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The Abused Inhalant Toluene Increases Dopamine Release in the Nucleus Accumbens by Directly Stimulating Ventral Tegmental Area Neurons

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Recreational abuse of toluene-containing volatile inhalants by adolescents is a significant public health problem. The mechanisms underlying the abuse potential of such substances remain unclear, but could involve increased activity in mesoaccumbal dopamine (DA) afferents innervating the nucleus accumbens (ACB). Here, using *in vitro* electrophysiology, we show that application of behaviorally relevant concentrations of toluene directly stimulates DA neurons in the ventral tegmental area (VTA), but not surrounding midbrain regions. Toluene stimulation of VTA neurons persists when synaptic transmission is reduced. Moreover, unlike *non*-DA neurons, the magnitude of VTA DA neuron firing does not decline during longer exposures designed to emulate 'huffing'. Using dual-probe *in vivo* microdialysis, we show that perfusion of toluene *directly* into the VTA increases DA concentrations in the VTA (somatodendritic release) and its terminal projection site, the ACB. These results provide the first demonstration that even brief exposure to toluene increases action potential drive onto mesoaccumbal VTA DA neurons, thereby enhancing DA release in the ACB. The finding that toluene stimulates mesoaccumbal neurotransmission by activating VTA DA neurons directly (independently of transynaptic inputs) provide insights into the neural substrates that may contribute to the initiation and pathophysiology of toluene abuse.
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INTRODUCTION

Inhalant abuse represents a major health problem because of its association with future drug use (NSDUH, 2003) and severe neurological/cognitive deficits (Filley, 2004). Many of these products (ie, spray paint, glue, etc.) including toluene and the abuse of pure toluene solvent, has been reported (Flanagan and Ives, 1994). Although overall drug use by teenagers has declined since 1998, inhalant use has increased (NSDUH, 2003). Inhalant use begins in preadolescence and continues well into adulthood (Rosenberg *et al*, 2002; NSDUH, 2003; Wu and Ringwalt, 2006; Cairney *et al*, 2005). Dopaminergic afferents arising from the ventral tegmental area (VTA) and projecting to the nucleus accumbens (ACB) are crucial elements in the neural circuits that mediate arousal, motivation, and reinforcement (Wise, 2002). Increased dopamine (DA) neurotransmission within this mesoaccumbal pathway mediates the reinforcing and psychomotor stimulant effects of drugs of abuse (Wise, 2002).

In experimental animals, toluene inhalation produces reinforcing effects (Bespalov *et al*, 2003; Blokhina *et al*, 2004; Funada *et al*, 2002; Weiss *et al*, 1979; Lee *et al*, 2006) and stimulates locomotor activity (Bowen, 2006; Riegel *et al*, 2003). Depletion of DA in the ACB attenuates this latter effect (Riegel *et al*, 2003). Toluene also induces c-FOS activation in both the VTA and ACB (Lo and Chen, 2005), and increases the firing and bursting of VTA DA neurons *in vivo* (Riegel and French, 1999a). Although acute toluene exposure increases TTX-sensitive DA release in the dorsal striatum (Stengard *et al*, 1994), similar effects have not been reported in the ACB (Kondo *et al*, 1995; Gerasimov *et al*, 2002b). Therefore, some investigators have concluded that a DA-independent (Spanagel and Weiss, 1999) or an, as yet, undetermined (Hyman and Malenka, 2001) mechanism mediates the actions of toluene. Such conclusions, if incorrect, may confound future work seeking to identify the mechanisms underlying toluene abuse and hamper the development of

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prevention strategies for toluene addiction (Beyer *et al*, 2001; Schiffer *et al*, 2006).

DA neurons are spontaneously active (Grace and Bunney, 1983) and their increased action potential firing stimulates DA release in the ACB (Wise, 2002). DA firing is influenced also by multiple mediators including various trans-synaptic inputs, local (GABAergic) interneurons, and somatodendritic DA released from VTA DA neurons (Adell and Artigas, 2004; Marinelli *et al*, 2006; Beckstead *et al*, 2004). Therefore, toluene may influence one or more neurotransmitter systems and the effects observed may vary depending upon the treatment regimen employed and the region or subregion examined. Indeed, the VTA is a heterogeneous structure composed of DA and non-DA (ie, γ-GABA) neurons (Ikemoto and Wise, 2004; Van Bockstaele and Pickel, 1995). Furthermore, recent work has shown that infusion of ethanol and other drugs into the posterior VTA elicits positive reinforcing effects, whereas anterior VTA infusion does not (Zangen *et al*, 2006; Rodd *et al*, 2004b; Ikemoto and Wise, 2004). Previous studies investigating toluene did not determine where toluene acts to alter the activity of midbrain DA neurons, and if these changes are sufficient to increase DA release in the ACB (Riegel and French, 1999a).

The present studies determined whether *in vivo* behaviorally relevant concentrations of toluene directly stimulate mesoaccumbal neurotransmission by a direct activation of VTA DA neurons. *In vitro* brain-slice experiments revealed that toluene displays anatomical specificity for VTA DA neurons that persists in treatments that reduce synaptic transmission and during longer, escalating exposures designed to emulate 'huffing'. Dual-probe microdialysis studies in awake animals revealed that perfusion of toluene into the VTA increases extracellular DA concentrations in both the VTA and distal ACB. Parts of this data have been presented previously in preliminary form (Riegel and French, 2002).

METHODS

Toluene

A stock solution of toluene in polyoxyethylene-sorbitan mono-oleate (Tween-80, final concentration 0.02%) was prepared immediately before use with care taken to avoid any solvent loss. For electrophysiological recordings, the stock solution was diluted in oxygenated aCSF (see below) and delivered (2 ml/min) to the recording chamber (35°C) by a gravity-feed (plasticizer-free) perfusion system. Toluene was bath applied using two separate protocols. In *single applications*, each concentration of toluene was applied to the bath for 3 min to determine reversibility. Existing studies indicate that inhalant abuse is characterized by a rapid intoxication (Meredith *et al*, 1989) and a related steep rise in brain toluene concentrations (Gerasimov *et al*, 2002a; Ameno *et al*, 1992; Kishi *et al*, 1988). Thus, as with other drugs (Balster and Schuster, 1973), the abuse liability of toluene may be affected by pharmacokinetic properties (Gerasimov *et al*, 2002a; Lammers *et al*, 2005) and supplemental 'huffs' may be required to preserve the 'high' reported by users (NSDUH, 2003). Therefore, to better model these conditions, a *staircase paradigm* was employed. In this paradigm, escalating concentrations of toluene (seven concentrations at 3 min/concentration) were applied to the bath and the total duration of toluene exposure was increased (21 min). Concentrations of toluene in perfusate samples taken from the recording chamber were determined by gas chromatograph (Riegel and French, 1999a) and were adjusted to match *in vivo* concentrations (4–79 μg/ml) observed in the blood of rodents (Riegel and French, 1999a) and humans exposed to toluene (Garriott *et al*, 1981). The drug reached the recording chamber in ~30 s, and the fluid in the chamber completely turned over in 3 min.

For microdialysis, known aliquots of the toluene stock solution were diluted in aCSF and sealed immediately just before reverse-dialysis. Data generated under conditions similar to those of the present study have shown that 10–30% of the drug concentration in the perfusion buffer reaches brain tissue surrounding the dialysis probe (Yim and Gonzales, 2000; Gonzales *et al*, 1998). Thus, perfusate concentrations of toluene (1, 3, 10 mM) are expected to generate *in situ* concentrations of 0.1, 0.3, and 1 mM, which are in accord with the *in vitro* concentrations employed (23–822 μM) and previous *in vivo* studies (for a discussion see Riegel *et al*, 2003).

In Vitro Extracellular Electrophysiology

Slice preparation. Brain slices were prepared from male Sprague–Dawley rats (PD 19-40) according to protocols approved by the University of Arizona IACUC (Wang and French, 1993b). Control experiments in brain slices prepared from older male Sprague–Dawley rats (PD 60) showed analogous (see below) toluene-induced changes in firing ($n=9$, data not shown). Coronal slices (400 μm) containing the VTA were incubated in oxygenated aCSF composed of (in mM) 124 NaCl, 2.5 KCl, 1.25 KH_2PO_4, 2.4 $MgSO_4$, 2.5 $CaCl_2$, 25.7 $NaHCO_3$, and 10 D-glucose. Where indicated, aCSF was altered by lowering Ca^{2+} and increasing Mg^{2+} to either 0.5 mM Ca^{2+}/10 mM Mg^{2+} (Media-1) or 0.25 mM Ca^{2+}/7.25 mM Mg^{2+} (Media-2). Flow rates were kept at 2 ml/min to insure adequate infusion of the altered media, consistent with previous published studies (Bondy and Harrington, 1979; Washio and Inouye, 1978; Alderdice and Volle, 1978; Scholfied, 1981). Incubation with such media for 5 min or greater has been shown to block synaptic transmission (Bondy and Harrington, 1979; Washio and Inouye, 1978; Alderdice and Volle, 1978; Scholfied, 1981; Hutter and Kostial, 1954). Extracellular electrophysiological recordings were made with glass microelectrodes filled with a solution of 2% pontamine sky-blue in 0.5 M sodium acetate (Yin and French, 2000). Action potential signals were amplified, and filtered (1–10 kHz) (Wang and French, 1993a; Marinelli *et al*, 2006).

Recordings. Recording sites were found with a dissection microscope and confirmed histologically *post hoc* (see below: 'Localization of recording sites and microdialysis probes'). All locations were anatomically differentiated by reference to the rat atlas (Paxinos and Watson, 1988). VTA neurons were identified according to conventional pharmacological and electrophysiological criteria (Grace and Bunney, 1983; Grace and Bunney, 1984; Johnson and North,

1992; Cameron *et al*, 1997; Marinelli *et al*, 2006). DA neurons possess: (1) bi- or tri-phasic action potentials with a somatodendritic notch, (2) action potential lasting >2 ms, (3) ~ 0.5 Hz firing rates, (4) inhibition to DA (50–100 μM), and (5) insensitivity to 5-HT (60 μM). Non-DA (type-II) neurons possess: (1) biphasic positive–negative action potentials lacking an S-D notch, (2) action potential lasting <2 ms, (3) 1–2 Hz firing rates, and (4) insensitivity to inhibition by DA (50–100 μM) and 5-HT (60 μM). *Non*-DA (type-III) neurons displayed: (1) positive–negative action potentials lacking a somatodendritic notch, (2) action potential lasting >2 ms, (3) ~ 1.5–2 Hz firing rates, and (4) inhibition to DA (50–100 μM) and 5-HT (60 μM). Consistent with previous electrophysiological studies, neurons in the interpeduncular nucleus (IPN) were classified by anatomical location (Lena *et al*, 1993; Takagi, 1984) and DA neurons in the retrorubral field (RRF) were identified by electrophysiological/pharmacological criteria analogous to those used for VTA DA neurons (Deutch *et al*, 1988). The identification of neurons in the rostral interstitial nucleus (RIN) was based upon *post hoc* histological analysis (below).

The *duration of inactivation* was defined as an episode of reduced action potential amplitude, below noise level, which sometimes occurred immediately after a period of pronounced neuronal excitation. Although the reversibility of toluene's actions permitted multiple challenges, only one cell was recorded from each brain slice.

In Vivo Microdialysis

Surgery. Male Sprague–Dawley rats (PD 70-80) were anesthetized with equithesin (1% pentobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propyleneglycol, 11% ethanol, 3 ml/kg i.p.). Microdialysis guide cannulae (CMA/11, CMA microdialysis, North Chelmsford, MA) were inserted stereotaxically (David Kopf instruments, Topanga, CA) and secured to the skull using stainless-steel screws and dental acrylic. Membrane probe length (VTA: 1 mm; ACB: 2 mm) was adjusted for stereotaxic location (VTA: AP −5.6, *L* −0.4, *V* −7.8; ACB: AP +1.7, *L* −1.4, *V* −6.4), where AP, *L*, and, *V* denote mm from bregma (Paxinos and Watson, 1988). After surgery, the animals were housed individually and allowed to recover for 2–4 days before experiments were started.

Microdialysis. Microdialysis was performed as described previously (Zapata and Shippenberg, 2005). The evening before the experiment, probes were inserted into the guide cannulae connected to a perfusion system consisting of 1 ml gastight syringes mounted on microdialysis pumps (CMA/102) and fitted with FEP tubing (CMA microdialysis) connecting the probes through dual-channel, quartz-lined, low-resistance swivels. Probes were perfused overnight (0.3 μl/min) and 1 h before experiments (1 μl/min) with aCSF (in mM: 145 NaCl, 2.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 0.25 ascorbic acid, 5.4 D-glucose, pH 6.5–7.0 adjusted with NaOH). During experiments, the VTA superfusate alternated between aCSF, vehicle (Tween-80, final concentration in aCSF 0.02%), or toluene (1, 3, and 10 mM; 1 h/concentration). Dialysis fractions were collected every 15 min, frozen (−80°C), and analyzed for DA content using HPLC-electrochemical detection within 48 h (Zapata and Shippenberg, 2005). Dialysate DA concentrations were quantified using an external standard curve. The detection limit of DA in dialysate was 0.3 nM.

Localization of Recording Sites and Microdialysis Probes

The anterior (−5.2 to −5.6 mm posterior to bregma) and posterior (−5.6 to −6.04 mm posterior to bregma) divisions of the VTA were defined as in other studies (Rodd *et al*, 2005; Zangen *et al*, 2006; Ikemoto and Wise, 2004; Carlezon *et al*, 2000; Carlezon and Nestler, 2002; Rodd-Henricks *et al*, 2000). For electrophysiology, recording sites were confirmed by iontophoresis of pontamine sky-blue dye at experiment completion (Wang and French, 1993a). Then, as described elsewhere (Johnson *et al*, 1996), brain slices were resectioned (5–10 μm), stained with cresyl violet, and examined by light microscopy. Microdialysis probe placement was confirmed after brains were removed, frozen, and sectioned on a cryostat (30 μm) as previously described (Zapata and Shippenberg, 2005). Only when probe location was confirmed in both the VTA and ACB (Paxinos and Watson, 1988), was data from that animal included for further analysis.

Data Analysis

For electrophysiology, only one neuron per slice was tested. Changes in neuron firing rates are expressed as a change from baseline (Hz). Significant differences between treatments were determined by a Student's paired *t*-test. All depicted action potential waveforms are averaged ($\geqslant 4$ spikes/trace). For microdialysis, basal DA levels in dialysate were determined in each animal and represent the average of four samples collected before vehicle perfusion and toluene exposure. For each concentration of toluene, four consecutive 15 min dialysate samples were obtained. The averaged values at each toluene concentration were compared to the effect of vehicle. Statistical significance was determined using a repeated measure one-way ANOVA and the Newman–Keuls test for *post hoc* comparisons.

Drugs and Reagents

Toluene (analytical grade), benzene (reagent grade), and carbon disulfide (reagent grade) were purchased from Fischer Scientific (Tustin, CA). DA and serotonin were purchased from Sigma Chemicals (St Louis, MO) and baclofen from RBI (Natick, MA).

RESULTS

In Vitro Extracellular Electrophysiology

Initial experiments using single applications determined whether toluene affects the excitability of VTA DA neurons in an isolated brain slice preparation. Short 3 min applications of toluene stimulated VTA DA neurons. In all cases, the increase in firing was concentration-dependent and firing rates returned to baseline upon washout (Figure 1a) (maximum 3.4 ± 0.3 Hz above baseline; $n = 17$; $p < 0.05$, data not shown). The maximum increase in firing was greater in

the anterior VTA (Figure 1d: 4.6 ± 0.4 Hz above baseline; $n = 7$), than the posterior VTA (Figure 1d: 2.8 ± 0.3 Hz above baseline; $n = 10$; $p < 0.01$). These *in situ* results resembled previous *in vivo* studies, with respect to both the basal properties of DA neurons (Table 1) and the reversible, toluene-induced increase in firing (Riegel and French, 1999a). In non-DA neurons, toluene stimulated the firing of type-II (Figure 1b; $n = 5$) and type-III cells ($n = 7$) (data not shown, but a sample type-III cell is shown in Figure 3b). At both anterior and posterior VTA locations, toluene induced similar increases in non-DA neuron firing. So, the data were grouped (Figure 1d: maximum 3.21 ± 0.63 Hz above baseline; $n = 12$). The stimulation of non-DA cells *in vitro* differs from the inhibition of firing previously observed *in vivo* (Riegel and French, 1999b), and may reflect the loss of monosynaptic glutamate inputs to neurons in a slice preparation (Tzschentke, 2001).

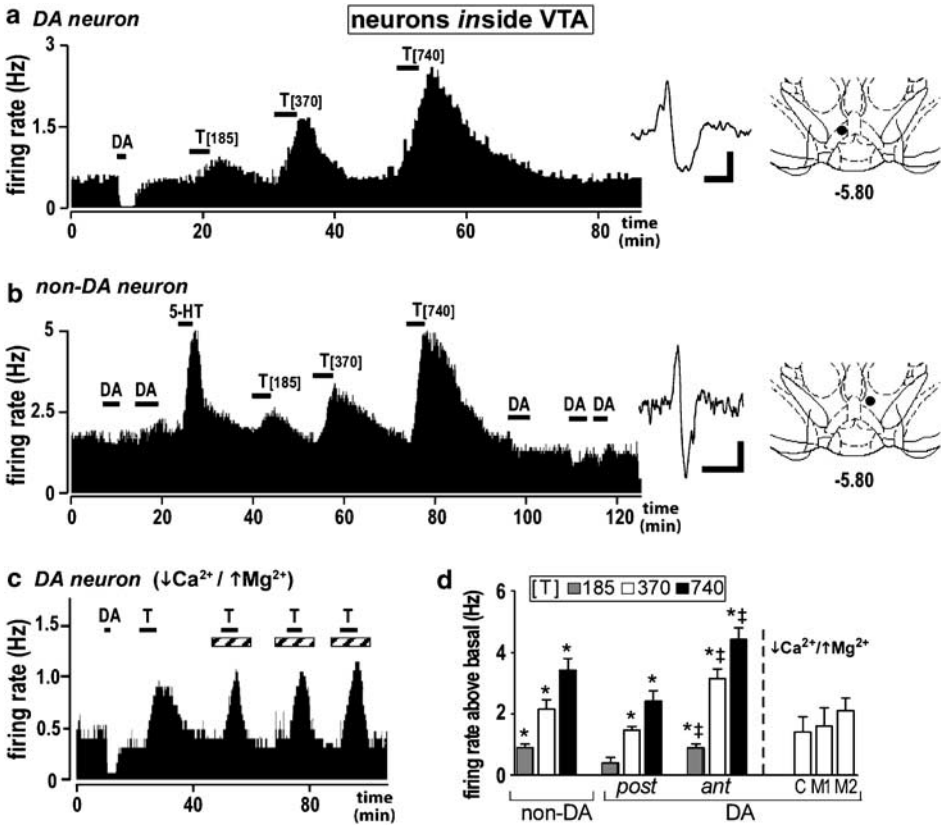

Figure 1 Toluene stimulates VTA neurons *in vitro* (single applications). Sample ratemeter histograms of a VTA (a) DA and (b) non-DA neuron during toluene (3 min/concentration). *Insets* on the right show matching action potential waveforms (calibration: 1 ms & 0.2 mV) and far right, the anatomical location of recording electrode (number is mm posterior from bregma). Numbers above bars in (a) and (b) indicate concentration (μM) of drugs (T: toluene; DA: 50–100 μM DA; 5-HT: 60 μM serotonin). (c) Toluene application (black bar, 370 μM) stimulates firing in a VTA DA neuron, even when inhibiting synaptic transmission by perfusion with high Mg^{2+}, low Ca^{2+} aCSF (hatched bars). (d) Summary of the toluene-induced changes in firing of VTA non-DA ($n = 12$) and DA neurons in the anterior (ant) VTA ($n = 10$) or posterior (post) VTA ($n = 7$). White bars on far right summarize results of high Mg^{2+}, low Ca^{2+} aCSF media (C: control, M1: media-1, M2: media-2). Error bars signify SEM. * Signifies significance at $p < 0.05$ *vs* vehicle; ‡ signifies significance at $p < 0.05$ *vs* posterior.

Table 1 Properties of Different Midbrain Neurons

						Change in firing (%) from baseline in the presence of:	
Neuron (*n*)	**Challenges (*n*)**	**Brain region**	**Neuron subtype**	**AP duration (ms)**	**Basal firing (Hz)**	**60 μM 5-HT**	**50–100 μM DA**
43	247	VTA	Type I DA	2.70±0.32	0.56±0.12	14.6±1.7	−96.4±3.1
11	63	VTA	Type II non-DA	1.80±0.17	1.90±0.11	8.4±4.1	−11.7±2.9
13	69	VTA	Type III non-DA	2.50±0.19	3.10±0.10	−94.1±4.2	−96.7±1.2
6	18	RRF	Type I DA	2.54±0.82	3.56±1.28	ND	−39.9±12.1
6	24	RIN	ND	1.61±0.12	3.90±0.12	ND	9.8±1.2
7	29	IPN	ND	1.21±0.12	11.00±2.70	ND	7.8±4.4

VTA, ventral tegmental area; RRF, retrorubral field; RIN, rostral interstitial nucleus; IPN, interpeduncular nucleus.

To determine whether toluene increased VTA firing by altering neurotransmitter release onto DA cells, we tested a low-Ca^{2+}, high-Mg^{2+} aCSF media, previously shown to inhibit synaptic transmission (Brodie *et al*, 1990; Katz, 1970; Katz and Miledi, 1970). The stimulatory properties of toluene (370 μM) were still apparent under these conditions (Figure 1c: 370 μM, single applications). As basal firing rates can vary under low Ca^{2+} conditions, initial results with toluene in media-1 (0.5 mM Ca^{2+}/10 mM Mg^{2+}; Figure 1d: 1.73 ± 0.86 Hz above baseline; $n = 5$) were confirmed with experiments in media-2 (0.25 mM Ca^{2+}/7.25 mM Mg^{2+}; Figure 1d: 2.32 ± 1.01 Hz above baseline; $n = 5$). The magnitude of the toluene-induced increase in firing did not differ between media (Figure 1d; $p > 0.05$, ANOVA), suggesting that toluene directly activates VTA neurons.

To determine if the stimulatory properties of toluene were unique to the VTA, three adjacent midbrain structures were tested using single applications. In the IPN, the basal firing rate was 11.0 ± 2.70 Hz (Table 1). Toluene inhibited the firing of neurons in the IPN (Figure 2a). The response was robust and concentration-dependent (Figure 2d: maximum, -10.2 ± 0.71 Hz below baseline; $n = 7$). However, transient (<30 s) and concentration-independent increases were also noted in some neurons in the IPN (Figure 2a: <4 Hz, $n = 2$). In contrast, toluene did not significantly alter neuronal activity in the RRF (Figure 2b) or RIN (Figure 2c) (Figure 2e; maximum, 0.43 ± 0.39 Hz above baseline, $n = 12$; $p > 0.05$). Basal firing characteristics of neurons in the IPN and RRF are summarized in Table 1, and resemble those reported previously (Takagi, 1984; Deutch *et al*, 1988). The characteristics of neurons in the RIN are shown (Table 1), although characterization of this region is lacking. Thus, these results demonstrate that behaviorally relevant concentrations of toluene (single applications) selectively stimulate VTA neurons and that the increase in firing is similar in both DA and non-DA neurons.

Behavioral responses to solvents are determined in part by the pattern of exposure (Lammers *et al*, 2005). Therefore, prolonged *in vitro* exposures were investigated. Using a staircase paradigm, we observed that cumulative applications of toluene stimulated VTA DA neurons (Figure 3a; 22–823 μM) and non-DA neurons (Figure 3b; 22–633 μM). The increases in firing were concentration-dependent and reversed upon washout (Figure 3a and b). At DA neurons, the efficacy of toluene did not change as a function of exposure paradigm. Thus, maximal activity under staircase conditions (Figure 3c: 4.07 ± 0.44 Hz above baseline at 822 μM toluene, first application, $n = 21$) resembled firing

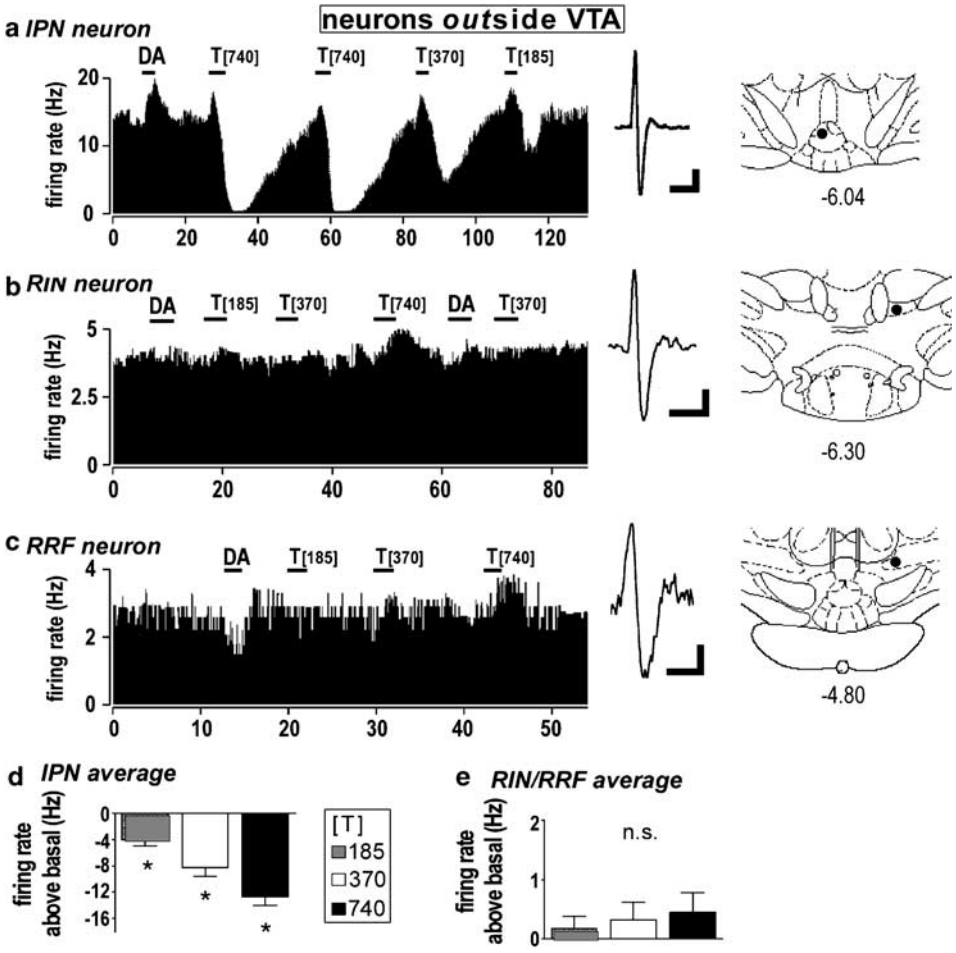

Figure 2 Toluene does not significantly stimulate the *in vitro* firing in other midbrain regions (single applications). Sample ratemeter histograms of neurons in the IPN (a), RIN (b), and RRF (c). *Insets* on the right show matching action potential waveforms (calibration: 1 ms and 0.2 mV) and on the far right, the anatomical location of recording electrode (mm posterior from bregma). (d, e) Summary of the toluene-induced changes in firing of neurons in the IPN ($n = 7$) (d) and both RIN ($n = 6$) and RRF ($n = 6$) (e). *Signifies significance at $p^* < 0.05$.

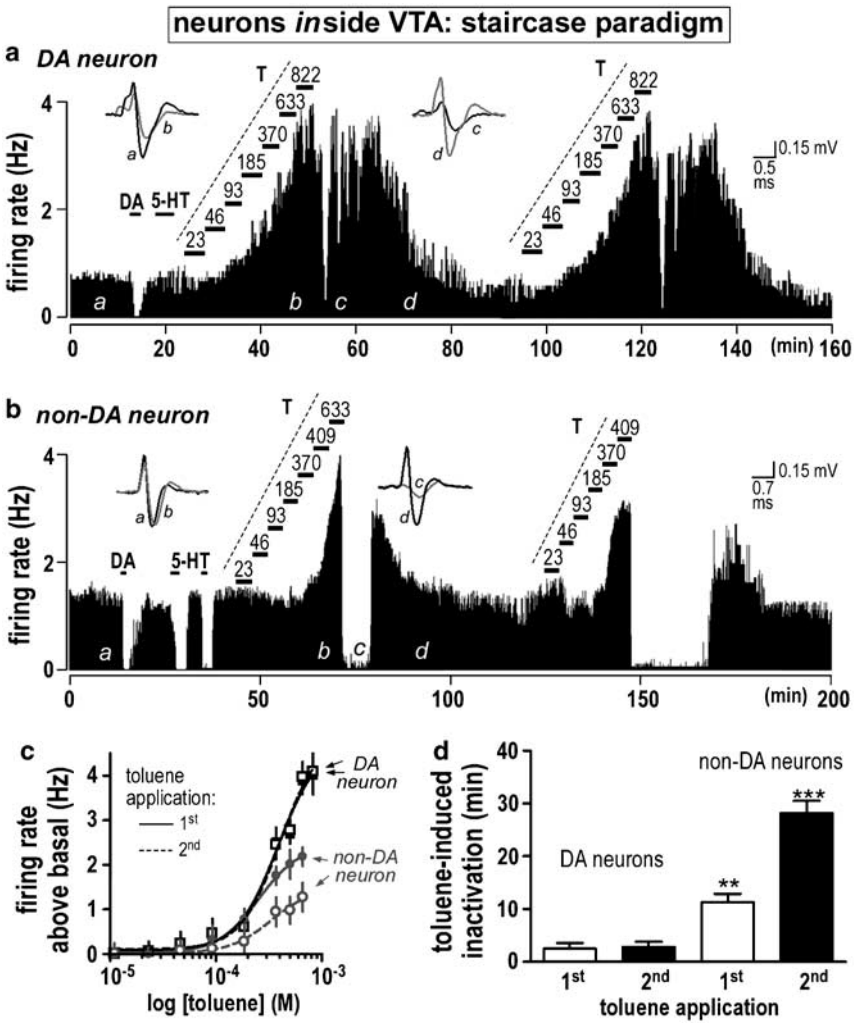

Figure 3 During longer repeat exposures, DA and non-DA neurons in the VTA respond differently to toluene (staircase paradigm). Sample ratemeter histograms of (a) DA and (b) non-DA neurons recorded *in vitro* during staircase application of toluene (3 min/concentration; seven concentrations). At the highest concentrations of toluene, firing briefly stopped (inactivated) and became undetectable. In (a), repeat applications of toluene generate similar periods of inactivation ($\sim$4 min). In contrast in (b), inactivation lengthened (application-1: $\sim$9 min; application-2: $\sim$29 min). *Insets* show action potential waveforms at the times (indicated with letters) in the rate histograms. (c) Concentration–response curves for toluene. (d) Summary graph illustrating the mean duration of inactivity. ***Signifies significance at $p<0.001$; **signifies significance at $p<0.01$.

under conditions using single applications (Figure 1d: 3.41 ± 0.37 Hz above baseline at 740 μM toluene, $n=17$). In contrast, in non-DA neurons under staircase conditions, the maximal efficacy of toluene declined $\sim$40% (Figure 3c: 2.19 ± 0.32 Hz above baseline; at 633 μM, first application, $n=9$) relative to single applications (Figure 1d: 3.21 ± 0.63 above baseline at 740 μM, $n=12$). The diminished efficacy is unlikely to be owing to tissue accumulation of toluene, because at comparable concentrations, the magnitude of the increase in DA cell firing was similar during both short (single applications: 3 min) and long (staircase: 21 min) exposures. Furthermore, the attenuation of firing was observed only in non-DA neurons during cumulative staircase conditions. More prolonged staircase exposure to toluene (>21 min) inactivated VTA DA (Figure 3a) and non-DA (Figure 3b) neurons and briefly limited further firing. As noted in previous studies (Riegel and French, 1999a), this stimulation-induced inactivation was not owing to acute, irreversible cell death, because in all instances, firing recovered to baseline when the perfusion media were replaced with toluene-free aCSF.

With first applications, toluene inactivated non-DA neurons 2–3 times longer than DA neurons (Figure 3a and b). To investigate if the toluene-induced inactivation of non-DA neurons was additive, the staircase paradigm was reapplied after firing resumed. The efficacy and duration of inactivation during the two repetitive episodes were noted for comparison. In DA neurons (Figure 3a), maximal firing (Figure 3c; episode-1: 4.00 ± 0.42 Hz above baseline, $n=17$; episode-2: 4.09 ± 0.49 Hz above baseline, $n=9$) and periods of inactivity (Figure 3d: episode-1: 2.50 ± 1.11 min, $n=17$; episode-2: 2.82 ± 0.96 min, $n=9$) remained the same. In non-DA neurons (Figure 3b), however, maximal efficacy declined (Figure 3c episode-1: 2.2 ± 0.2 Hz above baseline, $n=9$; episode-2: 1.3 ± 0.3 Hz above baseline, $n=3$) and the duration of inactivation increased (Figure 3d; episode-1: 11.3 ± 1.6 min, $n=9$, $p<0.01$; episode-2: 28.2 ± 2.3 min, $n=3$; $p<0.001$). Any assessment of toluene during a third

episode was not possible, because of the limitation of recording from a single neuron for longer than 2.5 h. Higher concentrations of toluene were not investigated because of the plateauing of neuronal stimulation. The decline in efficacy and increase in the period of inactivation was only observed in non-DA neurons. Toluene (<1 mM) stimulated VTA DA neuron firing repeatedly without a significant decline in efficacy.

In Vivo Microdialysis

Given these findings, we determined whether toluene can stimulate VTA DA neurons directly *in vivo*, and if so, whether this effect is sufficient to increase mesoaccumbal DA release. Dual-probe microdialysis was used to measure concentrations of somatodendritic DA release in the VTA, an indirect marker of VTA DA neuron activity, and DA released from DA terminals in the ACB.

DA release in the VTA. Toluene was infused directly into the VTA by reverse dialysis at concentrations of 1, 3, or 10 mM (see Methods). In the VTA, infusion of vehicle resulted in a non significant shift ($<15\%$) in basal dialysate DA concentrations (Figure 4a: 0.67 ± 0.09 nM; $n=9$). In contrast, infusion of toluene increased concentrations of somatodendritic VTA-DA (Figure 4a and b: $F_{3,24}=13.21$, $p<0.0001$). A significant increase was observed in response to 3 and 10 mM toluene (Figure 4b; $p<0.001$ *vs* vehicle). Significant increases in VTA DA were observed when toluene was infused into anterior or posterior VTA regions (Figure 4b; antVTA: $F_{3,12}=5.376$, $p=0.014$; post VTA: $F_{3,9}=8.561$, $p=0.0055$ ANOVA). The anatomical location of all dialysis probes is shown in Figure 5.

DA release in the ACB. The basal concentration of ACB DA in dialysate was 1.82 ± 0.43 nM. DA concentrations in the ACB did not change following intra-VTA vehicle perfusion. However, reverse-dialysis of toluene into the VTA significantly increased DA levels in the ACB (Figure 4c and d; $n=11$, $F_{3,43}=3.561$, $p=0.026$, ANOVA). All toluene-induced increases in DA reversed within 60 min. The ACB-DA increases were significant, when toluene was infused into the posterior VTA (Figure 4d; $F_{3,18}=4.772$, $p=0.013$, ANOVA), but not the anterior VTA (Figure 4c and d; $F_{3,9}=1.613$, $p=0.250$, ANOVA). This topographical selectivity (posterior VTA *vs* anterior VTA) parallels that previously observed in behavioral studies in which microinjection techniques were used to assess the effects of other drugs of abuse including both alcohol, and cannabinoids (Zangen *et al*, 2006; Zangen *et al*, 2002; Ikemoto and Wise, 2004; Rodd-Henricks *et al*, 2000, 2002; Rodd *et al*, 2005). To confirm functional connectivity between the posterior VTA and ACB probe placements, baclofen was infused into the VTA and DA levels after toluene washout and ACB DA levels were determined (Westerink *et al*, 1996). Baclofen produced a robust decline in ACB DA concentrations ($35 \pm 8\%$ of vehicle; $n=11$; data not shown). Perfusion of toluene into regions anterior ($n=5$), dorsal ($n=7$), lateral ($n=2$), or ventral ($n=2$) to the VTA failed to alter ACB-DA (Figure 4e and f) demonstrating the regional selectivity of this effect. Collectively, these data show that the effects of toluene on ACB-DA levels are specific to the posterior VTA.

DISCUSSION

The present results indicate that toluene directly stimulates DA cell firing, thereby facilitating DA release in the ACB. Dopaminergic innervation of the ACB is a key substrate for the reinforcing effects of other drugs of abuse (Wise, 2002). Nevertheless, despite intensive study, the neural substrates mediating the CNS actions of toluene have remained elusive. Previous behavioral studies indicated that the locomotor activating effects of toluene require intact mesoaccumbal DA transmission (Riegel *et al*, 2003). Moreover, toluene increases the activity of VTA DA neurons *in vivo* (Riegel and French, 1999a). To date, however, it remained unclear whether toluene stimulated these neurons by direct actions on neurons *within the VTA* and if such activation was sufficient to increase DA release from distal DA terminals in the ACB.

Toluene Stimulates VTA Neurons, Leading to DA Release in the ACB

Our data support the premise that toluene-containing inhalants activate mesoaccumbal neurotransmission via direct actions on VTA DA neurons. First, toluene stimulated DA neurons in the VTA slice, a preparation in which transynaptic inputs to DA cells are severed (Tzschentke, 2001). Similar effects were observed in slices incubated in aCSF containing low Ca^{2+}/high Mg^{2+}, conditions in which synaptic transmission is inhibited (Katz, 1970; Katz and Miledi, 1970). Identical media were used previously to study the site of action for other drugs of abuse (Brodie *et al*, 1990; Sanghera *et al*, 1984; Trulson *et al*, 1987) including toluene (in other regions) (Magnusson *et al*, 1998). These findings suggest that toluene increases DA neuronal activity post-synaptically and independently of afferent input.

Second, *in vivo* microdialysis showed that direct intra-VTA perfusion of toluene enhanced DA concentrations in the VTA and ACB. As VTA DA neuron cell bodies are the only known source of DA in this region (Adell and Artigas, 2004), the former effect most likely reflects the well-described increase in somatodendritic DA release that occurs as a consequence of increased firing of DA neurons (Adell and Artigas, 2004). Other drugs of abuse also increase somatodendritic DA release (Adell and Artigas, 2004). Work, however, from several laboratories has shown that the VTA is functionally heterogeneous (Ikemoto and Wise, 2004; Ford *et al*, 2006). Experimental animals will work to obtain infusions of ethanol, opiates, cannabinoids, or psychostimulants into the posterior VTA, whereas infusions into the anterior VTA are without effect (Rodd-Henricks *et al*, 2000, 2002; Rodd *et al*, 2005; Rodd *et al*, 2004b; Zangen *et al*, 2006). Interestingly and consistent with this heterogeneity, microdialysis revealed that toluene was more effective in increasing ACB-DA concentrations when infused directly into the posterior than in the anterior VTA.

Third, the effects of toluene were anatomically specific. Unlike VTA DA cells, bath application of toluene did not alter DA neuronal firing in the RRF or RIN. Furthermore, firing of GABAergic neurons in the IPN was inhibited. Similarly, reverse-dialysis of toluene into regions adjacent to the VTA did not alter ACB-DA levels. Anterior VTA perfusion of toluene failed to change ACB-DA, a finding

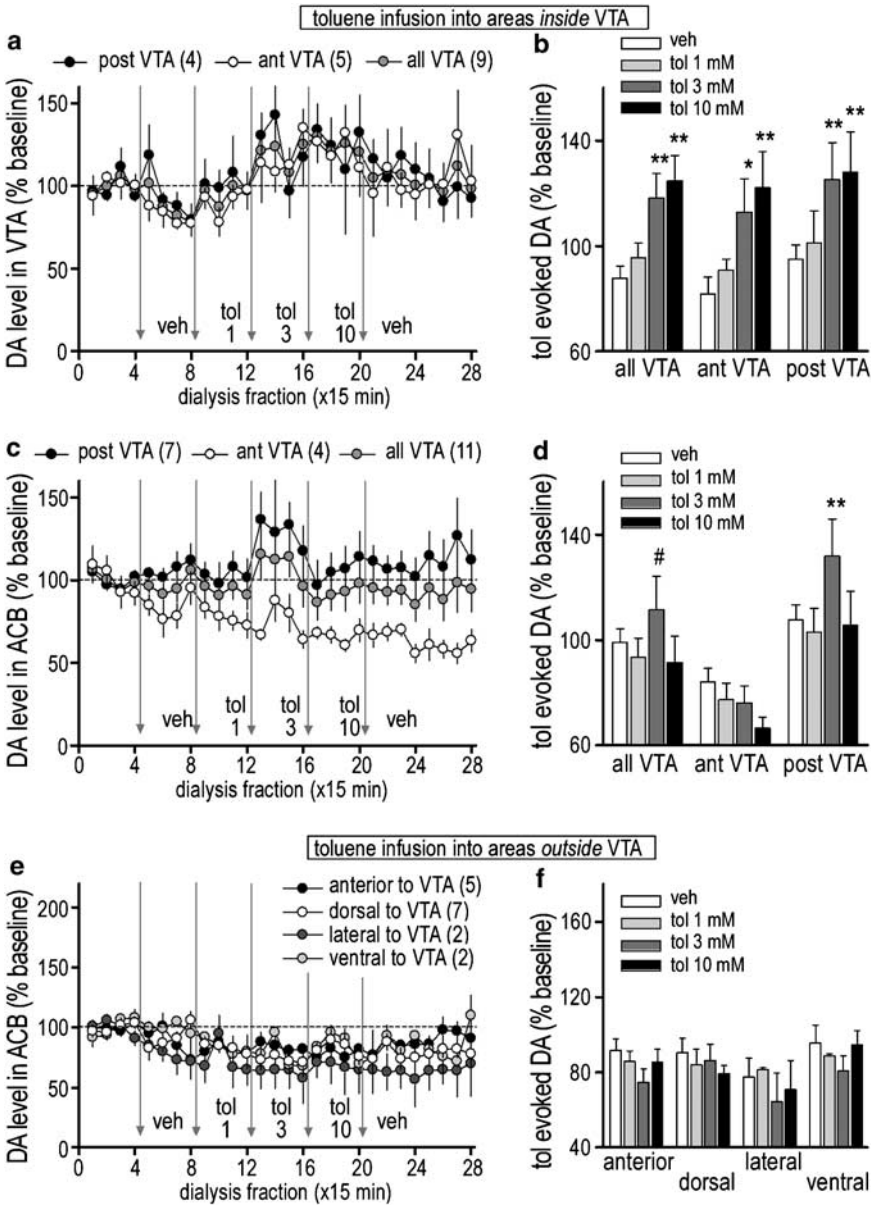

Figure 4 *In vivo*, the direct infusion of toluene into the VTA increases DA in the VTA and ACB; infusion of toluene outside the VTA does not. Time courses illustrate changes in mean extracellular concentrations of DA dialysate concentrations in the VTA (a) and ACB (c, e). Bar graphs summarize mean changes in DA dialysate concentrations in the VTA (b) and ACB (d, f). (a–d) Changes in DA dialysate following vehicle or toluene infusion inside the VTA. (e, f) Changes in DA dialysate in the ACB following vehicle or toluene infusion outside the VTA. Numbers in parentheses signify the number of animals. Data are the mean and error bars signify SEM. The symbols *, **, and # signify significance at $p<0.05$, $p<0.02$, and $p=0.07$, respectively.

consistent with anatomical studies showing that most DA neurons in this region do not innervate the ACB (Westerink *et al*, 1998; Tzschentke, 2001; Brog *et al*, 1993). However, there is an apparent trend to a delayed response, the magnitude of which may have been masked by a downward baseline drift in this experimental group. This delayed response may reflect diffusion of toluene from the probe to more posterior VTA regions. The toluene-induced stimulation of anterior VTA DA neurons may be related to the reported toluene-induced increase in DA release in the prefrontal cortex (Gerasimov *et al*, 2002b). Taken together, these findings indicate that by stimulating posterior VTA DA cells, toluene increases extracellular DA concentrations in the distal ACB.

Concentration Dependence and Neurobehavioral Implications

Toluene is rapidly absorbed, distributed, and eliminated from brain tissue (Gerasimov *et al*, 2002a; Kishi *et al*, 1988). Behavioral effects of toluene depend on both the brain concentrations and the pattern of exposure (Kishi *et al*, 1993; Lammers *et al*, 2005). Although inhalation exposure is relevant to inhalant abuse, the site (or sites) of action can be difficult to interpret. In both of our experimental approaches, toluene was delivered directly to the VTA. The concentrations of toluene employed were those previously shown to be achieved in humans (Garriott *et al*, 1981) and experimental animals (see discussion in Riegel *et al*, 2003).

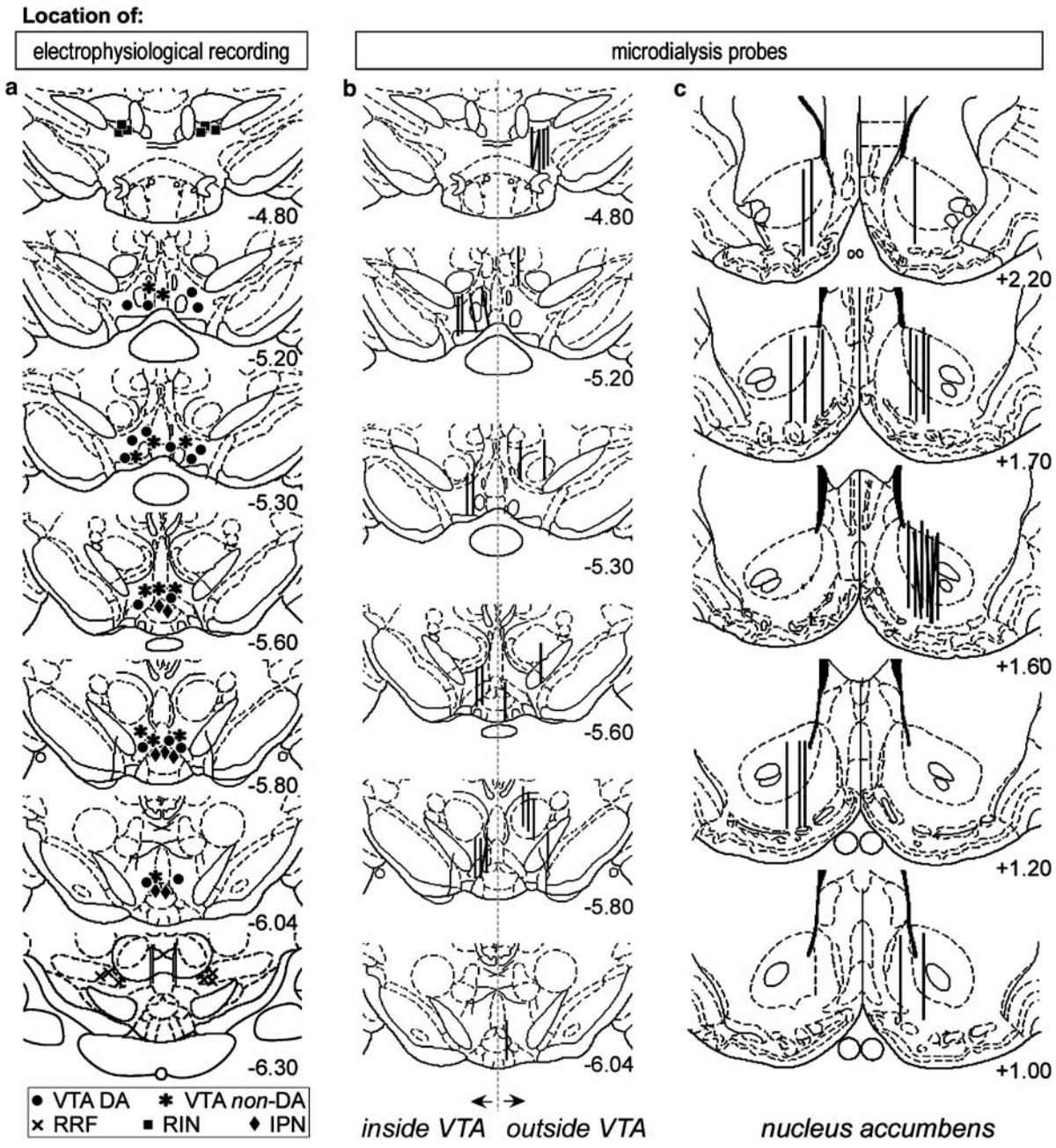

Figure 5 Summary of all anatomical locations for (a) electrophysiological recordings in the midbrain, (b) dialysis probes in the midbrain, and (c) dialysis probes in the ACB. In (b), tracts left of midline represent probe locations of animals with a positive VTA location. Tracts right of midline represent probe locations from animals with a negative VTA location, which served as negative anatomical controls. Numbers on the atlas plate indicate the distance from bregma in mm. Adapted from Paxinos and Watson (1998).

To simulate this exposure pattern *in vitro*, VTA slices were perfused with repeated, rapidly rising, escalating concentrations of toluene (staircase paradigm). Extracellular recordings preserve action potential firing for long periods of time and are correlated with DA release in the ACB (Marinelli *et al*, 2006; Westerink *et al*, 1996). We observed that VTA firing increased only over a narrow concentration range. At lower concentrations ($<100\,\mu M$), toluene increased DA cell firing rates, but ACB DA concentrations were unaltered. As noted elsewhere (Wise, 2002; Yeomans, 1989), the stimulation of unmyelinated fibers may have been insufficient to detect increased synaptic overflow of DA in distal terminals. At intermediate concentrations, toluene increased both DA neuron firing and DA concentrations in the ACB. As discussed above (see Methods), retro-dialysis results in an estimated 10-fold reduction in drug tissue concentration. Therefore, there is good concordance between the current results and previous *in vivo* experiments, where blood toluene concentrations of $300\,\mu M$ stimulated VTA DA neurons (Riegel and French, 1999a).

The firing of VTA DA neurons inactivated temporarily when toluene concentrations reached ~ 1 mM. This inactivation persisted until concentrations decreased, and is consistent with previous *in vivo* inhalation studies showing inactivation of DA neurons when blood toluene

concentrations exceeded ~900 μM (Riegel and French, 1999a). Thus, it is plausible that the lack of toluene-induced DA release in previous *in vivo* studies results from an inactivation of VTA DA neurons induced by higher toluene concentrations (Kondo *et al*, 1995; Gerasimov *et al*, 2002b).

The lack of an accumbal DA response to 10 mM toluene may seem surprising given that this concentration increased extracellular DA concentrations in the VTA (ie, somatodendritic release). The mechanisms mediating the differential effects of the higher toluene concentration on VTA and ACB DA levels are unclear. Importantly, however, DA clearance in the VTA is less efficient than in terminal areas (Cragg and Greenfield, 1997). Thus, DA tissue concentrations may remain elevated even after suppression of cell firing in the VTA. It is known that depolarization inhibits DA transporter function (Sonders *et al*, 1997). Sustained depolarization by toluene may further inhibit VTA DA uptake resulting in impaired DA clearance in this region and an elevation of VTA DA levels. Interestingly, other organic solvents (eg, ethanol) inhibit DA uptake (Robinson *et al*, 2005). Therefore, direct effects of toluene on transporter activity cannot be ruled out.

In the staircase paradigm, VTA non-DA neurons were also stimulated. However, unlike DA cells, these neurons displayed an inactivation threshold that became lower with repeated stimulation. Some VTA non-DA cells are GABA projection neurons (Cameron *et al*, 1997; Van Bockstaele and Pickel, 1995), which innervate striatal cholinergic interneurons (Pickel and Chan, 1990) and cerebellar nuclei (Ikai *et al*, 1992). Although the functional role of these neurons is not completely understood (Van Bockstaele and Pickel, 1995), their activation by low concentrations of toluene may contribute to TTX-sensitive increases in extracellular levels of GABA in these regions (Stengard and O'Connor, 1994; Stengard *et al*, 1993) and to decreases in striatal ACh release (Stengard, 1994) during toluene inhalation. Other VTA non-DA cells are GABAergic interneurons (type-II) that tonically inhibit VTA DA neurons *in vivo* (Johnson and North, 1992). Although the mechanism underlying the shift in the inactivation point remains unclear, increases in the firing activity of DA over GABA neurons would be expected to facilitate mesoaccumbal output.

Finally, regarding the cellular targets, studies in cell expression systems indicate that toluene disrupts the activity of numerous ion channels (Cruz *et al*, 1998; Bale *et al*, 2005; Beckstead *et al*, 2000), Ca^{2+} signaling (Westerink and Vijverberg, 2002; Westerink *et al*, 2002; Meulenberg and Vijverberg, 2003), ATPases (Calderon-Guzman *et al*, 2005), and G-proteins (Tsuga *et al*, 1999; Tsuga and Honma, 2000; Tsuga *et al*, 2002). Additional studies will be needed to determine if one or more of these mechanisms may be contributing to the toluene-induced excitation of VTA DA neurons.

In conclusion, our results indicate that toluene selectively activates VTA DA neurons projecting to the ACB. They identify a narrow concentration range within which toluene directly stimulates mesoaccumbal neurotransmission. Such findings suggest that previous conclusions, that DA-independent mechanisms underlie the rewarding properties of inhalants, may be premature. Furthermore, given the alarming increase in inhalant abuse among youth (NSDUH, 2003), additional studies examining the interaction of toluene and other inhalants with the mesoaccumbal DA reward pathway are warranted.

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