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RESEARCH PAPER

Pharmacological recruitment of the GABAergic tail of the ventral tegmental area by acute drug exposure

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BACKGROUND AND PURPOSE
The tail of the ventral tegmental area (tVTA), also called the rostromedial tegmental nucleus, is a newly defined brain structure and a potential control centre for dopaminergic activity. It was identified by the induction of DeltaFosB following chronic cocaine exposure. In this work, we screened 20 drugs for their ability to induce FosB/DeltaFosB in the tVTA.

EXPERIMENTAL APPROACH
Immunohistochemistry following systemic drug administration was used to study FosB/DeltaFosB induction in the tVTA of adult rats. Double-staining was used to determine whether dopamine or GABA neurones are involved in this induction.

KEY RESULTS
The acute injection of the psychostimulant drugs cocaine, D-amphetamine, (+/–)-3,4-methylenedioxymethamphetamine (MDMA), methylphenidate or caffeine, induced the expression of FosB/DeltaFosB in the tVTA GABAergic cells. No induction was observed following exposure to ethanol, diazepam, γ-hydroxybutyric acid (GHB), morphine, ketamine, phencyclidine (PCP), Δ^9-tetrahydrocannabinol (THC), sodium valproic acid or gabapentin. To evaluate the role of monoamine transporters in the psychostimulant-induced expression of FosB/DeltaFosB, we tested the antidepressant drugs reboxetine, nortriptyline, fluoxetine and venlafaxine (which target the noradrenaline and/or the 5-hydroxytryptamine transporters), the 5-hydroxytryptamine releasing agent dexfenfluramine, and the dopamine transporter inhibitor GBR12909. Only GBR12909 was able to induce FosB/DeltaFosB expression in the tVTA, showing that this induction is mediated by dopamine.

CONCLUSIONS AND IMPLICATIONS
Newly described brain structures may help to increase our knowledge of brain function, pathology and targets for treatments. FosB/DeltaFosB induction in the tVTA is a common feature of drugs sharing psychostimulant properties but not of drugs sharing risk of abuse.

Abbreviations
DAT, dopamine transporter; GABA, γ-aminobutyric acid; GAD, glutamatic acid decarboxylase; GHB, γ-hydroxybutyric acid; i.p., intraperitoneal; MDMA, (±)-3,4-methylenedioxymethamphetamine; NET, noradrenaline transporter; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; RMTg, rostromedial tegmental nucleus; s.c., subcutaneous; SERT, 5-hydroxytryptamine transporter; TH, tyrosine hydroxylase; THC, Δ^9-tetrahydrocannabinol; tVTA, tail of the VTA; VTA, ventral tegmental area

Introduction

Newly discovered brain structures may help to improve our knowledge of brain functions and of brain disorders, and may provide new neuroanatomical targets for treatments. The tail of the ventral tegmental area (tVTA) is a recently defined structure (Perrotti *et al.*, 2005; Kaufling *et al.*, 2009; 2010),

which was discovered simultaneously by different research groups. This region is of interest as it may be a potential control centre for the dopaminergic system (Jhou *et al.*, 2009b; Kaufling *et al.*, 2010). In the literature, it is mainly referred to either as the tVTA (Perrotti *et al.*, 2005; Kaufling *et al.*, 2009; 2010) or as the rostromedial tegmental nucleus (Jhou *et al.*, 2009a,b). However, parts of this structure can be found under various other names: 'retro-VTA' (Scammell *et al.*, 2000), 'Area 2' in the description of GABAergic cell clusters within the VTA (Olson and Nestler, 2007), 'ventral tegmental tail' (Ikemoto, 2007), 'interstitial nucleus of the decussation of the superior cerebellar peduncle' for its most caudal extent (Paxinos and Watson, 2007), 'rostromedial pontine tegmentum' (Geisler *et al.*, 2008) or 'caudal pole of the VTA' (Ferreira *et al.*, 2008).

The boundaries and the connections of the tVTA have been recently described (Jhou *et al.*, 2009a; Kaufling *et al.*, 2009; 2010) (Supporting Information Figure S1). The tVTA was initially revealed by the accumulation of the transcription factor ΔFosB, a stable truncated variant of FosB (Nestler *et al.*, 2001; McClung *et al.*, 2004), after chronic exposure to cocaine or amphetamine (Perrotti *et al.*, 2005). In its most rostral section, the tVTA is restricted to a bilateral sub-region, posterior to the VTA paranigral nucleus and dorsolateral to the interpeduncular nucleus (Kaufling *et al.*, 2009). As it progresses caudally, the tVTA shifts dorsally and slightly laterally and becomes embedded within the decussation of the superior cerebellar peduncle. Its inputs are mainly similar to those of VTA (Jhou *et al.*, 2009a; Kaufling *et al.*, 2009), but its outputs are more restricted (Supporting Information Figure S1). A notable feature of tVTA outputs is the presence of a dense projection to the mesencephalic dopamine cells (Ferreira *et al.*, 2008; Jhou *et al.*, 2009a; Kaufling *et al.*, 2010). Because this tVTA output is mainly GABAergic (Kaufling *et al.*, 2010), the tVTA might be an inhibitory control centre for dopaminergic activity (Jhou *et al.*, 2009b; Kaufling *et al.*, 2010). As the dopaminergic system is involved in a wide variety of functions and pathologies (Le Moal and Simon, 1991; Iversen and Iversen, 2007), the tVTA is a structure of interest for fundamental and preclinical research.

One possible approach to study the function of a particular brain region is to look for stimuli that recruit this region. Hence, in the present work, we screened 20 different drugs for their ability to induce the expression of FosB/ΔFosB in the tVTA. We showed that only psychostimulant drugs were able to recruit these Fos proteins locally. This action affects tVTA GABAergic cells and is probably mediated through the recruitment of the dopaminergic system as it was observed after treatment with the specific dopamine reuptake inhibitor GBR12909.

Methods

The nomenclature for drugs and for their molecular targets conforms to the *British Journal of Pharmacology Guide to Receptors and Channels* (Alexander *et al.*, 2009).

Animals

Animal care and procedures were performed in accordance with the European Communities Council Directive 86/6609/EEC. The animal facilities are legally registered for animal housing and experimentation (veterinary Animal House Agreements B67-482-1 and C67-482-1). The scientists in charge of the experiments possess the French certificate authorizing experimentation on living animals, obtained from the government veterinary office. Male Sprague Dawley rats were used in all the experiments (n = 130 rats for all the studies, 280–340 g, Janvier, France), housed under standard conditions (22°C, lights on 07 h 00 min–19 h 00 min) with food and water available *ad libitum*.

Drug injections

Drug doses were chosen based on behavioural and/or molecular studies in rodents that are relevant to the related human use of these drugs. Drugs were injected i.p. or s.c. at the following doses: cocaine hydrochloride, 2.5, 5, 10, 20 or 40 $mg \cdot kg^{-1}$ i.p. (Cooper, Melun, France); D-amphetamine sulphate, 1 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich, St Quentin Fallavier, France) (Gruner *et al.*, 2009); (+/–)-3,4-methylenedioxymethamphetamine hydrochloride (MDMA), 5 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich) (Stephenson *et al.*, 1999); methylphenidate hydrochloride, 10 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich) (Gruner *et al.*, 2009); caffeine, 2.5, 10, 25, 60 or 100 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich) (Deurveilher *et al.*, 2006); 1-(2-[bis-(4-fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl) piperazine dihydrochloride (GBR12909), 15 $mg \cdot kg^{-1}$ i.p. (Biotrend, Zurich, Switzerland) (Gruner *et al.*, 2009); ethanol (15% solution), 1.5 or 5 $g \cdot kg^{-1}$ i.p. (Vilpoux *et al.*, 2009); diazepam, 1.5 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich) (Chaouloff *et al.*, 1997); γ-hydroxybutyric acid sodium salt (GHB), 1 $g \cdot kg^{-1}$ i.p. in a 2 $mL \cdot kg^{-1}$ volume (Sigma-Aldrich) (Maitre, 1997); morphine sulphate, 10 or 50 $mg \cdot kg^{-1}$ s.c. (Francopia, Paris, France); ketamine hydrochloride, 50 $mg \cdot kg^{-1}$ i.p. (Centravet, Taden, France) (Tose *et al.*, 2009); phencyclidine hydrochloride (PCP), 3 or 10 $mg \cdot kg^{-1}$ i.p. (Sigma-Aldrich) (Castellani and Adams, 1981); Δ^9-tetrahydrocannabinol solution

(THC) in 30% (2-hydroxypropyl)-β-cyclodextrin, 3 mg·kg^{-1} i.p. (Sigma-Aldrich) (Lepore *et al.*, 1995); sodium valproic acid, 20 mg·kg^{-1} i.p. (Sigma-Aldrich); gabapentin, 50 mg·kg^{-1} i.p. (Teva Santé, Paris la Défense, France) (Pedersen and Blackburn-Munro, 2006); reboxetine mesylate, 0.8 mg·kg^{-1} i.p. (Edronax®, Pharmacia Gmbh, Karlsruhe, Germany) (Yalcin *et al.*, 2009); nortriptyline hydrochloride, 15 mg·kg^{-1} i.p. (Sigma-Aldrich) (Beck, 1995); fluoxetine hydrochloride, 10 mg·kg^{-1} i.p. (Biotrend) (Cryan *et al.*, 2005); venlafaxine hydrochloride, 5 mg·kg^{-1} i.p. (Effexor®, Wyeth, Paris la Défense, France) (Millan *et al.*, 2001); S-(+)-fenfluramine hydrochloride (dexfenfluramine), 4 mg·kg^{-1} i.p. (Sigma-Aldrich) (Vickers *et al.*, 1996). Unless otherwise indicated, the drugs were prepared in 0.9% NaCl and injected in a volume of 1 mL·kg^{-1}. For the three highest doses of caffeine, this drug was injected using a 20 mg·mL^{-1} solution due to limits of solubility. Control animals received an injection of 0.9% NaCl. As the tVTA is a brain region with no or very few FosB/ΔFosB staining under basal conditions (Perrotti *et al.*, 2005; Kaufling *et al.*, 2009; 2010), the induction of these transcription factors is easy to detect locally. It allowed us to use only three animals (triplicate) for most of the drugs tested.

Histochemistry

Three hours following the drug injection, the rats were perfused under deep chloral hydrate anaesthesia (800 mg·kg^{-1}; Sigma, St. Louis, MO, USA) with 100 mL phosphate buffer (0.1 M, pH 7.4) followed by 500 mL of a paraformaldehyde solution (4% in phosphate buffer). Brains were removed and post-fixed overnight in the same fixative. Forty µm mesencephalic sections were subsequently cut following the frontal plane on a vibratome (VT 1000S, Leica, Rueil-Malmaison, France). Sections were serially collected in phosphate buffer with 0.9% NaCl [phosphate-buffered saline (PBS)]. For the time course of cocaine response, perfusions were done 30 min and 1, 1.5, 3, 6, 24, 48 and 96 h post-injection.

Sections for immunohistochemistry were washed in PBS (3 × 10 min), incubated 15 min in a 1% H_2O_2/50% ethanol solution if used for a peroxidase reaction, washed in PBS (3 × 10 min) and incubated in PBS containing Triton X-100 and 5% donkey serum for 45 min. Sections were then incubated overnight at room temperature in PBS with Triton X-100, 1% donkey serum and the primary antibody(ies). Triton X-100 was used at 0.5%, except for glutamic acid decarboxylase (GAD) immunohistochemistry for which it was reduced to 0.2% to limit the background noise and facilitate identification of the cell body.

The rabbit anti-FosB polyclonal antibody (Santa-Cruz Biotechnology, Santa Cruz, CA, USA; catalogue number SC-48; 1:2000 for peroxidase reaction, 1:200 for immunofluorescence) was raised against an internal region of the transcription factor FosB (Santa-Cruz data sheet). It recognizes both FosB and its splice variant ΔFosB, but no other member of the Fos proteins (Perrotti *et al.*, 2004; 2005; Luis-Delgado *et al.*, 2006). The sheep anti-tyrosine hydroxylase (TH) polyclonal antibody (Chemicon, Temecula, CA, USA; catalog number AB1542; 1:1000) was raised against a sodium dodecyl sulphate-denaturated TH from rat phaeochromocytoma (Chemicon data sheet). The mouse anti-GAD 67 kDA monoclonal antibody (Chemicon, catalogue number MAB5406; 1:10 000) was raised against a recombinant fusion protein containing N-terminal regions of GAD 67 kDA not shared by GAD 65 kDA (Chemicon data sheet).

Sections for immunohistofluorescence were washed in PBS (3 × 10 min), incubated with a donkey Cy3 or FITC fluorophore-labelled secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:400) for 1 h 30 min, and washed in PBS (3 × 10 min) before being mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). Sections for the peroxidase reaction were washed in PBS (3 × 10 min), incubated with a biotinylated donkey anti-rabbit secondary antibody (Amersham Biosciences, Orsay, France; 1:200 in PBS containing Triton X-100, 1% donkey serum) for 1 h 30 min, washed in PBS (3 × 10 min) and incubated with PBS containing the avidin-biotin-peroxidase-complex (ABC) (ABC Elite, 0.2% A and 0.2% B; Vector Laboratories) for 1 h 30 min. After being washed in Tris-HCl buffer (0.05 M, pH 7.5; 3 × 10 min), bound peroxidase was revealed by incubation in 0.025% 3.3′-diaminobenzidine tetrahydrochloride, 0.0006% H_2O_2 (Sigma) in Tris-HCl buffer. Sections were incubated for approximately 10 min and washed again. Sections were serially mounted on gelatine-coated slides, air dried, dehydrated in graded alcohols, cleared in Roti-Histol (Carl Roth GmbH & Co., Karlsruhe, Germany) and coverslipped with Eukitt.

Analysis and illustrations

Coded slides were used to analyse the number of FosB/ΔFosB-positive nuclei throughout the tVTA. Blind counting was done using a Nikon Eclipse 80i microscope with the Neurolucida® 8.0 software (MicroBrightField Inc., Williston, VT, USA). We counted the number of FosB/ΔFosB-positive nuclei bilaterally in the tVTA, between –5.80 and –7.30 mm from the bregma, analysing a 40 µm section every 160 µm. Section drawings presenting the FosB/ΔFosB signal were done using a microscope

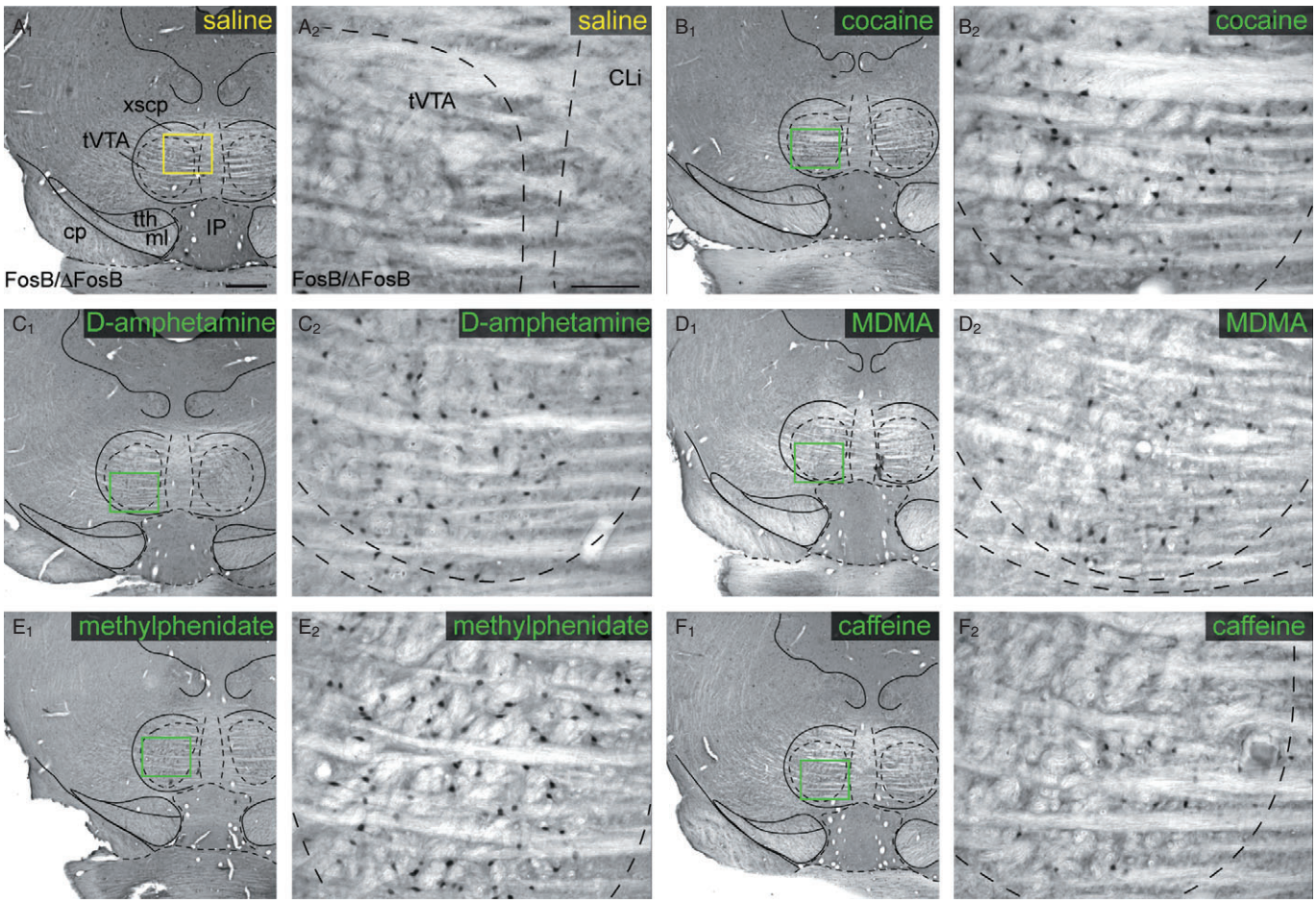


Figure 1

Psychostimulant drugs induce FosB/ΔFosB in the tVTA. Control saline injection had no effect on FosB/ΔFosB in the tVTA (A). The acute injection of cocaine 20 mg·kg^{-1} (B), D-amphetamine 1 mg·kg^{-1} (C), MDMA 5 mg·kg^{-1} (D), methylphenidate 10 mg·kg^{-1} (E) or caffeine 10 mg·kg^{-1} (F) induced FosB/ΔFosB in the tVTA. The rats were perfused 3 h post-injection. $n = 3$ per group. Scale bars = 500 μm in A_1 (applies to B_1–F_1); 100 μm in A_2 (applies to B_2–F_2). The coloured squares in A_1-F_1 indicate the regions shown at higher magnification in A_2–F_2. CLi, caudal linear nucleus of the raphe; cp, cerebral peduncle; IP, interpeduncular nucleus; ml, medial lemniscus; tth, trigeminothalamic tract; tVTA, tail of the ventral tegmental area; xscp, superior cerebellar peduncle decussation.

equipped with a *camera lucida* (Nikon Eclipse E600, Nikon Instruments, Kingston, UK). Data for FosB/ΔFosB analysis are expressed as mean ± SEM of positive nuclei per hemi-tVTA. Statistical analysis was performed with STATISTICA 7.1 (Statsoft, Tulsa, OK, USA), using Student's *t*-test to compare the drug action with the control. The significance level was set at $P < 0.05$.

The analysis of double-labelling fluorescence was done on 3 to 6 frontal sections per animal using an epifluorescence microscope (Leica DMRD). Pictures were taken by using a microscope (Leica) with a digital camera (Cool-snap, Princeton, NJ, USA). Adobe Photoshop 7.0 was used to adjust contrast, brightness and sharpness. The colour channels were individually adjusted for the merged pictures. Abbreviations and structure limits are based on the frontal diagrams from the atlas of Paxinos and Watson (1998).

Results

Expression of FosB/ΔFosB in the tVTA by psychostimulant drugs

We previously reported that chronic exposure to cocaine induced the transcription factor ΔFosB in the tVTA (Perrotti *et al.*, 2005; Kaufling *et al.*, 2010) and we recently observed a similar induction with acute cocaine (Kaufling *et al.*, 2009; 2010). We thus used acute cocaine as a positive control for the present study. Few FosB/ΔFosB-positive nuclei were observed in the tVTA following saline administration (Figure 1A), which is in agreement with previous

reports (Perrotti *et al.*, 2005; Kaufling *et al.*, 2009). In contrast, acute injections of the psychostimulants cocaine, D-amphetamine, MDMA, methylphenidate or caffeine resulted in a strong expression of the transcription factor FosB/ΔFosB in the tVTA (Figure 1B–F). This induction was observed in each of the animals receiving the psychostimulant injection.

This bilateral induction started in the most posterior part of the VTA and the cluster of nuclear staining extended more caudally, shifting dorsally and slightly laterally to become embedded within the decussation of the superior cerebellar peduncle (Figures 2 and 3A). The overall quantification of this tVTA staining (Figure 2) and the quantification along the anteroposterior axis (Figure 3A) revealed that all the drugs induced FosB/ΔFosB with a similar anteroposterior profile. The induction obtained with caffeine was significant but much lower than that observed with the other psychostimulants (Figures 2E and 3A).

A time-course analysis of the cocaine response (20 mg·kg^{-1}) revealed a rapid and long-lasting induction of FosB/ΔFosB (Figures 3B and S2) ($F_{7,23}$ = 9.2, P < 0.00001). This protein induction could be detected within 30 min post-injection (insert of Figure 3B, P < 0.001) and FosB/ΔFosB was still present within the tVTA 4 days after cocaine exposure. The rapid induction and its peak at 3 h are in agreement with current knowledge regarding psychostimulant-induced FosB expression in other brain regions such as the nucleus accumbens (Nestler *et al.*, 2001; McClung *et al.*, 2004). These data confirmed this timepoint as adequate for our experiments. The long-lasting effect suggests that stable variants of ΔFosB were also locally expressed (Nestler *et al.*, 2001; McClung *et al.*, 2004; Perrotti *et al.*, 2005). No induction of FosB/DeltaFosB was observed over time after saline injection ($F_{2,6}$ = 0.6, P > 0.5).

The expression of FosB/DeltaFosB induced by both cocaine and caffeine was dose dependent (cocaine: $F_{5,12}$ = 43.7, P < 0.00001; Figures 3C and S3) (caffeine: $F_{5,12}$ = 11.4, P < 0.001; Figures 3D and S3). For cocaine, the maximal effect was reached with 20 mg·kg^{-1}. For caffeine, it increased up to the highest tested dose, 100 mg·kg^{-1}. Even at this high dose of caffeine, the induction of FosB/ΔFosB remained much smaller than that observed with the psychostimulant drugs that directly target amine uptake sites (Figures 2 and 3C,D).

The double-labelling by immunofluorescence (Figures 4 and S4) revealed that FosB/ΔFosB induced by the acute injection of these psychostimulants was in each case almost always present in the GABAergic neurones (98–100% depending on the drug, Figure 4G$_2$), with no detectable expression in dopaminergic neurones (Figure 4G$_1$).

Lack of expression of FosB/ΔFosB in the tVTA after exposure to other drugs

Drugs stimulating GABAergic transmission were unable to induce FosB/ΔFosB in the tVTA. This lack of induction was observed with ethanol (Figures 5A and S5A); with the anxiolytic drug diazepam (Figure 5B), an agonist with allosteric activity at the benzodiazepine site on $GABA_A$ receptors; and with GHB (Figure 5C), a GABA metabolite acting through both its own receptors and GABA receptors, predominantly $GABA_B$ (Carter *et al.*, 2009). The opiate analgesic and drug of abuse morphine (Figures 5D and S5B), an agonist of opioid receptors, was also unable to induce FosB/ΔFosB in the tVTA. Dissociative drugs that primarily act through NMDA antagonism, such as the anaesthetic ketamine (Figure 5E) and PCP (Figures 5F and S5C) did not induce FosB/ΔFosB in the tVTA. The cannabinoid agonist THC (Figure 5G) also had no effect on FosB/ΔFosB expression in the tVTA. All these drugs are however liable to induce drug abuse.

Lastly, the anticonvulsant drug valproic acid (Figure 5H) which inhibits histone deacetylases and also favours GABA transmission through an indirect mechanism, and the anticonvulsant drug gabapentin (Figure 5I), which binds the α_2-δ protein subunits of voltage-gated calcium channels ($Ca_V\alpha_2$-δ), were unable to induce FosB/ΔFosB in the tVTA.

The lack of FosB/ΔFosB induction with all of the said drugs was quantified (Figure 5J).

Expression of FosB/ΔFosB in the tVTA by inhibition of the DAT

The most prominent induction of FosB/ΔFosB in the tVTA was observed with psychostimulant drugs acting on monoamine transporters: cocaine, D-amphetamine, MDMA, methylphenidate. To evaluate the role of the different monoamines in this induction, we tested inhibitors of the various transporters: dopamine transporter (DAT), noradrenaline transporter (NET) and/or 5-hydroxytryptamine transporter (SERT). The antidepressant drug reboxetine (Figure 6A), which is a specific NET inhibitor, the tricylic antidepressant drug nortriptyline (Figure 6B), a NET/SERT inhibitor with a predominant NET action, the antidepressant drug fluoxetine (Figure 6C), a SERT inhibitor, and the antidepressant drug venlafaxine (Figure 6D), a non-tricyclic NET/SERT inhibitor, were unable to induce the expression of FosB/ΔFosB in the tVTA. The anorexigenic drug dexfenfluramine (Figure 6E),

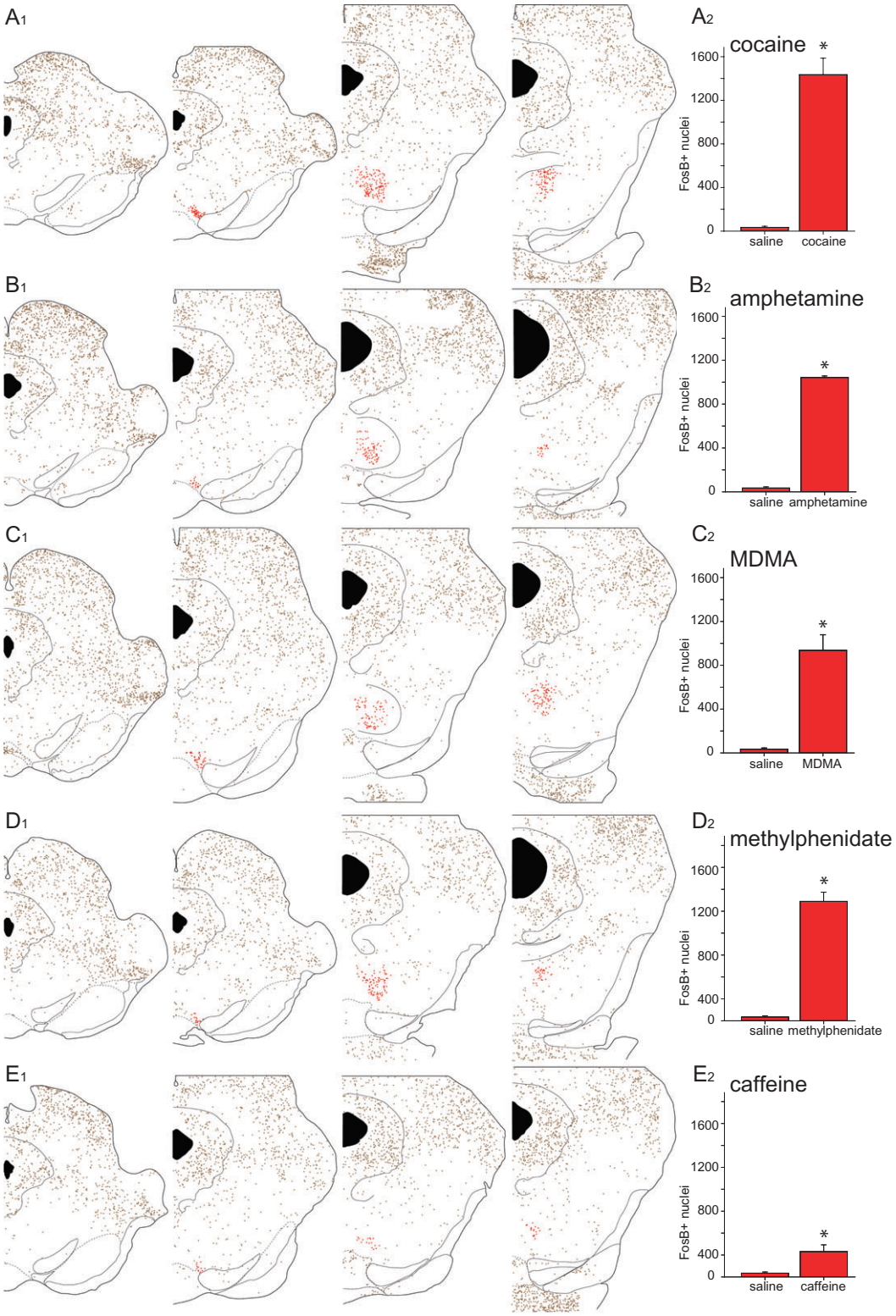


Figure 2

Quantification and *camera lucida* drawings of FosB/ΔFosB-positive nuclei in the tail of the ventral tegmental area (tVTA). The acute injection of cocaine 20 mg·kg^{-1} (A), D-amphetamine 1 mg·kg^{-1} (B), MDMA 5 mg·kg^{-1} (C), methylphenidate 10 mg·kg^{-1} (D) or caffeine 10 mg·kg^{-1} (E) induced FosB/ΔFosB in the whole of the tVTA. For each drug, drawings are ordered from rostral to caudal. Each dot represents a positive nucleus for FosB/ΔFosB immunohistochemistry. The positive nuclei within tVTA are in red; the positive nuclei outside tVTA are in brown. The induction of FosB/ΔFosB is bilateral; for each drug, the total number of FosB/ΔFosB-positive nuclei per hemi-tVTA is presented as mean ± SEM; $n = 3$ per group. *$P < 0.05$, *t*-test against control data (saline).

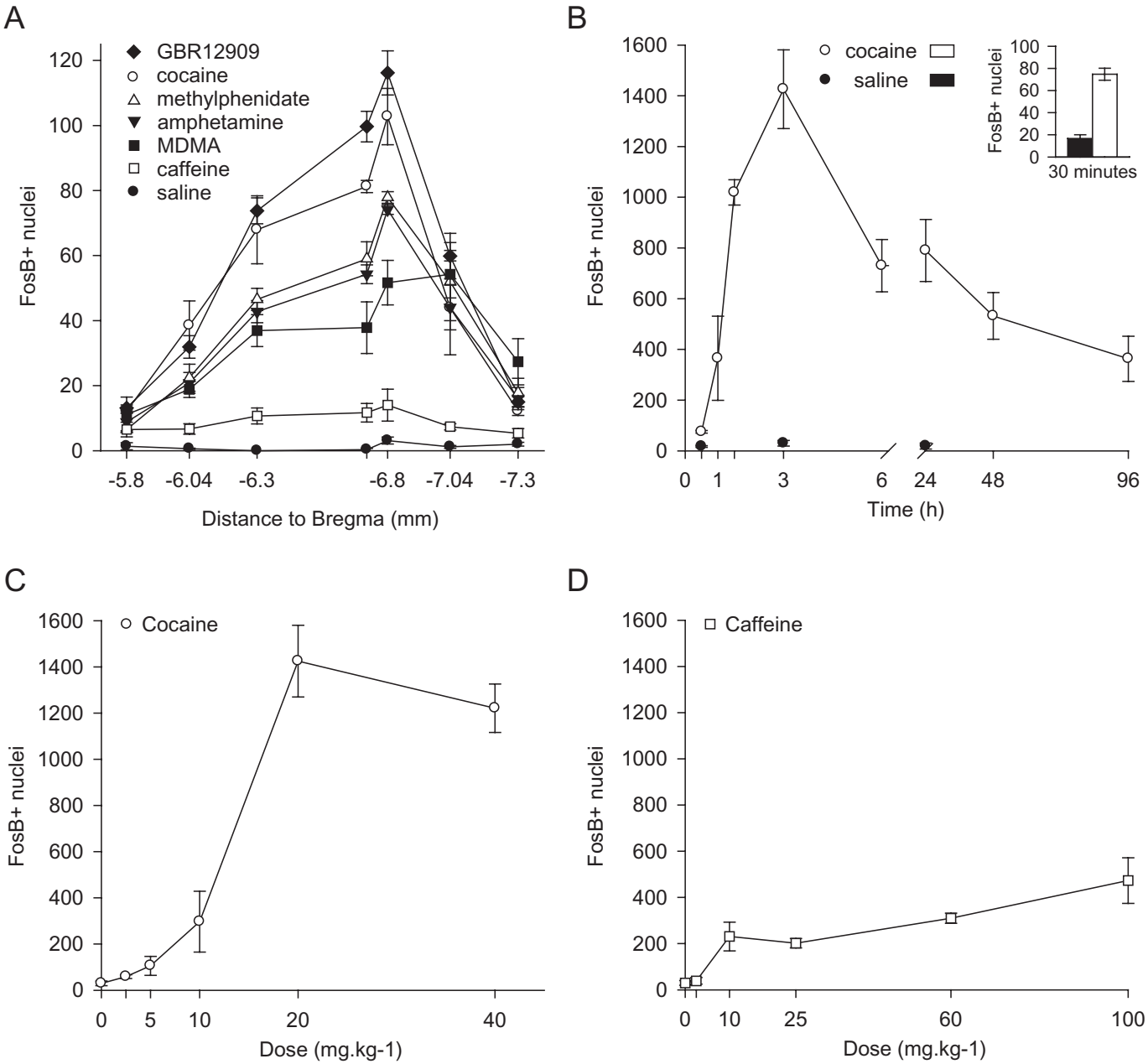
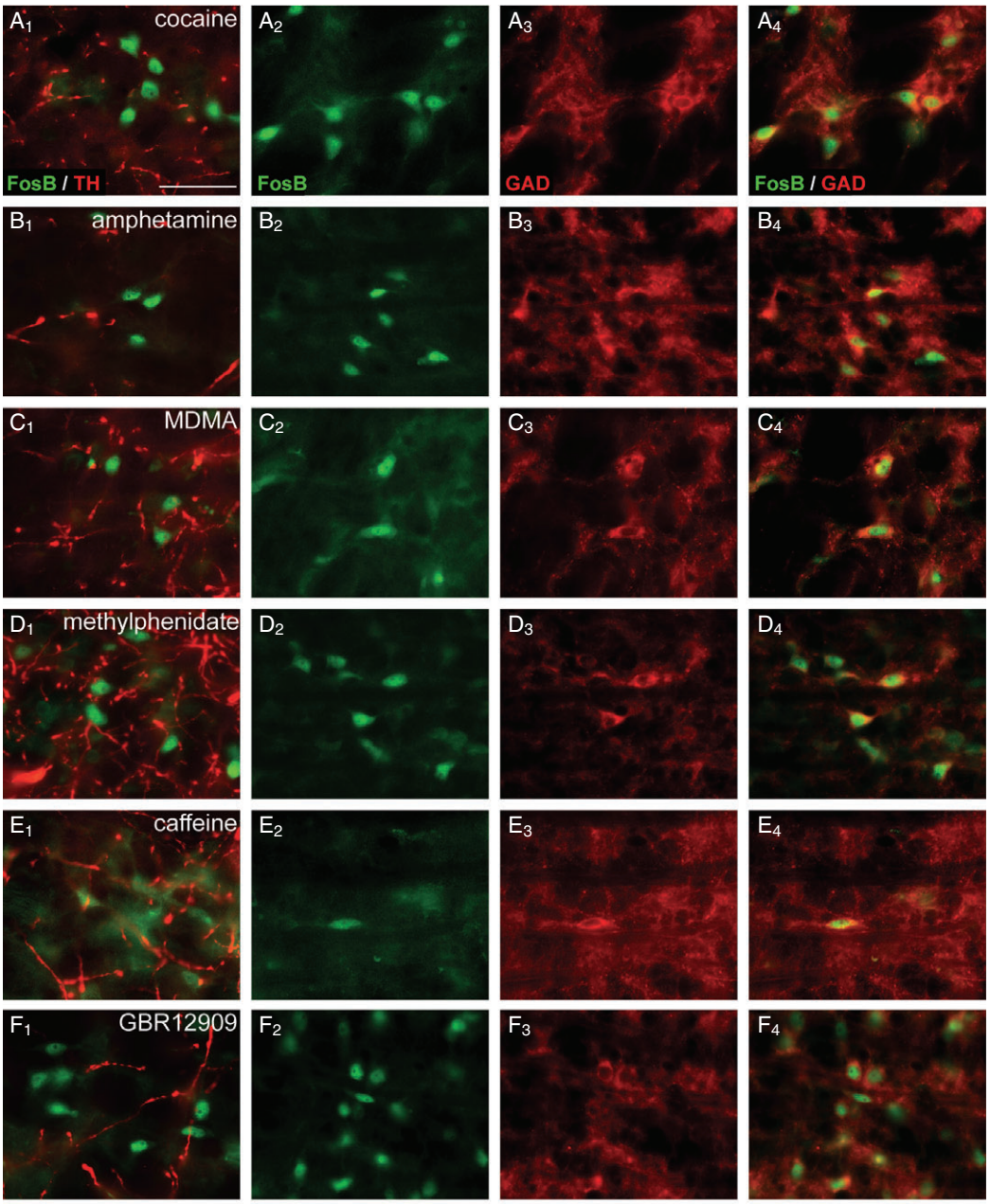


Figure 3

Quantification of FosB/ΔFosB-positive nuclei along the anteroposterior extent of the tail of the ventral tegmental area (tVTA) and dose-responses. (A) Rats received an acute injection of cocaine 20 mg·kg^{-1}, D-amphetamine 1 mg·kg^{-1}, MDMA 5 mg·kg^{-1}, methylphenidate 10 mg·kg^{-1}, caffeine 10 mg·kg^{-1}, GBR12909 15 mg·kg^{-1} or 0.9% NaCl (saline) as control. They were perfused 3 h post-injection. $n = 3$ per group. The mean number of FosB/ΔFosB-nuclei is given per bregma level, according to the atlas of Paxinos and Watson (1998). (B) Rats were perfused at various timepoints following an acute injection of cocaine 20 mg·kg^{-1} ($n = 3$–5 per timepoint). Data indicate the number of FosB/ΔFosB-positive nuclei over the whole of the tVTA. The top right insert shows with a larger scale the increase in the number of FosB/ΔFosB-positive nuclei at 30 min. Dose-responses to cocaine (C) or to caffeine (D) were done, perfusing the animals 3 h post-injection ($n = 3$ per dose).

which is a 5-hydroxytryptamine releasing agent, was also unable to induce FosB/ΔFosB in the tVTA. In contrast, the DAT inhibitor GBR12909, at a dose that promotes an awake state (Gruner *et al.*, 2009), induced a strong expression of FosB/ΔFosB in the tVTA (Figures 3A and 6F,G). This induction of FosB/ΔFosB following GBR12909 exposure displayed the same anteroposterior profile as the psychostimulant drugs (Figure 3A), and was also mainly present in GABAergic cells (98%, Figure 4F,G$_2$).



G_1

FosB$^+$ cells	TH$^-$	TH$^+$
cocaine	259	0
amphetamine	307	0
MDMA	264	0
methylphenidate	480	0
caffeine	52	0
GBR12909	320	0

G_2

FosB$^+$ cells	GAD$^-$	GAD$^+$
cocaine	4	427
amphetamine	2	263
MDMA	0	218
methylphenidate	1	374
caffeine	1	55
GBR12909	5	315

Figure 4

Psychostimulants induced the expression of FosB/ΔFosB in GABAergic neurones of the tail of the ventral tegmental area (tVTA). Following acute injection of cocaine 20 mg·kg^{-1} (A), D-amphetamine 1 mg·kg^{-1} (B), MDMA 5 mg·kg^{-1} (C), methylphenidate 10 mg·kg^{-1} (D), caffeine 10 mg·kg^{-1} (E) or GBR12909 15 mg·kg^{-1} (F), FosB/ΔFosB-positive nuclei are found in GABAergic but not in dopaminergic neurones in the tVTA. The merged image for FosB/ΔFosB (green) and tyrosine hydroxylase (TH) (red) double labelling is presented in the 1st column. The merged image for FosB/ΔFosB (green, 2nd column) and glutamic acid decarboxylase (GAD) (red, 3rd column) is presented in the fourth column. Quantification of double-labelling immunofluorescence revealed that none of the FosB/ΔFosB-positive cells co-expressed TH (G_1) whereas 98–100% were GAD-positive (G_2).

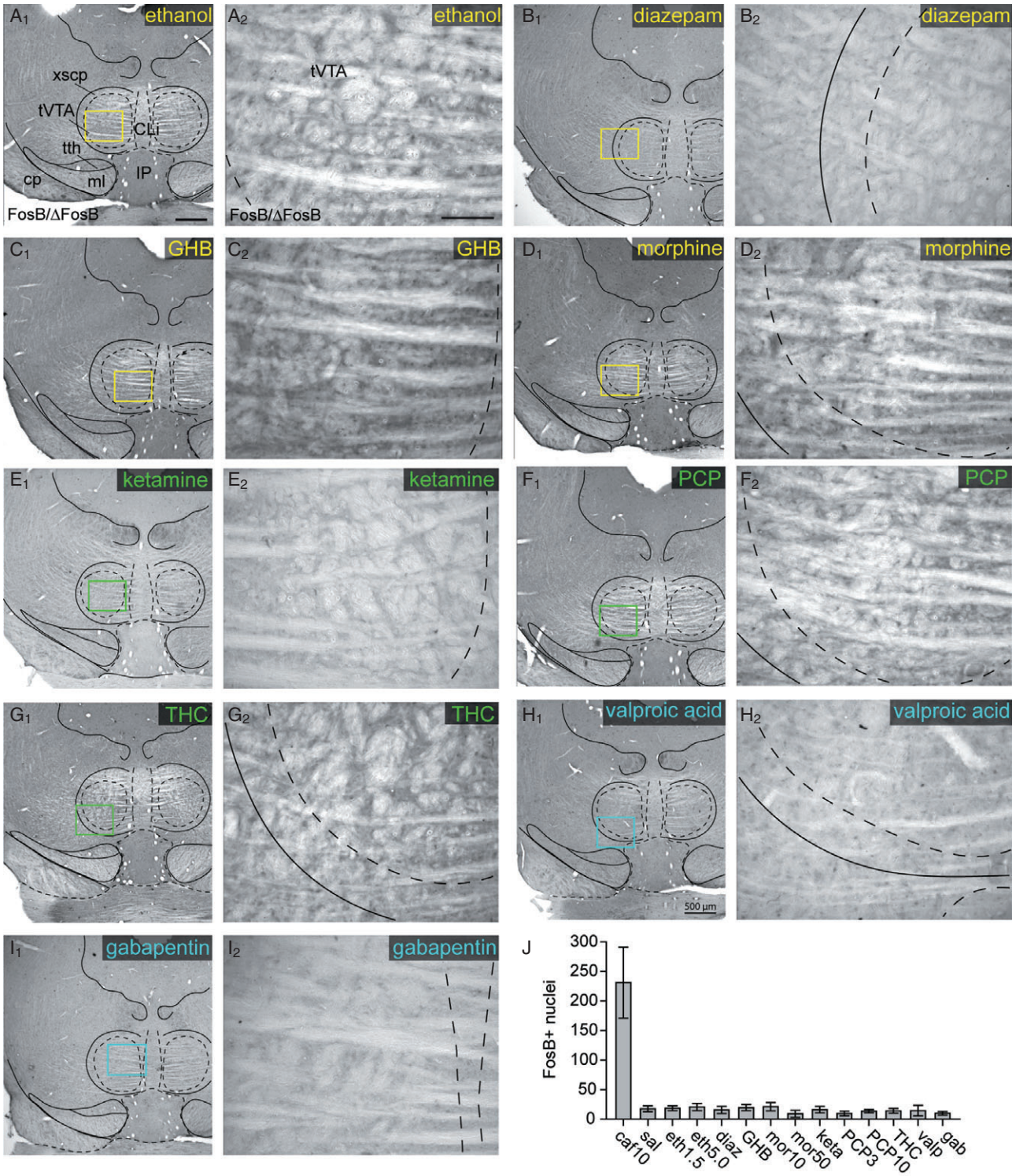


Figure 5

Sedative, dissociative or anticonvulsant drugs did not induce FosB/ΔFosB in the tVTA. The acute injection of ethanol 1.5 g·kg^{-1} (eth1.5) (A), diazepam 1.5 mg·kg^{-1} (diaz) (B), γ-hydroxybutyric acid sodium salt 1 g·kg^{-1} (GHB) (C), morphine 10 mg·kg^{-1} (mor10) (D), ketamine 50 mg·kg^{-1} (keta) (E), phencyclidine 3 mg·kg^{-1} (PCP3) (F), Δ^9-tetrahydrocannabinol 3 mg·kg^{-1} (THC) (G), valproic acid 20 mg·kg^{-1} (valp) (H) or gabapentin 50 mg·kg^{-1} (gab) (I) did not induce FosB/ΔFosB in the tVTA. (J) Quantification of FosB/ΔFosB-positive nuclei over the whole extent of the tVTA, caffeine data (10 mg·kg^{-1}, caf10) and saline data (sal) are given as references. $n = 3$ per group. Scale bars = 500 µm in A_1 (applies to B_1–I_1); 100 µm in A_2 (applies to B_2–I_2). The coloured squares in A_1–I_1 indicate the regions shown at higher magnification in A_2–I_2. eth5.0, ethanol 5 g·kg^{-1}; mor50, morphine 50 mg·kg^{-1}; PCP10, PCP 10 mg·kg^{-1}; CLi, caudal linear nucleus of the raphe; cp, cerebral peduncle; IP, interpeduncular nucleus; ml, medial lemniscus; tth, trigeminothalamic tract; tVTA, tail of the ventral tegmental area; xscp, superior cerebellar peduncle decussation.

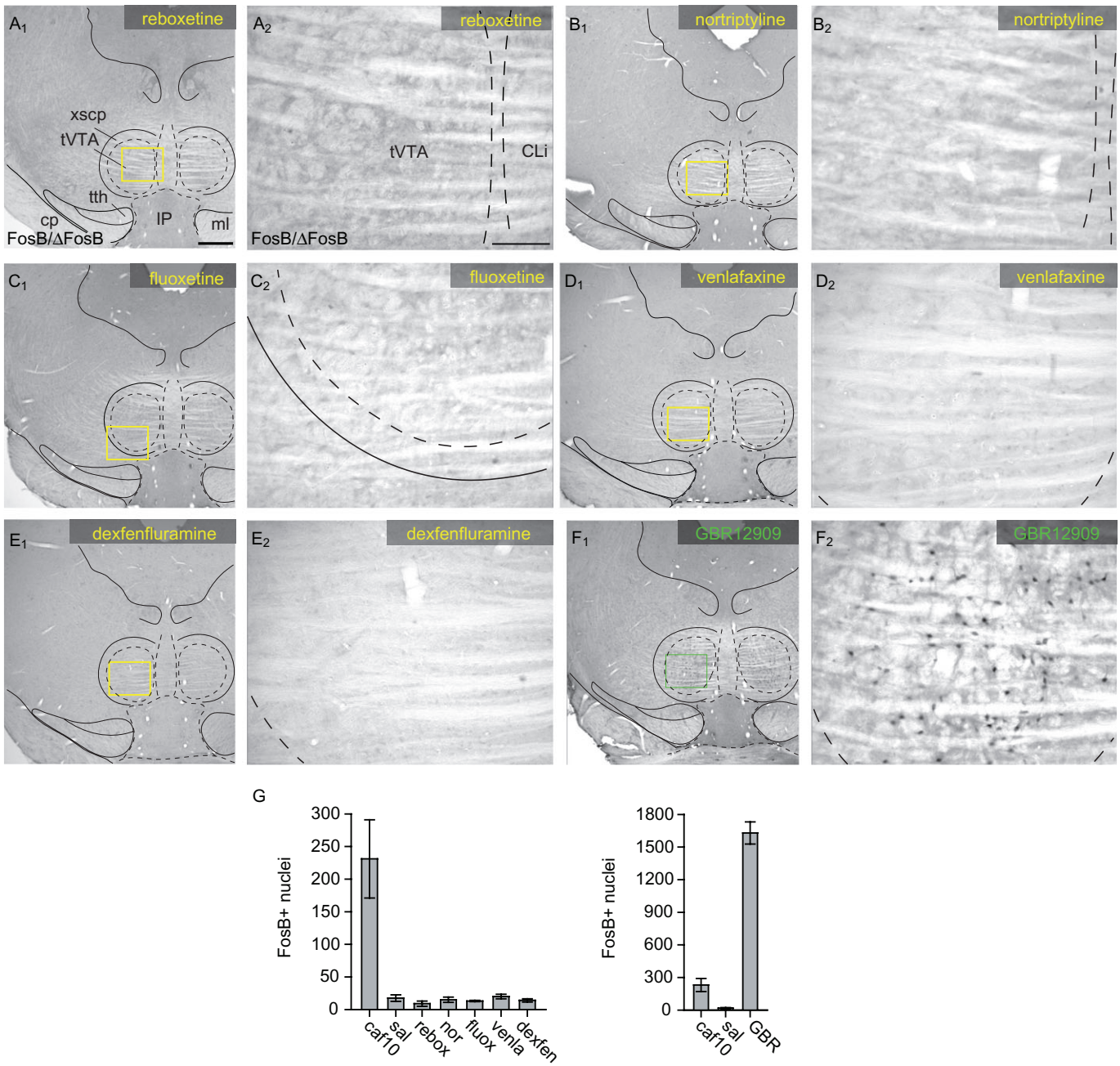


Figure 6

FosB/ΔFosB was induced in the tVTA by a DAT inhibitor, but not by antidepressant drugs or by a 5-hydroxytryptamine releasing agent. The acute injection of reboxetine 0.8 mg·kg^{-1} (rebox) (A), nortriptyline 15 mg·kg^{-1} (nor) (B), fluoxetine 10 mg·kg^{-1} (fluox) (C), venlafaxine 5 mg·kg^{-1} (venla) (D) or S-(+)-fenfluramine (dexfenfluramine) 4 mg·kg^{-1} (dexfen) (E) did not induce FosB/ΔFosB in the tVTA. The acute injection of GBR12909 15 mg·kg^{-1} (GBR) (F) induced FosB/ΔFosB in the tVTA. (G) Quantification of FosB/ΔFosB-positive nuclei over the whole of the tVTA, caffeine data (10 mg·kg^{-1}, caf10) and saline data (sal) are given as references. GBR12909 data are presented separately due to the high level of induction of FosB/ΔFosB; $n = 3$ per group. Scale bars = 500 μm in A_1 (applies to B_1–F_1); 100 μm in A_2 (applies to B_2–F_2). The coloured squares in A_1–F_1 indicate the regions shown at higher magnification in A_2–F_2. CLi, caudal linear nucleus of the raphe; cp, cerebral peduncle; IP, interpeduncular nucleus; ml, medial lemniscus; tth, trigeminothalamic tract; tVTA, tail of the ventral tegmental area; xscp, superior cerebellar peduncle decussation.

Discussion and conclusions

In this study, we tested pharmacological compounds for their ability to recruit a recently discovered brain region, the tVTA. We show that an acute injection of the psychostimulant drugs cocaine, D-amphetamine, MDMA, methylphenidate and caffeine, induced the expression of FosB/ΔFosB in tVTA

cells that were identified as GABAergic by double immunostaining. No induction was observed following exposure to ethanol, diazepam, GHB, morphine, ketamine, PCP, THC, sodium valproic acid or gabapentin. By testing various drugs targeting the aminergic systems (reboxetine, nortriptyline, fluoxetine, venlafaxine, dexfenfluramine, GBR12909), we found that the DAT inhibitor GBR12909 was the only one able to induce FosB/ΔFosB expression in the tVTA. This shows that the dopaminergic system has a critical role in the recruitment of tVTA induced by pharmacological compounds. Our results suggest that the tVTA is a common target for drugs sharing psychostimulant properties.

The VTA is key structure for adaptive and goal-directed behaviours, motivation, reward and mood, and it is thus implicated in various psychopathological disorders (Le Moal and Simon, 1991; Nestler and Carlezon, 2006; Grace *et al.*, 2007; Iversen and Iversen, 2007; Schultz, 2007). Its functional heterogeneity along the anteroposterior axis was first observed 30 years ago (Arnt and Scheel-Krüger, 1979). This functional heterogeneity then remained unstudied until the last 10 years. Behavioural studies showed that cocaine (Rodd *et al.*, 2005a), nicotine (Ikemoto *et al.*, 2006), cannabinoids (Zangen *et al.*, 2006), opioid peptides such as the endogenous ligand of μ-opioid receptors endormorphin-1 (Zangen *et al.*, 2002), but also ethanol (Rodd-Henricks *et al.*, 2000; Rodd *et al.*, 2005b) or its metabolite acetaldehyde (Rodd-Henricks *et al.*, 2002), are self-administered if delivered into the posterior VTA; but they are not or poorly self-administered if delivered into the anterior VTA. The functional distinction between anterior and posterior VTA is also supported by studies using local viral-mediated gene transfer to manipulate the AMPA receptor subunit GluR1 (Carlezon *et al.*, 2000), the phospholipase Cγ (Bolanos *et al.*, 2003) or the transcription factor CREB (Olson *et al.*, 2005). This last study identified the functional transition between anterior and posterior VTA around –5.5 mm from the bregma in the rat. However, the anterior versus posterior subdivisions of the VTA only cover the rostral and central tiers of this structure. There is less information available on the caudal-most tier, corresponding to the tVTA which is mainly GABAergic and can be revealed by ΔFosB induction following cocaine exposure (Perrotti *et al.*, 2005; Kaufling *et al.*, 2009). Our results show that various psychostimulant drugs recruit the tVTA after acute administration, as indicated by the induction of the transcription factor FosB/ΔFosB. Behaviourally, future studies are now needed to evaluate whether tVTA also supports drug self-administration and to functionally differentiate the posterior VTA from the tVTA. However, the partial overlap between the posterior VTA and tVTA (Kaufling *et al.*, 2009) might make the latter studies challenging.

FosB and its stable truncated splice variant ΔFosB are expressed in different regions of the nervous system in response to various pharmacological or non-pharmacological stimuli: drugs of abuse (Hope *et al.*, 1994a; Pich *et al.*, 1997; Perrotti *et al.*, 2008), NMDA antagonists (de Olmos *et al.*, 2009), antipsychotic drugs (Hiroi and Graybiel, 1996), antidepressant drugs (Hope *et al.*, 1994a), L-3,4-dihydroxyphenylalanine (Berton *et al.*, 2009), but also seizures (Hope *et al.*, 1994b), stress (Perrotti *et al.*, 2004; Berton *et al.*, 2007), ischaemia (McGahan *et al.*, 1998), inflammatory pain (Luis-Delgado *et al.*, 2006), compulsive running (Werme *et al.*, 2002) or natural rewards (Wallace *et al.*, 2008). With regard to drugs of abuse, this induction was observed with cocaine, amphetamine, methamphetamine, PCP, morphine, nicotine, ethanol and THC (Pich *et al.*, 1997; McDaid *et al.*, 2006; Perrotti *et al.*, 2008). Some brain regions, such as the striatal complex, appear to be common targets for molecular plasticity induced by all these drugs. However, this is not the case for the tVTA. Our survey of various pharmacological stimuli showed that, despite their abuse potential, ethanol, diazepam, GHB, morphine, ketamine, PCP or THC do not induce FosB/ΔFosB in the tVTA. The induction of FosB/ΔFosB in the tVTA may thus be related to the stimulant/arousing properties of psychostimulant drugs rather than to their potential for abuse. In support of this hypothesis, it has been previously shown that modafinil (Provigil®, Cephalon, Maisons-Alfort, France), a non-addictive psychostimulant used against narcolepsy, induces a strong Fos expression specifically in the caudal-most tier of the VTA, which corresponds to the tVTA (Scammell *et al.*, 2000).

Cocaine, D-amphetamine, MDMA and methylphenidate target monoamine transporters. However, the NET inhibitor reboxetine, the NET/SERT inhibitors nortriptyline and venlafaxine, the SERT inhibitor fluoxetine, and the 5-hydroxytryptamine releasing agent dexfenfluramine are unable to induce FosB/ΔFosB in the tVTA. In contrast, FosB/ΔFosB can be induced in the tVTA by the DAT inhibitor GBR12909. A marked increase in dopamine transmission thus appears to be sufficient to induce the local expression of this transcription factor. The dopamine involved may originate from the sparse dopaminergic cells or fibres within the tVTA itself. These fibres may arise from local cell bodies or from dopaminergic cell bodies in VTA and substantia nigra pars compacta, which are tVTA afferents (Kaufling *et al.*, 2009).

However, the dopamine may also be of somatodendritic origin from the VTA itself (Kalivas and Duffy, 1988) and diffuse by volume transmission to the nearby tVTA. Another hypothesis that should not be discarded is the possibility of a system-wide polysynaptic recruitment of tVTA. Indeed, tVTA receives inputs from various brain regions recruited by psychostimulants. This includes, but is not restricted to, prefrontal cortical areas, nucleus accumbens or lateral habenula (Geisler *et al.*, 2008; Jhou *et al.*, 2009a; Kaufling *et al.*, 2009; Brinschwitz *et al.*, 2010).

An increase in somatodendritic and axonal dopamine has also been observed after morphine exposure (Kalivas and Duffy, 1988), whereas we found that morphine did not induce the expression of FosB/ΔFosB in the tVTA. Two explanations may be proposed to account for this discrepancy. Firstly, the somatodendritic release of dopamine induced by morphine has a lower capacity for diffusion than that induced by the previously cited psychostimulants. Indeed, DAT are still effective in animals administered with morphine, preventing an important diffusion of the dopamine. Secondly, it has been shown that tVTA neurones express the μ-opioid receptors (Jhou *et al.*, 2009a) and direct stimulation of these opioid receptors by morphine may inhibit the cAMP/PKA pathway and prevent FosB/ΔFosB induction in tVTA neurones. It is also important to remember that the induction of FosB/ΔFosB does not necessarily reflect an electrophysiological activity of the corresponding cell and that a lack of induction does not reflect a lack of electrophysiological effect.

The FosB/ΔFosB induction in the tVTA after caffeine is mild but significant at various doses. Caffeine is not an inhibitor of the monoamine transporter; it is a non-selective adenosine receptor antagonist and a competitive non-selective phosphodiesterase inhibitor (Nehlig *et al.*, 1992). Due to caffeine's low affinity for phophodiesterases, its *in vivo* action, particularly its psychostimulant effect, is thought to be mediated through its inhibitory effects on adenosine receptors (Ferré, 2008). Studies on the striatal complex have revealed that both pre- and post-synaptic mechanisms, which depend on A_1 and A_{2A} receptors and on their interactions with dopamine receptors, are involved in the psychostimulant effect of caffeine (Ferré, 2008). Future work is however needed to evaluate whether similar mechanisms are implicated in the recruitment of tVTA cells by caffeine.

In conclusion, among the 20 drugs tested, only the psychostimulant drugs induced the expression of FosB/ΔFosB in the tVTA. Newly described brain structures may help to increase our knowledge on brain functions, pathology and pharmacological targets. The tVTA, a potential control centre for dopaminergic activity, appears to be a common target for drugs sharing psychostimulant properties rather than for drugs sharing a risk of abuse. Future work is needed to understand the functional implication of tVTA in the stimulant and/or arousing properties of these drugs, and to determine whether this newly defined brain area is a critical neuroanatomical substrate for such properties.

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Statement of conflicts of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Summary of tVTA connections in the rat brain. The structures connected to the tVTA are schematically presented according to their distance to tVTA (1 mm per circle) along the anteroposterior (top-bottom) and lateral (left-right) axis. Colours indicate the relative density of the connections. The afferents are presented on left half of the schematic and the efferents on the right half. Abbreviations: 3, oculomotor ncl; AcbC, accumbens ncl, core; AcbSh, accumbens ncl, shell; AI, agranular insular cortex; a/pVTA, anterior/posterior VTA; BST, bed ncl of the stria terminalis; Cg, cingulate cortex; Cl, claustrum; CPu, caudate putamen; DA, dorsal hypothalamic area; DMTg, dorsomedial tegmental area; DP, dorsal peduncular cortex; DpMe, deep mesencephalic ncl; DR, dorsal raphe ncl; F, ncl of the fields of Forel; HBD, ncl of the horizontal limb of the diagonal band; IL, infralimbic cortex; ILT, intralaminar thalamic ncl; IP, interpeduncular ncl; IPAC, interstitial ncl of the posterior limb of the anterior commissure; LC, locus coeruleus; LDTg, laterodorsal tegmental ncl; LH, lateral hypothalamic area; LHb, lateral habenula; LPO, lateral preoptic area; LS, lateral septum; MCPO, magnocellular preoptic ncl; MHb, medial habenular ncl; MnR, median raphe ncl; MPO, medial preoptic ncl; ncl, nucleus/nuclei; PAG, periaqueductal gray; PB, parabrachial ncl; PF, parafascicular thalamic ncl; PH, posterior hypothalamic area; PnC, pontine reticular ncl, caudal part; PnO, pontine reticular ncl, oral part; PMnR, paramedian raphe ncl; PPTg, pedunculopontine tegmental ncl; PrL, prelimbic cortex; PV, paraventricular thalamic ncl; R, retrorubral field; SC, superior colliculus; SI, substantia innominata; SNC, substantia nigra, compact part; SNR, substantia nigra, reticular part; Sub, submedius thalamic ncl; TC, tuber cinereum area; tVTA, tail of the VTA; VP, ventral pallidum; VT, ventral thalamic ncl; VTA, ventral tegmental area; ZI, zona incerta.

Figure S2 Time course of FosB/ΔFosB induction in the tVTA. The microphotographs illustrate the induction of FosB/ΔFosB at various time-points following an injection of cocaine 20 $mg \cdot kg^{-1}$ (A) or saline (B). The quantification of FosB/ΔFosB-positive nuclei over the whole of the tVTA is given in Figure 3 of the main article. Scale bar = 100 μm in A_1 (applies to all pictures).

Figure S3 Dose-responses of FosB/ΔFosB induction in the tVTA. The microphotographs illustrate the induction of FosB/ΔFosB by various doses of cocaine (A) or caffeine (B). The quantification of FosB/ΔFosB-positive nuclei over the whole of the tVTA is given in Figure 3 of the main article. Scale bar = 100 μm in A_1 (applies to all pictures).

Figure S4 FosB/ΔFosB expression in GABAergic and non-GABAergic neurones from the tVTA. (A) While most of FosB/ΔFosB-positive nuclei (A1, green) are found in glutamic acid decarboxylase (GAD)-expressing neurones (A2, red), as seen in the merged image (A3), arrowheads point two FosB/ΔFosB-positive nuclei in GAD-negative neurones. (B) The merged image of another section shows a FosB/ΔFosB-positive nucleus in a GAD-negative neurone (white arrowhead), a GAD-positive neurone without detectable expression of FosB (red arrowhead), and a GAD-positive neurone expressing FosB in its nucleus (yellow arrowhead). These tVTA pictures are from an animal that received GBR12909 15 $mg \cdot kg^{-1}$. Scale bars: 50 μm in A1 (applies to A2 and A3) and B.

Figure S5 FosB/ΔFosB induction in the tVTA. High doses of ethanol (5 $g \cdot kg^{-1}$, A), morphine (50 $mg \cdot kg^{-1}$, B) or phencyclidine (PCP, 10 $mg \cdot kg^{-1}$, F) did not induce FosB/ΔFosB in the tVTA. The quantification of FosB/ΔFosB-positive nuclei over the whole extent of the tVTA is given in Figure 5J of the main article; n = 3 per group. Abbreviations: cp, cerebral peduncle; IP, interpeduncular nucleus; ml, medial lemniscus; tth, trigeminothalamic tract; tVTA, tail of the ventral tegmental area; xscp, superior cerebellar peduncle decussation. Scale bars = 500 μm in A_1 (applies to B_1 and C_1); 100 μm in A_2 (applies to B_2 and C_2). The coloured squares in A_1-C_1 indicate the regions shown at higher magnification in A_2–C_2.

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