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Activation of afferents to the ventral tegmental area in response to acute amphetamine: a double labeling study

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Abstract

The ventral tegmental area (VTA), primary source of the mesocorticolimbic dopaminergic system, is regarded as a critical site for initiation of behavioural sensitization to psychostimulants. The present study was undertaken to identify the neural pathways converging on the VTA that are potentially implicated in this process. Rats were sensitized by a single exposure to amphetamine (5 mg/kg, s.c.). The distribution of VTA-projecting neurons activated by amphetamine was examined by combining retrograde transport of the cholera toxin β subunit (CTb), injected into the VTA, with immunodetection of Fos. The quantitative analysis of CTb–Fos double labelling demonstrates that amphetamine induced a rapid activation of Fos in a large number of brain areas projecting to the VTA. More than half of the CTb–Fos double-labelled neurons were located in the prefrontal cortex, lateral preoptic area–lateral hypothalamus, pontomesencephalic tegmentum, dorsal raphe nucleus, ventral pallidum and nucleus accumbens. In addition, scattered CTb–Fos double-labelled cells were observed in many other VTA afferent structures, such as claustrum, lateral septum, diagonal band–magnocellular preoptic nucleus, deep mesencephalic nucleus, oral part of pontine reticular nucleus and dorsomedial tegmental area. This suggests that systemic amphetamine activates a wide population of neurons projecting to the VTA that may be important for the modulation of neurobehavioural plasticity produced by this psychostimulant.

Keywords

cholera toxin; Fos; psychostimulant; rat; retrograde transport

Introduction

Exposure to drugs of abuse can result in long-lasting adaptations within specific critical neural circuits leading to long-term changes in behaviour. For example, single or repeated administration of psychostimulants such as amphetamine or cocaine produces an enduring sensitization to their psychomotor activating effects (Robinson et al., 1982; Robinson & Becker, 1986; Vanderschuren et al., 1999; Grignaschi et al., 2004), a phenomenon directly related to an enhancement in drug-motivated behaviour (De Vries et al., 1998, 2002; Vezina et al., 2002; Robinson & Berridge, 2003; Vezina, 2004; Ferrario et al., 2005).

The ventral tegmental area (VTA), the primary source of the mesocorticolimbic dopaminergic (DA) system, is regarded as a critical site for initiation of behavioural

sensitization to psychostimulants (Vanderschuren & Kalivas, 2000, for review). Administration of amphetamine into the VTA, but not in the nucleus accumbens (NAc), elicits a sensitized response to subsequent systemic injections of amphetamine or cocaine (Kalivas & Weber, 1988; Hook et al., 1992; Vezina, 1993; Perugini & Vezina, 1994; Cador et al., 1995). Conversely, administration of NMDA or DA D1 receptor antagonists into the VTA prevents the initiation of psychostimulant-induced behavioural sensitization (Bjijou et al., 1996; Vezina, 1996; Vezina & Queen, 2000). Psychostimulants have also been found to trigger a transient potentiation of glutamatergic transmission in the VTA (White et al., 1995; Zhang et al., 1997; Giorgetti et al., 2001; Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004; Faleiro et al., 2004) that is blocked by NMDA receptor antagonists (Ungless et al., 2001), further supporting the relevance of VTA glutamatergic transmission in the initial steps leading to sensitization.

However, several lines of evidence suggest that the specific molecular and cellular events that initiate behavioural sensitization to psychostimulants result from a complex interplay between converging signals onto VTA neurons. In addition to DA and glutamate, various neurotransmitters and neuromodulators regulating DA neurons in the VTA, including acetylcholine (Schoffelmeer et al., 2002; Chantpiaux et al., 2006), neuropeptides (Panayi et al., 2005; Borgland et al., 2006) and trophic factors (Flores et al., 2000), appear critical for the induction of behavioural sensitization and synaptic plasticity. Moreover, it has been suggested that specific subsets of VTA afferents contribute to the initiation of this process. Lesion of the medial prefrontal cortex (PFC) blocks the initiation of behavioural sensitization to psychostimulants (Wolf et al., 1995; Cador et al., 1999; Bjijou et al., 2002) whereas its tetanic stimulation sensitizes animals to subsequent cocaine administration (Schenk & Snow, 1994). Other brain regions have received little attention in this context, although it has been reported that the amygdala (Wolf et al., 1995; Bjijou et al., 2002), the laterodorsal tegmental nucleus (LDTg) and the pedunculopontine tegmental nucleus (PPTg; Alderson et al., 2003; Nelson et al., 2007) are likely to contribute to this process.

Given the complexity of the interconnected network of afferents to the VTA, i.e. brain regions projecting directly to the VTA but also to structures that in turn project to the VTA (Geisler & Zahm, 2005), it has been difficult to clearly define the circuitry underlying initiation of sensitization. The present study was therefore designed to identify the neural pathways potentially implicated in the induction of sensitization to amphetamine. To that aim, rats were sensitized by a single exposure to amphetamine, as we and others previously described (Robinson et al., 1982; Vanderschuren et al., 1999; Faleiro et al., 2003; Colussi-Mas et al., 2005; Panayi et al., 2005). The distribution of the neurons projecting to the VTA and activated by amphetamine was then examined by combining retrograde transport of an axonal tracer, cholera toxin β subunit (CTb), injected into the VTA with immunodetection of Fos, a marker of neuronal activation.

Material and methods

Animals

Male Sprague–Dawley rats (Harlan, Gannat, France) weighing 250–275 g upon arrival were housed four per cage in a temperature controlled (22°C) colony room under a 12-h light–dark cycle (lights on at 07.00 h), with ad libitum access to food and water. Rats were habituated to these conditions for 1 week prior to their use. All experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the French Guidelines (Act 87–848. Ministère de l’Agriculture) for the care and use of laboratory animals.

CTb injections into the VTA

The retrogradely transported marker CTb (List Biological Laboratories, Campbell, CA, USA) was unilaterally injected by iontophoresis into the VTA of 16 rats. CTb is considered to be a very sensitive retrograde tracer when visualized by immunohistochemistry. It is taken and retrogradely transported by all neurons of the central nervous system and, unlike viral tracers, not transported transynaptically (Luppi et al., 1990). Animals were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, South Natick, MA, USA) with the incisor bar set at -3.3 mm; body temperature was maintained at 37°C using a homeothermic heated pad (Harvard Apparatus, Edenbridge, UK). A hand drill was used to expose the brain surface above the VTA and a borosilicate glass micropipette (tip diameter $5\text{--}10\text{ }\mu\text{m}$) filled with a low-salt CTb solution (0.5% made in 0.1 M phosphate buffer, pH 6.0) was lowered into the brain at the following stereotaxic coordinates: AP, -5.5 mm from bregma; L, -0.7 mm from the midline; and V -7.2 mm from the dural surface (Paxinos & Watson, 2005). CTb was delivered by applying positive pulses of $1\text{--}4\text{ }\mu\text{A}$ (7 s on, 7 s off) for 10 min through a chlorinated silver wire placed in the micropipette and connected to a current generator (Bionic Instruments, France). The micropipette was withdrawn 10 min after the CTb ejection, the scalp incision was sutured and rats were returned to their home cages for recovery.

Experimental design

After a 5- to 10-day postoperative survival, rats received a subcutaneous injection of 5 mg/kg amphetamine (d-amphetamine sulphate; Sigma-Aldrich, France) or saline. In order to record locomotor activity, the experiment was conducted in an activity cage equipped with an array of four parallel horizontal infrared beams (Imetronic, Pessac, France). Rats were injected in the activity cage after a 2-h habituation period. Locomotor activity was continually recorded by computer for the subsequent 2 h by quantification of successive photocell beam breaks (crossovers). Data are expressed as the mean $\pm$ SEM number of crossovers cumulated over 2 h.

Two hours after the amphetamine or saline injection, rats were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 200 mL of saline containing 0.1% heparin followed by 400 mL of freshly prepared 4% paraformaldehyde solution in 0.1 M phosphate buffer (PB), pH 7.4. Brains were then rapidly removed, placed in a fresh fixative overnight, cryoprotected in 0.1 M PB containing 30% sucrose at 4°C , and frozen for 3 min in isopentane at -50°C .

Coronal sections ($30\text{ }\mu\text{m}$ thick) were cut on a freezing microtome along the entire brain, immersed in 10 mM PB, pH 7.4, containing 0.9% NaCl (PBS) and 0.1% sodium azide, then stored at 4°C for subsequent processing. A 1-in-8 series of sections was reacted to reveal neurons expressing the retrograde tracer CTb and/or Fos. Briefly, free-floating sections were washed with PBS containing 0.3% Triton X-100 (PBST; 3×10 min) and incubated overnight with the primary rabbit anti-Fos antibody raised against amino acids 4–17 of the human Fos protein (Ab-5; Oncogene Research Products, Darmstadt, Germany) diluted 1: 10 000 in PBST and 1% bovine serum albumin. This primary antibody reacts with rat Fos protein but not with Jun protein. After three rinses in PBST, sections were incubated for 90 min at room temperature with biotinylated goat antirabbit antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1: 1000 in PBST, then for 60 min with preformed avidin-biotinylated horseradish peroxidase complex diluted 1: 100 in PBST. Visualization of bound peroxidase was achieved by reaction in a solution of 50 mM Tris-HCl, pH 7.4, containing 0.02% 3-3'-diaminobenzidine, 0.8% nickel chloride and 0.003% H_2O_2 to yield a blue-black precipitate. The reaction was stopped by three washes with PBST and the bound peroxidase was inactivated by 10 min incubation in PBS containing 1% H_2O_2 . Sections were then

exposed to a goat anti-CTb primary antibody (1: 10 000; List Biological Laboratories, Campbell, CA, USA) for 24 h at room temperature, rinsed with PBST and taken through all steps described above, except that a biotinylated horse antigoat secondary antibody (Vector Laboratories, Burlingame, CA, USA) was used. Tissue was exposed to 0.02% 3-3'-diaminobenzidine and 0.003% H₂O₂ which stained CTb-containing neurons in brown. Double-labelled cells (Fos-CTb) were easily identified by the presence of blue-black nuclear Fos staining and brown cytoplasmic CTb labelling. Sections were then mounted on gelatine-coated slides, stained with neutral red and cover-slipped with DePeX mounting medium (Gurr, Poole, UK).

For each rat, adjacent series of sections were coprocessed for single immunolabelling of CTb- or Fos-containing neurons. Controls omitting primary antibodies were routinely run with single- and double-labelling procedures to ensure the absence of nonspecific single or double immunostaining in the material.

Cell counts and analysis of the immunohistochemical data

Immunostained sections were analysed using a light microscope (Axioskop, Zeiss) equipped with a motorized X-Y sensitive stage and a video camera connected to a computerized image analysis system (Mercator; ExploraNova, La Rochelle, France). Quantitative analysis of double-labelled cells was performed on sections taken at 240- μ m intervals along the brain (84 sections from +6.00 to -14.16 mm relative to bregma; Paxinos & Watson, 2005). CTb-Fos double-labelled cells were plotted and counted per structure with a $\times 20$ objective, using the software Mercator. When a structure was present on more than one section, the neurons counted on all sections were summed. For each structure of interest, the mean $\pm$ SEM numbers of CTb-Fos double-labelled cells were counted on the sides ipsilateral and contralateral to the CTb injection and summed. In addition, single-immunostained sections for CTb were analysed in the medial PFC, one of the main projection to the VTA, to determine whether the retrograde transport was similar in groups of rats receiving amphetamine or saline. CTb-labelled neurons were counted on five sequential sections and the sum of ipsilateral and contralateral counts was expressed as mean $\pm$ SEM.

For construction of the atlas figures, drawings of the outlines of the sections and the major structures were made at low magnification ($\times 2.5$ objective) and CTb single-labelled and CTb-Fos double-labelled cells were plotted at higher magnification ($\times 20$ objective) using the mapping software Mercator. The figures were then imported into Adobe Illustrator for plate production. Photomicrographs were captured with a digital Nikon Coolpix camera and the images were imported into Adobe Photoshop and edited minimally, and then into Adobe Illustrator for plate production.

Statistical analysis

The mean numbers of crossovers was analysed with an unpaired Student's t-test. Comparisons of the mean numbers of the CTb-Fos double-labelled cells in each structure of interest and of CTb single-labelled cells in the medial PFC were made using the Mann-Whitney U-test. The level of significance was set at $P < 0.05$.

Results

Behavior

Figure 1 shows the locomotor activity exhibited by rats administered with amphetamine or saline. As expected, an injection of 5 mg/kg of amphetamine produced a strong increase in locomotor activity relative to an injection of saline (saline, 20 ± 5 vs. amphetamine, $247 \pm$

60 crossovers in 2 h; $P < 0.05$). This locomotor activation was associated with the occurrence of focused stereotypies (data not shown).

Injection sites and general localization of CTb retrogradely labeled cells

All the injection sites were targeted to the middle third of the VTA (bregma -5.2 to -5.5 mm). They typically consisted of a densely stained CTb core centred on the parabrachial pigmented and paranigral nuclei, and surrounded by a halo of lighter and more diffuse staining (Fig. 2). The spread of CTb usually filled almost the entire rostrocaudal extent of the VTA on one side of the brain. Animals with CTb invading the medial substantia nigra and resulting in labelling of cells in the medial striatum were excluded from the study. In no case was electrolytic lesion of the tissue detected as determined on sections only stained with neutral red.

Analysis of sections single-labelled for CTb revealed that the overall pattern of retrogradely labelled cells was consistent with previous studies characterizing afferent projections to the VTA with retrograde tracers (Phillipson, 1979; Geisler & Zahm, 2005, 2006). As illustrated in Fig. 3, retrogradely labelled neurons were distributed throughout the rostrocaudal axis of the brain. They were mostly centred on the medial forebrain bundle, with the exception of the PFC, septal nuclei, lateral habenula and periaqueductal grey. In the case of the descending afferents, the majority of CTb-positive neurons were localized ipsilaterally to the injection site. In the case of the ascending afferents, retrogradely labelled cells were observed in similar numbers on the ipsilateral and contralateral sides.

Distribution of Fos-labeled cells in saline- and amphetamine-treated rats

Animals injected with saline showed a very weak expression of Fos in the brain. The Fos-labelled cells were often associated with brain sensory systems (visual, auditory and piriform cortices, anterior olfactory and suprachiasmatic nuclei, superior and inferior colliculi) and a few other nuclei such as the paraventricular thalamic and pontine nuclei.

As expected, acute amphetamine considerably increased Fos expression in numerous regions of the brain (Klitenick et al., 1995; Badiani et al., 1998; Uslaner et al., 2001). Two hours after amphetamine, abundant Fos-labelled cells were found in most neocortical and allocortical regions, cerebellum, basal ganglia (except the external globus pallidus), the islands of Calleja and the extended amygdala, as well as in a number of thalamic and hypothalamic nuclei. Fos-labelled cells were also observed in the mesencephalon (superior colliculi, periaqueductal grey and caudal VTA, for example) and the pons and medulla oblongata [dorsal raphe nucleus (DR), parabrachial nuclei and nucleus of the solitary tract]. The expression of Fos remained elevated in most structures up to 7 h after amphetamine (data not shown).

Distribution and quantification of neurons projecting to the VTA and expressing Fos after amphetamine

The quantitative analysis of CTb–Fos-labelled cells was performed in saline- and amphetamine-treated rats that presented similar localization and spread of the tracer ($n = 5$ in each group). In these two groups of selected rats, single-immunostained sections for CTb revealed no significant difference in the total number of retrogradely labelled cells quantified in the medial PFC.

As shown in Table 1, amphetamine increased almost seven-fold the total number of neurons projecting to the VTA and expressing Fos. The CTb–Fos double-labelled cells were distributed, in low to high numbers, in a large number of structures that extended throughout the brain as schematically depicted in Fig. 3 and Table 1.

Forebrain

Amphetamine strongly increased the number of CTb–Fos double-labelled cells in specific areas of the forebrain such as the PFC (14 ± 4 vs. 224 ± 57 cells in saline and amphetamine groups, respectively; $P < 0.01$), the NAc (6 ± 2 vs. 44 ± 13 , $P < 0.01$), the ventral pallidum (VP; 3 ± 2 vs. 60 ± 19 , $P < 0.01$) and a continuous band encompassing the lateral preoptic area (LPO) and the lateral hypothalamus (LH; 32 ± 7 vs. 137 ± 30 , $P < 0.05$; Table 1). A small but significant increase in the number of CTb–Fos double-labelled cells was also observed in the dorsal tenia tecta, the claustrum, the dorsal endopiriform nucleus, the olfactory tubercle, the bed nucleus of the stria terminalis, the caudal part of the lateral septum and some other structures in the hypothalamus (Fig. 3C–I and Table 1). In general, double labelling occurred bilaterally with a clear ipsilateral predominance (from 78% in the claustrum to 98% in the NAc). On the contralateral side, double-labelled neurons were seen in homotypic loci.

Prefrontal cortex—As shown in Table 1, the PFC contained the greatest number of CTb–Fos double-labelled cells after amphetamine, accounting for 18.5% of the CTb–Fos cells observed in the entire brain. They were mostly found in the medial PFC, predominantly distributed in the prelimbic (PrL), cingulate (Cg) and medial orbital (MO) cortices and with a limited number in the infralimbic cortex (IL; Fig. 3A–C, Table 1). Interestingly, 30–50% of the CTb–Fos neurons of the medial PFC were located in its most rostral part, corresponding to the MO and anterior part of the PrL. A substantial number were also observed in the ventral–lateral orbital cortex (Fig. 3A, Table 1) and some in the dorsal peduncular cortex.

Most of the CTb–Fos double-labelled cells displayed a typical pyramidal morphology, i.e. a triangular soma with a long apical dendrite orientated perpendicular to the pial surface (Fig. 4). These cells were present almost exclusively in layer V of the Cg, PrL, IL and MO. Occasionally, nonpyramidal double-labelled cells were found in layer VIb of the PrL and IL. In the MO, CTb–Fos double-labelled cells were round, multipolar and with a large-sized nucleus (Fig. 4).

Nucleus accumbens—The NAc contained 3.6% of the double-labelled cells observed in the brain after amphetamine. These cells were located in the core and the medial part of the shell region. In the shell, a clear rostrocaudal gradient was observed, with the CTb–Fos double-labelled neurons being found mostly in the caudal and dorsal part of the structure, ventrally to the lateral ventricle (Fig. 3C and D). These cells appeared medium-sized, round, multipolar with short and thin dendrites, a morphology typical of medium spiny neurons (Fig. 4).

Ventral pallidum—In the VP, CTb–Fos double-labelled neurons were mostly found in the medial and anterior parts of the structure (Fig. 3E and F), accounting for 5% of the double-labelled cells observed in the brain after amphetamine. These CTb–Fos neurons were large-sized and fusiform in shape (Fig. 4).

Hypothalamus—In the hypothalamus, CTb–Fos double-labelled cells were mostly observed in the LPO–LH, in a band centred on the medial forebrain bundle (Fig. 3E–H). Taken together, the LPO and LH exhibited 11.3% of the total CTb–Fos double-labelled cells observed after amphetamine and thus contained the second greatest number of Fos-expressing neurons projecting to the VTA. The CTb–Fos cells were elongated and medium-sized, and regularly distributed inside and all along the structure (Fig. 4).

A few scattered CTb–Fos double-labelled neurons were also found in the diagonal band–magnocellular preoptic nucleus and the posterior hypothalamus, and their number was significantly enhanced after amphetamine ($P < 0.05$; Fig. 3E and F, Table 1). Occasional CTb–Fos double-labelled neurons were observed in the medial preoptic area and dorsomedial hypothalamic nucleus but their numbers were not affected by amphetamine.

Thalamus and epithalamus—In the thalamus and epithalamus, CTb–Fos double-labelled cells were observed in the lateral habenula and the zona incerta but their numbers were not altered by amphetamine.

Midbrain

A group of non-DA cells, i.e. Fos-positive but tyrosine hydroxylase-negative cells (data not shown), was found to contain both CTb and Fos after amphetamine; this group was just caudal to the injection site and ventral to the tegmental decussation. However, due to the proximity to the injection site, the specificity of the CTb uptake should be interpreted with caution. A significantly higher number of CTb–Fos double-labelled neurons was found in the deep mesencephalic nucleus after amphetamine ($P < 0.05$; Fig. 3K, Table 1). CTb–Fos double-labelled cells were also observed clustered in the supraocular periaqueductal grey and the superior colliculi. Although their numbers were slightly higher after amphetamine, the increase did not reach statistical significance.

Pons and medulla

Amphetamine increased considerably the number of CTb–Fos double-labelled cells in the pontomesencephalic tegmentum (5 ± 3 vs. 133 ± 30 cells, $P < 0.01$) and to a lesser extent in the DR (4 ± 2 vs. 66 ± 14 cells, $P < 0.01$; Fig. 3L and M, Table 1). A significantly higher number of CTb–Fos neurons was also observed after amphetamine in the dorsomedial tegmental area ($P < 0.01$), the oral part of the pontine reticular nucleus ($P < 0.05$) and the central grey ($P < 0.05$; Fig. 3L and M, Table 1). Very few CTb–Fos double-labelled cells were seen in the median and paramedian raphe nuclei and the parabrachial nuclei, and their numbers were not altered with amphetamine.

Dorsal raphe nucleus—In the DR, CTb–Fos double-labelled cells were found in the medial and lateral parts of the nucleus, from the rostral to the caudal part, and accounted for 5.5% of all CTb–Fos double-labelled cells observed after amphetamine. They were bilaterally distributed, with an ipsilateral predominance (Figs 3L and 4).

Pontomesencephalic tegmentum—The pontomesencephalic tegmentum, like the LPO–LH, contained the second greatest number of Fos-expressing neurons projecting to the VTA after amphetamine (11% of all CTb–Fos double-labelled cells). These double-labelled cells were mostly observed in the caudal PPTg and subpeduncular tegmental nucleus (SPTg; Fig. 3L, Table 1). These CTb–Fos cells were large-sized with large dendrites in the PPTg (Fig. 4); they were elongated in shape and horizontally orientated in the SPTg (Fig. 4). They were equally distributed on ipsilateral and contralateral sides. The PPTg and SPTg presented a unique feature. In contrast to the forebrain structures where CTb–Fos double-labelled cells generally represented less than one-fifth of the CTb-labelled cells, they accounted for a third of the CTb-labelled cells in the SPTg and nearly half of the CTb-labelled cells in the PPTg.

CTb–Fos double-labelled neurons were also increased in the LDTg (Fig. 3M, Table 1), although to a lesser extent than in the PPTg and SPTg. Contrary to the PPTg and SPTg, the relative proportion of CTb–Fos neurons accounted for less than one-tenth of all CTb-labelled cells of the LDTg.

Discussion

The present study was conducted to identify the projection pathways to the VTA that potentially contribute to the initiation of amphetamine behavioural sensitization. Our results demonstrate that a single exposure to amphetamine that has previously been shown to cause long-lasting behavioural sensitization (Robinson et al., 1982; Vanderschuren et al., 1999; Faleiro et al., 2003; Colussi-Mas et al., 2005; Panayi et al., 2005) activated a wide population of neurons projecting to the VTA which are distributed from the PFC to the medulla oblongata. More than half of all neurons expressing Fos after amphetamine and projecting to the VTA were located in a limited number of structures including the PFC (18.5%), the LPO-LH (11.3%), the pontomesencephalic tegmentum (11%), the DR (5.5%), the VP (5%) and the NAc (3.6%). The remaining of CTb-Fos double-labelled cells observed after amphetamine were found scattered in a large number of VTA afferent structures such as the claustrum, the bed nucleus of the stria terminalis, the lateral septum, the diagonal band-magnocellular preoptic nucleus, the deep mesencephalic nucleus, the oral part of the pontine reticular nucleus and the dorsomedial tegmental area. This pattern of distribution of the activated VTA afferents is consistent with the high level of connectivity of the VTA that underlies its high capacity to integrate various forms of information (Geisler & Zahm, 2005).

Considering the crucial role of the VTA in the early steps that lead to psychostimulant sensitization, these data provide new insights into the circuitry potentially involved in the initiation of this complex process. The detection of Fos in pyramidal cells projecting to the VTA located in the rostral medial PFC, predominantly in the MO and the rostral part of the PrL, brings support for the activation of a direct cortical-VTA glutamatergic pathway after amphetamine. This is in accordance with evidence that glutamatergic neurotransmission in the VTA is important for neuroadaptations related to the initiation of behavioural sensitization (see Introduction). Activation of glutamatergic transmission is thought to participate in the enhancement of excitatory synaptic strength onto VTA DA neurons (Saal et al., 2003; Faleiro et al., 2004) that leads to a transient excitability and increase in the firing rate of these neurons during early withdrawal (Zhang et al., 1997; Marinelli et al., 2006 for review). This short-lived increase is believed to be critical in initiating and transferring sensitization to forebrain regions. In this context, a necessary role of the medial PFC, one of the main sources of glutamatergic inputs to the VTA (Sesack & Pickel, 1992; Geisler et al., 2007), in the initiation of sensitization has been postulated. This was supported by the observation that the development of sensitization was prevented by ibotenic acid lesion of the medial PFC (Wolf et al., 1995; Cador et al., 1999) and that, conversely, its tetanic stimulation sensitized animals to subsequent cocaine administration (Schenk & Snow, 1994).

However, recent anatomical studies analysing the synaptic links between cortical afferents and VTA DA neurons raised the question of how direct cortical glutamatergic input to the VTA exerts its excitatory influence on mesolimbic DA neurons. Indeed, Carr & Sesack (2000) showed that, within the VTA, terminals of excitatory projections from the medial PFC do not form synapses onto mesolimbic DA neurons but rather on mesocortical DA neurons or GABAergic neurons projecting to the NAc. One possibility is that cortical inputs making synapses onto mesoaccumbens DA neurons arise predominantly from the rostral part of the medial PFC and the medial orbital cortex, two areas that were not totally included in the tracer injection sites of Carr & Sesack (2000) and that might have been underestimated in this tract-tracing study. Another possibility is that the medial PFC influences mesolimbic DA neurons via polysynaptic pathways involving intrinsic neurons of the VTA such as the mesolimbic GABAergic neurons or mesocortical DA neurons. Of course, it remains likely that in addition to its direct connection to the VTA the medial PFC

contributes to the induction of sensitization through neurons extrinsic to the VTA that in turn send glutamatergic projections to the VTA. Such an indirect mechanism is supported by the recent demonstration of widespread subcortical glutamatergic inputs to the VTA from regions that receive PFC projections (Sesack et al., 1989; Geisler et al., 2007; Omelchenko & Sesack, 2007) and the observation that PFC stimulation produces excitatory responses in VTA cells with latency inconsistent with a monosynaptic projection (Tong et al., 1996).

Although having not received much attention in sensitization, many other VTA afferents we found activated by amphetamine are known to activate or inhibit DA neurons and, by this means, may differently modulate the initiation of behavioural sensitization. This is the case in the pontomesencephalic tegmentum, particularly the PPTg and the LDTg, which provides the main cholinergic input to the VTA (Oakman et al., 1995; Omelchenko & Sesack, 2006) as well as a glutamatergic input (Clements & Grant, 1990; Charara et al., 1996; Geisler et al., 2007). It is interesting to note that, contrary to what has been observed with the medial PFC glutamatergic output, the excitatory neurons of the LDTg, and presumably the PPTg, synapse on the mesolimbic DA neurons of the VTA (Omelchenko & Sesack, 2005, 2006). The stimulation of both the PPTg and LDTg has been shown to activate VTA DA neurons (Floresco et al., 2003; Lodge & Grace, 2006a, b). Moreover, behavioural studies indicate that lesions of the PPTg or the LDTg (Laviolette et al., 2000; Alderson et al., 2003; Nelson et al., 2007), as well as intra-VTA nicotinic and glutamatergic antagonists (Vezina & Queen, 2000; Schoffelmeer et al., 2002), prevent behavioural sensitization. Thus, one can suggest that cholinergic and/or glutamatergic projections from the pontomesencephalic tegmentum contribute to the initiation of behavioural sensitization to amphetamine by a direct excitatory drive to mesolimbic DA neurons. Also, because LDTg and PPTg receive a substantial input from the medial PFC (Sesack et al., 1989), these structures may provide an indirect route for the PFC to affect mesolimbic DA neuron activity.

The LPO–LH is another major VTA afferent activated by amphetamine. The VTA-projecting neurons in this area contain a large variety of neurotransmitters, notably glutamate (Geisler et al., 2007), and peptides such as neurotensin (Geisler & Zahm, 2006) and orexin (Fadel & Deutch, 2002). These two peptides increase the firing rate of VTA neurons and DA release in the NAc (Shi & Bunney, 1991; Sotty et al., 1998; Korotkova et al., 2003; Vittoz & Berridge, 2006) and are likely to contribute to the cellular and behavioural adaptations induced by psychostimulants (Panayi et al., 2005; Borgland et al., 2006). Although the LPO–LH is regarded as a critical element in motivation and reward circuits activated by drugs of abuse (DiLeone et al., 2003), the participation of these specific pathways in sensitization has not been clearly determined.

Interestingly, we also found that amphetamine activated neurons in the NAc and the VP, two brain structures that are predominantly GABAergic and provide the main inhibitory afferents to the VTA (Maeda & Mogenson, 1980; Zahm, 1989; Kalivas et al., 1993). The presence of GABA within these neurons raises the possibility that GABA release in the VTA constitutes a means to dampen drug-induced neurobehavioural plasticity (Gardner et al., 2002; Liu et al., 2005) or to determine, in concert with other afferents, the subsets of DA neurons that respond to excitatory inputs. Indeed, recent data suggest that GABAergic afferents to the VTA are in a position to select the population of DA neurons capable of exhibiting activation in response to PPTg stimulation (Floresco et al., 2003; Lodge & Grace, 2006a). However, the lack of information about the neurochemical identity of the activated neurons impedes further interpretation of the data. This is also the case for Fos-expressing neurons of the DR, which are probably nonserotonergic because psychostimulants, in releasing serotonin, activate somatodendritic impulse-regulating autoreceptors that inhibit neuronal activity (Rebec et al., 1982; Cunningham & Lakoski, 1990). Clearly, many additional studies are required not only to identify the phenotype of neurons activated by

amphetamine and their synaptic link with subpopulations of DA, GABAergic and possibly glutamatergic neurons of the VTA (Yamaguchi et al., 2007), but also to determine whether altered activity within these projections participate in or modulate the initiation of behavioural sensitization.

The distribution of Fos-CTb neurons in a large number of brain areas indicates that the VTA receives feedback signals after amphetamine that are likely to supply a different kind of information. The medial PFC is involved in a variety of cognitive and emotional processes (Heidbreder & Groenewegen, 2003, for review), and human and animal studies support a general role for the MO in the processing of the reward value of natural reinforcers like food (Arana et al., 2003; Rolls, 2004) and drugs of abuse (Porrino & Lyons, 2000; Volkow et al., 2005). Other subcortical afferents such as the LPO-LH, NAc, PPTg-LDTg and DR are more likely to convey less processed signals that encode internal physiological states, environment saliency or behavioural arousal. Although each population of VTA afferents may provide unique information, the fact that they are embedded in a complex network of interconnected structures (Geisler & Zahm, 2005) suggests that they may act in concert to convey a wide range of information on the internal and external environment. Such organization of these activated inputs to the VTA may contribute to the modulation of the pharmacological effect of amphetamine. Indeed, a large body of evidence demonstrates that drug-induced neurobehavioural plasticity is the result of complex drug-environment interactions. In particular, minor differences in the environmental context in which amphetamine is administered, home cage vs. an environment distinctive from the home cage, for example, have a profound effect on their ability to induce psychomotor sensitization, on its discriminative properties and on the circuitry solicited by this drug (Robinson et al., 1998; Badiani & Robinson, 2004; for reviews). In this regard, one may hypothesize that the recruitment of amphetamine-activated VTA afferents differs according to the context of its administration and constitutes part of the neural bases of the modulatory actions of the environment.

In conclusion, these results represent the first systematic study on the localization of VTA afferents activated by acute amphetamine in order to further the investigation and understanding of the circuitry of sensitization. It reveals that the PFC, the pontomesencephalic tegmentum and the LPO-LH, as well as the DR, the VP and the NAc to a lesser extent, are the main sources of amphetamine-stimulated neurons projecting to the VTA. Moreover, by pointing to structures not commonly associated with behavioural sensitization to psychostimulants such as the PPTg-LDTg, LPO-LH and DR, it brings a new light to the neuronal network potentially involved in the early events that lead to amphetamine-induced neuroplasticity.

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Abbreviations

Cg	cingulate cortex
CTb	cholera toxin b subunit
DA	dopamine or dopaminergic
DR	dorsal raphe nucleus
IL	infralimbic cortex

LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamus
LPO	lateral preoptic area
MO	medial orbital cortex
NAc	nucleus accumbens
PB	phosphate buffer
PBS	10 mM PB, pH 7.4, containing 0.9% NaCl
PBST	PBS containing 0.3% Triton X-100
PFC	prefrontal cortex
PPTg	pedunculopontine tegmental nucleus
PrL	prelimbic cortex
SPTg	subpeduncular tegmental nucleus
VP	ventral pallidum
VTA	ventral tegmental area

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Fig. 1.

Effect of amphetamine on locomotor activity. After a 2-h period of habituation to the activity cage, animals were given a single injection of amphetamine (5 mg/kg, s.c., $n = 4$) or saline ($n = 4$). The time course of crossovers (mean + SEM) measured during the whole behavioural experiment is presented on the left. The locomotor response expressed as mean (+ SEM) crossovers cumulated during the 2-h period following amphetamine or saline injection is presented on the right. * $P < 0.05$ compared to saline.

Fig. 2. Photomicrograph of a coronal section of the brain illustrating a representative iontophoretic deposit of CTb in the VTA with a 1- μ A pulsed positive current.

Fig. 3.

Schematic representation of the distribution of CTb-immunoreactive cells (grey dots) and CTb-Fos double-labelled cells (see blue squares on-line) after amphetamine (5 mg/kg, s.c.) in a representative animal unilaterally injected with CTb in the VTA. Abbreviations: III, oculomotor nucleus; VII, facial nucleus; 3V, 3rd ventricle; 4V, 4th ventricle; AcbS, nucleus accumbens, shell; AH, anterior hypothalamic area; A-O, schematic representation; AOP, anterior olfactory nucleus, posterior part; Aq, aqueduct; BLA, basolateral amygdaloid nucleus, anterior part; BNST, bed nucleus of the stria terminalis; Cb, cerebellum; Ce, central amygdaloid nucleus; Cg, cingulate cortex; Cl, claustrum; CPu, caudate putamen; D3V, dorsal 3rd ventricle; DC, dorsal cochlear nucleus; DM, dorsomedial hypothalamic nucleus; DpMe, deep mesencephalic nucleus; DR, dorsal raphe nucleus; DTgC, dorsal tegmental nucleus, central part; DTT, dorsal tenia tecta; E/OV, ependymal and subependymal layer/olfactory ventricle; EGP, external globus pallidus; f, fornix; fi, fimbria of the hippocampus; fr, fasciculus retroflexus; g7, genu of the facial nerve; GABA, gamma aminobutyric acid; Gi, gigantocellular reticular nucleus; IC, inferior colliculus; ic, internal capsule; icp, inferior cerebellar peduncle; IL, infralimbic cortex; IP, interpeduncular nucleus; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; LHb, lateral habenula; lo, lateral olfactory tract; LPO, lateral preoptic area; LSI, lateral septal nucleus, intermediate part; LV, lateral ventricle; Me, medial amygdaloid nucleus; ml, medial lemniscus; MO, medial orbital cortex; MPA, medial preoptic area; MPO, medial preoptic nucleus; MRe, mammillary recess of the 3rd ventricle; MVe, medial vestibular nucleus; NAc, nucleus accumbens; PAG, periaqueductal grey; PFC, prefrontal cortex; Pir, piriform cortex; PM, premammillary nucleus; PMCo, posteromedial cortical amygdaloid nucleus; Pn, pontine nuclei; PnC, pontine reticular nucleus, caudal part; PnO, pontine reticular nucleus, oral part; PPTg, pedunculopontine tegmental nucleus; PrL, prelimbic cortex; py, pyramidal tract; R, red nucleus; RIP, raphe interpositus nucleus; s5, sensory root of the trigeminal nerve; SC, superior colliculus; scpd, superior cerebellar peduncle, descending limb; SFO, subfornical organ; sm, stria medullaris of the thalamus; SNR, substantia nigra, reticular part; SO,

superior olive; sp5, spinal trigeminal tract; SPTg, subpeduncular tegmental nucleus; sumd, supramammillary decussation; Tu, olfactory tubercle; VDB, nucleus of the vertical limb of the diagonal band; VM, ventromedial thalamic nucleus; VO, ventral orbital cortex; VP, ventral pallidum; VTA, ventral tegmental area; ZI, zona incerta.

Fig. 4. Photomicrographs of Fos- and CTb-immunostained sections after amphetamine (5 mg/kg, s.c.) within the prelimbic cortex (A), the medial orbital cortex (B), the nucleus accumbens (C), the ventral pallidum (D), the lateral hypothalamus (E), the dorsal raphe nucleus (F) the pedunculopontine tegmental nucleus (G) and the subpeduncular tegmental nucleus (H). Fos-positive nuclei appear black and CTb-immunoreactive cells appear brown; arrows indicate Fos-CTb double-labelled neurons.

Table 1

Distribution and quantification of CTb-Fos double-labelled cells in the brain after saline or amphetamine injection (s.c.) in animals unilaterally injected with CTb in the VTA.

Area	Number of CTb-Fos double- labelled cells	
	Saline	Amphetamine [†]
Forebrain		
Prefrontal cortex	14 ± 4	224 ± 57 **
Ventral/lateral orbital cortex	2 ± 1	71 ± 28 **
Medial prefrontal cortex	10 ± 3	133 ± 28 **
Dorsal peduncular cortex	2 ± 1	17 ± 5 **
Dorsal tenia tecta	1 ± 1	10 ± 3 *
Clastrum	3 ± 1	20 ± 6 *
Dorsal endopiriform nucleus	2 ± 1	8 ± 3 *
Nucleus accumbens	6 ± 2	44 ± 13 **
Core	2 ± 1	11 ± 5 *
Shell	4 ± 2	30 ± 8 *
Olfactory tubercle	0 ± 0	4 ± 1 *
Bed nucleus of the stria terminalis	3 ± 1	12 ± 3 *
Amygdala	3 ± 1	9 ± 4
Lateral septum	1 ± 0	16 ± 7 **
Medial septal nucleus – Diagonal band	3 ± 1	14 ± 6
Ventral pallidum	3 ± 2	60 ± 19 **
Hypothalamus		
Medial preoptic area	3 ± 1	8 ± 3
Median preoptic nucleus	0 ± 0	2 ± 1
Medial preoptic nucleus	0 ± 0	1 ± 1
Diagonal band – Magnocellular preoptic nucleus	2 ± 1	17 ± 5 *
Paraventricular hypothalamic nucleus	0 ± 0	2 ± 1
Anterior hypothalamic area	2 ± 1	3 ± 1
Lateral preoptic area – lateral hypothalamus	32 ± 7	137 ± 30 *
Lateral preoptic area	9 ± 4	38 ± 10 *
Lateral hypothalamus	23 ± 4	99 ± 24 **
Dorsal hypothalamic area	1 ± 1	3 ± 2
Dorsomedial hypothalamic nucleus	3 ± 2	7 ± 3
Posterior hypothalamus	4 ± 2	11 ± 3 *
Zona incerta	7 ± 3	17 ± 4
Thalamus		

Area	Number of CTb-Fos double- labelled cells	
	Saline	Amphetamine [†]
Lateral habenula	3 ± 1	28 ± 10
Paraventricular thalamic nucleus	0 ± 0	1 ± 1
Midbrain		
Periaqueductal gray	19 ± 9	51 ± 18
Deep mesencephalic nucleus	5 ± 2	28 ± 10 *
Superior colliculus	9 ± 4	30 ± 14
Pons and medulla		
Dorsal raphe nucleus	4 ± 2	66 ± 14 **
Median/paramedian raphe nucleus	2 ± 1	7 ± 3
Pontomesencephalic tegmentum	5 ± 3	133 ± 30 **
Pedunculopontine tegmental nucleus	1 ± 1	47 ± 10 **
Subpeduncular tegmental nucleus	1 ± 0	61 ± 19 **
Laterodorsal tegmental nucleus	3 ± 2	25 ± 7 *
Dorsomedial tegmental area	0 ± 0	14 ± 5 **
Pontine reticular nucleus, oral part	1 ± 1	24 ± 10 *
Parabrachial nuclei	2 ± 1	13 ± 5
Central gray	1 ± 1	13 ± 5 *
Other structures	38 ± 13	197 ± 58
Total	179 ± 54	1209 ± 298 **

[†] Dose of amphetamine, 5 mg/kg, s.c. Data are expressed as mean ± SEM of the sum of ipsilateral and contralateral double-labelled cells contained in a structure.

* P < 0.05,

** P < 0.01 relative to saline group.