–F

mPFC: $F(2, 41) = 8.213$, $p = 0.001$] (Fig. 5G–5I). *Post hoc* tests revealed that after 10 days, *c-fos* mRNA expression was significantly increased in the cocaine self-administration group compared with the sucrose self-administration and the control group in three brain regions (VS: $p < 0.001$, DS: $p < 0.001$, mPFC: $p < 0.001$; Fig. 5G–5I). After 60 days, significant differences between the cocaine self-administration and other two groups were only seen in DS ($p < 0.001$; Fig. 5H). In VS and mPFC, *c-fos* mRNA expression in the cocaine self-administration group was significantly higher than in the control group (VS: $p = 0.004$, mPFC: $p = 0.041$), but not different from the sucrose self-administration group (VS: $p = 0.097$, mPFC: $p = 0.089$) (Fig. 5G and 5I). In addition, *c-fos* mRNA after 60 days of cocaine self-administration was significantly lower than that after 10 days in VS, DS, and mPFC (VS: $t = 3.834$, $df = 7.897$, $p = 0.005$; DS: $t = 2.66$, $df = 13$, $p = 0.02$; mPFC: $t = 4.355$, $df = 10.775$, $p = 0.001$). In contrast, only in mPFC were *c-fos* mRNA levels significantly higher after 60 days of sucrose self-administration compared with 10 days ($t = 3.028$, $df = 14$, $p = 0.009$) (Fig. 5G–5I).

Bdnf ISH signal was increased in mPFC after cocaine self-administration (Fig. 6A–6F). Two-way ANOVA indicated a significant main effect of treatment [F (2, 41) = 26.521, $p < 0.001$] and a main effect of session

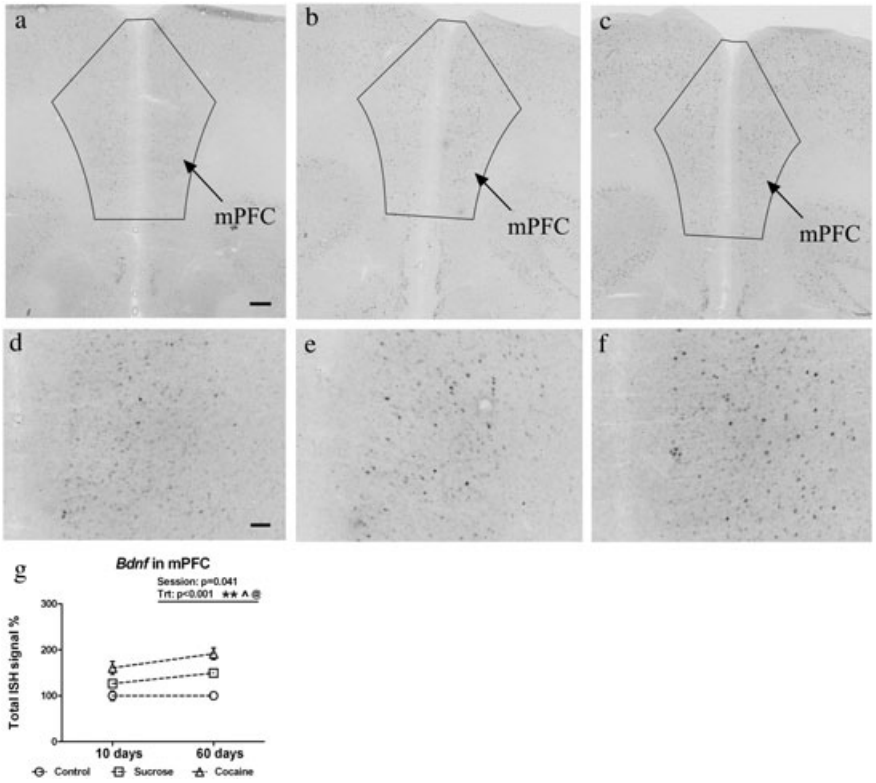

Figure 6 *Bdnf* transcript in mPFC. Representative micrographs of hybridized coronal sections through mPFC (Bregma +2.8) showing distribution of *Bdnf* mRNA-positive cells after 60-day self-administration (A, control; B, sucrose; C, cocaine). At high magnification (D, Control; E, Sucrose; F, Cocaine), differences in number and staining intensity of cortical neurons can be seen. In (G), quantification of *Bdnf* probe hybridization (total ISH signal) in mPFC is expressed as percentage of control. Values are presented as mean ± SEM after normalizing to the control group. P values refer to two-way ANOVA, symbols refer to significant differences between experimental groups in *post hoc* testing: *, cocaine and control groups, $*p < 0.05$, $**p < 0.001$; ^, cocaine and sucrose groups, $^p < 0.05$; $^{@}$, sucrose and control groups, $^{@}p < 0.05$. Scale bar: 400 μm in A–C, 100 μm in D–F

[F (1, 41) = 4.457, $p = 0.041$] on the total amount of *Bdnf* ISH signal in mPFC. However, the interaction between treatment and session was not significant [F (2, 41) = 1.186, $p = 0.316$] (Fig. 6G). *Post hoc* tests on the treatment effects revealed that the *Bdnf* mRNA level in the cocaine self-administration group was significantly higher than in the control ($p < 0.001$) and sucrose groups ($p = 0.001$). The *Bdnf* mRNA level in the sucrose self-administration group was significantly higher than in the control group ($p = 0.001$) (Fig. 6G).

DISCUSSION

In the present study, we demonstrate profound changes after cocaine self-administration in the expression of a subset of IEGs from a panel of 17 that was largely the same in striatum and mPFC. Comparison of the effects of short- versus long-term cocaine self-administration showed no major differences in the IEG expression profiles, except for two IEGs: *Egr2* in mPFC and *Fosb*/*ΔFosb* in DS. In addition, in the mPFC, induction of certain IEGs was also observed after long-term sucrose self-administration.

Neuronal reactivity in striatum and prefrontal cortex

A group of six IEGs (*c-fos*, *Mkp1*, *Fosb*/*ΔFosb*, *Egr2*, *Arc* and *Egr4*) was found to increase expression in DS as a corollary of cocaine self-administration. In VS, an identical response was established for these genes (except for *Egr4*), with an additional set of four IEGs (*Egr3*, *Egr4*, *Jun* and *Fosl1*) showing significant differences between cocaine self-administration and control but not between exposure to the drug and the natural reinforcer. For *c-fos*, *Fosb*/Δ*Fosb* or *Arc* in striatum, similar changes have been observed after cocaine self-administration (Fumagalli *et al.* 2009; Larson *et al.* 2010; Zahm *et al.* 2010). The response to cocaine self-administration in *Mkp1*, *Egr2* and *Egr4* expression—genes that are involved in transcriptional regulation—is a novel finding, however. The induction of IEGs acting as transcription factors or effectors in neuronal networks is considered to mediate the neuroplastic changes that take place as a consequence of repeated drug use (Lüscher & Malenka 2011; Robison & Nestler 2011). Interestingly, in the present study, the group of IEGs with increased reactivity specifically to cocaine self-administration was the same in DS and VS. These striatal

regions are thought to be involved in different aspects of drug seeking behaviour (Everitt & Robbins 2005; Pierce & Vanderschuren 2010), but the present findings suggest that cocaine self-administration-induced neuroplasticity in these two striatal sectors is affected in a similar fashion (refer also Besson *et al.* 2013; Coffey *et al.* 2015).

In mPFC, along with the same set of genes that was up-regulated in striatum, *Bdnf*, *Homer1*, *Sgk1* and *Rgs2* were found to respond to cocaine self-administration. Drug-induced changes in the expression of *Rgs2* have hitherto been described in cortex and VTA (Burchett *et al.* 1999; Kuntz-Melcavage *et al.* 2009; Hearing, Zink, & Wickman 2012). For *Sgk1*, the upregulation response appeared to be present in both neurons and glial cells. Because *Sgk1* has been identified in oligodendrocytes (Miyata *et al.* 2011), the present findings might be of relevance for the white matter abnormalities detected in cocaine-dependent subjects (O'Neill, Cardenas, & Meyerhoff 2001; Moeller *et al.* 2005; Ma *et al.* 2009). For *c-fos* and *Bdnf*, similar results as in the present study have been previously reported after short-term cocaine self-administration (Zahm *et al.* 2010; Fumagalli *et al.* 2013). Both BDNF and Homer1 are thought to be involved in cortico-striatal glutamatergic neurotransmission (Berglind *et al.* 2009; Ary *et al.* 2013), while BDNF and all members of the group of six IEGs that was upregulated after cocaine self-administration in striatum and mPFC are known to play a role in regulatory networks subserving synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Messaoudi *et al.* 1998; Messaoudi *et al.* 2002; Coba *et al.* 2008; McGinty, Whitfield, & Berglind 2010). Effects of cocaine exposure on LTP as well as LTD have been described (for review, see Wolf (2010)). There is evidence to suggest blunted induction of LTP and LTD in the VS after cocaine self-administration (Moussawi *et al.* 2009; Kasanetz *et al.* 2010), which might be related to the transcript upregulation observed in the present study. Furthermore, in PFC impairment of LTD after cocaine self-administration has been found that might facilitate induction of LTP (Kasanetz *et al.* 2013). In conclusion, the fact that largely the same subset of IEGs as in striatum was affected in mPFC suggests considerable similarites between the neuroplastic adaptations in the two brain regions to cocaine self-administration. In addition, the upregulation of four additional IEGs in mPFC points to cortex-specific responses.

Effects of cocaine and sucrose self-administration

Four IEGs, namely, *Bdnf*, *Homer1*, *Fosb*/Δ*Fosb*, and *Egr2*, were found to respond both to cocaine and to sucrose self-administration, and these responses were restricted to the mPFC. For *Egr2*, this is a novel finding. With respect to *Bdnf* and *Homer1*, the observed changes may point to altered corticostriatal excitatory neurotransmission, as noted in the preceding texts. Because the sucrose-associated effects on these IEGs were not significantly different from those induced by cocaine self-administration, it is reasonable to assume that *Bdnf* and *Homer1* in mPFC are involved in neuronal plasticity induced by both natural and drug rewards. The same may hold true for *Fosb*/*ΔFosb* (Pitchers *et al.* 2010).

The present results demonstrate that self-administration of cocaine altered IEG expression to a far greater extent than did sucrose. After cocaine, a greater number of IEGs were upregulated and two (*Egr2* and *Fosb*/Δ*Fosb*) of the four (*Egr2*, *Fosb*/*ΔFosb*, *Bdnf* and *Homer1*) IEGs that were affected by self-administration of both cocaine and sucrose show a higher response to the drug. These findings indicate that exposure to a substance of abuse in reward-dependent learning has a greater effect on IEG-associated neuroadaptations than the instrumental learning process *per se*. This is noteworthy for mPFC as well as DS and VS, which have all been implicated in instrumental conditioning (Kelley *et al.* 2003; Yin *et al.* 2005). In interpreting the present data, it is important, however, to note that the IEG responses were brought about by an acute 'pharmacological' effect of cocaine (and sucrose, for that matter) together with long(er)-term substance-induced neuroadaptive changes. The choice to include cocaine and sucrose reward in the final self-administration session was deliberate, because (in our view) it is not quite possible to disentangle the effects of instrumental conditioning and cocaine exposure. In fact, omitting the reward in the final session would render it an extinction session, which likely impacts by itself on cortical and striatal processing (Self *et al.* 2004; Peters, Kalivas, & Quirk 2009). Similarly, the use of a yoked control would have led to unwanted effects. Considerable differences have been reported between IEG responses to drug self-administration or yoked administration. (Kuntz-Melcavage *et al.* 2009; Larson *et al.* 2010; Zahm *et al.* 2010; Fumagalli *et al.* 2013; Radley *et al.* 2015). However, these differences do not allow us to dissect out a substance effect.

Stable cocaine self-administration behaviour and IEG responses

The IEG up-regulation patterns in mPFC and striatum did not markedly differ after short and long periods of cocaine self-administration except for *Egr2* and *Fosb*/Δ*Fosb*. *Egr2* in mPFC was the only gene with a significantly different, i.e. augmented, response to cocaine self-administration after long-term compared with short-term exposure. This suggests that *Egr2* up-regulation might be related to loss of cognitive control over drug seeking and taking, possibly leading to compulsive behaviour (refer in the succeeding

texts for further discussion of behavioural findings). As outlined in the preceding texts, the increased expression of *Egr2* might implicate synaptic plasticity changes. A role for *Egr2* in stabilization of LTP has indeed been suggested (Williams *et al.* 1995). With respect to *Fosb*/Δ*Fosb*, an increase of significantly smaller magnitude was seen after extended compared with short-term cocaine exposure. A similar blunting of effect on *Fosb*/*ΔFosb* mRNA levels has been reported after chronic self-administration of cocaine, possibly as a consequence of tolerance induced by persistently high *Fosb*/Δ*Fosb* protein levels (Larson *et al.* 2010). Such changes will affect AP-1 binding activity and gene expression in DS, which might be implicated in the (hypothesized) intensified functional engagement of DS as the development of addiction behaviour progresses (Larson *et al.* 2010).

Other than for *Egr2* and *Fosb*/Δ*Fosb*, in all three brain regions, the increases in gene expression that were seen after 10 days of self-administration were maintained after 60 days. In this respect, it should be noted that in contrast to the RT-PCR analysis, the ISH experiments did show differences between the responses of *c-fos* and *Bdnf* after 10 and 60 days. This discrepancy might be caused by the fact that RT-PCR analysis was performed on much bigger portions of cortex and striatum than ISH; alternatively, it might indicate higher sensitivity of the ISH technique compared with RT-PCR. Using RT-PCR, no exposure time-dependent differences were detected in VS or DS, which is largely consistent with the findings of Besson *et al.* (2013) who reported no major differences between the expression of *Egr1* in VS, DS and mPFC after short-term (10 days) or long-term (50 days) cocaine self-administration. By contrast, Porrino *et al.* (2004b) showed that five sessions of cocaine self-administration reduced glucose utilization in the monkey VS and limited portions of the DS, whereas the effects after extended self-administration (up to 100 days) had intensified and spread to the most dorsal part of the striatum. At least two, not mutually exclusive explanations for this discrepancy can be put forward. It might be that changes in IEG expression levels are restricted to subregions of the brain areas that were dissected in the present RT-PCR experiments. For instance, the present results of the c-fos ISH experiment showed stronger responses in posterior dorsomedial striatum after both short-term and long-term cocaine self-administration suggesting (sub)regional differentiation of effects. A further methodological issue concerns the fact that the cocaine self-administration regimens in our study and that of Porrino *et al.* (2004b) were not the same. In the latter study, animals obtained a maximum number of cocaine infusions allowed in each session for 100 days. In contrast, in the present study cocaine intake was stable yet never reached maximum preset levels. This may be of consequence for the various (re)activity patterns observed, and the similarity of the IEG responses after 10 and 60 days of cocaine self-administration in the present study probably reflects the stable cocaine self-administration. Be that as it may, the total amount of cocaine intake was vastly different between the 10 and 60 days cocaine-exposed animals (averages of 49.5 and 405.5 mg/kg, respectively), and it is reasonable to expect that this would have resulted in major differences in molecular plasticity (Taylor & Jentsch 2001; Koob & Le Moal 2005; Lüscher & Malenka 2011; Robison & Nestler 2011). The fact that such differences were not observed as a function of prolonged cocaine self-administration indicates that at this level of analysis, stable long-term cocaine taking evokes stable responses in neural reactivity.

We, and others, have previously shown that prolonged exposure to self-administered cocaine results in compulsive, addiction-like patterns of behaviour (Deroche-Gamonet *et al.* 2004; Vanderschuren & Everitt 2004; Pelloux, Everitt, & Dickinson 2007; Limpens *et al.* 2014b). Recent studies have implicated functional changes in VS (Kasanetz *et al.* 2010; Bock *et al.* 2013), DS (Zapata *et al.* 2010; Jonkman *et al.* 2012a) and mPFC (Chen *et al.* 2013; Kasanetz *et al.* 2013; Limpens *et al.* 2014a) in this behaviour. In view of these findings, it is remarkable that we observed no major differences in IEG responsivity patterns in these brain regions between animals with 10 and 60 days of cocaine self-administration experience. Indeed, it has been shown that it is extended drug exposure that evokes compulsive cocaine seeking (Jonkman, Pelloux, & Everitt 2012b), albeit that the development of addiction-like changes in behaviour does not seem to be directly related to the absolute amount of self-administered drug (Deroche-Gamonet *et al.* 2004; Belin *et al.* 2009). Consistent with our findings, a recent study found no major differences in *Egr1* expression in VS, DS and mPFC in animals with 10 and 50 days of cocaine self-administration experience (Besson *et al.* 2013), suggesting that the development of compulsive cocaine seeking is not mirrored by changes in cellular reactivity to the drug. At first glance, the resemblance of the IEG responses after 10 and 60 days of cocaine self-administration suggests a similarity in behavioural drive, i.e. controlled responding continuing for up to 60 days. The resemblance, however, might be deceptive if we take into account that a consistently increased reactivity was not readily foreseeable on basis of the literature (McCoy *et al.* 2011). For instance, for the Fos IEG family it has been shown that extended cocaine self-administration (2–3 weeks) in comparison to acute exposure leads to a reduced response and not to a persistent increase (Larson *et al.* 2010; Zahm *et al.* 2010). Possibly, it is the sustained reactivity and not necessarily a further sensitization of responsivity that provides the basis for

neuroadaptive changes related to the development of compulsive cocaine use.

In conclusion, our findings demonstrate cocaine self-administration-induced changes in a similar subset of IEGs in mPFC, DS and VS. This response does not build up or dwindle over time in any of the three anatomical regions for up to 60 days of continued drug taking. Thus, the steady changes in IEG expression are associated with stable self-administration behaviour rather than the total dosage of cocaine obtained. Over this protracted period of drug taking, regulatory impulses to the IEGs are sustained, which might be associated with plastic changes in neural networks underlying compulsive drug seeking and taking.

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Authors Contribution

PV and LJMJV were responsible for the study concept and design. JHWL contributed to the acquisition of behavioural data. PG performed the RT-PCR and ISH experiments and data analysis. SS assisted with RT-PCT experiment and data analysis. PV, LJMJV and PG participated in data interpretation. PG drafted the manuscript. PV, LJMJV and SS provided critical revision of the manuscript. All authors critically reviewed content and approved final version for publication.

Abbreviations

IEGs immediate early genes
DS dorsal striatum
VS ventral striatum
mPFC medial prefrontal cortex
RT-PCR real-time PCR
ISH *in situ* hybridization

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used in the RT-PCR experiment.

Table S2. Summary of relative transcript expression levels of 17 IEGs after 10 or 60 days self-administration. Fold changes in the cocaine and sucrose groups were calculated relative to the control group and presented as mean ± SEM. Bold typing indicates significant changes. * indicates significant p level (< 0.05). DS, dorsal striatum; VS, ventral striatum; mPFC, medial prefrontal cortex.

Figure S3. *Sgk1* transcript in gray and white matter visualized with ISH. Representative micrographs of hybridized coronal sections including cortex, corpus callosum and striatum (Bregma +1.2) show *Sgk1* mRNA-positive cells after 60 days of cocaine self-administration. (A) Arrows point to small-sized, heavily labeled cells. (B) At high magnification, large (arrow head) –and small-sized (arrow) types of cells can be identified. Scale bars: 100 μm in A, 50 μm in B.