Original Paper

Harmine augments electrically evoked dopamine efflux in the nucleus accumbens shell

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Abstract

Harmine is a β-carboline alkaloid and major component of *ayahuasca*, a traditional South American psychoactive tea with anecdotal efficacy for treatment of cocaine dependence. Harmine is an inhibitor of monoamine oxidase A (MAO-A) and interacts in vitro with several pharmacological targets which modulate dopamine (DA) neurotransmission. In vivo studies have demonstrated dopaminergic effects of harmine, attributed to monoamine oxidase inhibitor (MAOI) activity, however none have directly demonstrated a pharmacological mechanism. This study investigated the acute effects, and pharmacological mechanism(s), of harmine on electrically evoked DA efflux parameters in the nucleus accumbens both in the absence and presence of cocaine. Fast cyclic voltammetry in rat brain slices was used to measure electrically evoked DA efflux in accumbens core and shell. Harmine (300 nM) significantly augmented DA efflux (148±8% of baseline) in the accumbens shell. Cocaine augmented efflux in shell additive to harmine (260±35%). Harmine had no effect on efflux in the accumbens core or on reuptake in either sub-region. The effect of harmine in the shell was attenuated by the $5\text{-HT}_{2A/2C}$ antagonist ketanserin. The MAOI moclobemide (10 μM) had no effect on DA efflux. These data suggest that harmine augments DA efflux via a novel, shell-specific, presynaptic 5-HT_{2A} receptor-dependent mechanism, independent of MAOI activity. A DA-releasing 'agonist therapy' mechanism may thus contribute to the putative therapeutic efficacy of *ayahuasca* for cocaine dependence.

Keywords

Harmine, cocaine, voltammetry, dopamine, nucleus accumbens, ketanserin, moclobemide, ayahuasca, monoamine oxidase, 5-HT2A receptor

Introduction

Cocaine addiction is a chronically relapsing mental disorder for which no pharmacotherapeutic treatments are currently licensed. Development of effective pharmacological treatments has been identified as a major public health priority (Leiderman et al., 2005). Cocaine dependence is a complex pathology involving multiple neuronal processes and thus it provides numerous prospective targets for pharmacological intervention. The reinforcing effects of cocaine are now understood to be primarily mediated by increased dopamine (DA) levels in the nucleus accumbens (NAc), as a result of blockade of the DA reuptake transporter (DAT) on pre-synaptic terminals (Schmitt and Reith, 2010) and enhanced release probability of DA from a synapsin-dependent reserve vesicle pool (Venton et al., 2006). A significant body of evidence now supports the hypothesis that acquisition and expression of addictive behaviours occurs through maladaptive learning mechanisms caused by hijacking of the mesolimbic reward circuit by drugs such as cocaine, which induce persistent forms of synaptic plasticity which drive drug-seeking behaviour (Chen et al., 2010).

Several DA-related targets have been identified as substrates for the botanical alkaloid harmine, a heterocyclic β-carboline alkaloid found in several plants, including *Banisteriopsis caapi*. Harmine is a major component of the Amazonian traditional medicine *ayahuasca* (McKenna et al., 1984) which has been suggested as having beneficial effects on substance dependent individuals (Grob et al., 1996). *Ayahuasca* use has spread throughout the world, with published reports of users in traditional and urban settings maintaining abstinence from previous cocaine dependence with significantly more success than matched controls, and without exhibiting psychological problems associated with chronic use of drugs of abuse (Fabregas et al., 2010; Grob et al., 1996). Further anecdotal reports of *ayahuasca* use in drug rehabilitation centres in Peru claim abstinence rates of over 70% of patients who complete treatment (Lizarzaburu, 2003). Experimental studies on harmine, as an isolated compound, show significant neurobiological effects on a number of molecular targets with established or emerging relevance to dopaminergic modulation of cocaine dependence. These include inhibition of the dual-specificity tyrosine phosphorylation-regulated kinase family member DYRK1A (Bain et al., 2007), inhibition of monoamine oxidase A (MAO-A) (Schwarz et al., 2003), affinity for the 5-HT_{2A} receptor (Grella et al., 1998), affinity for the imidazoline (I_2) binding site (Husbands et al., 2001) and inhibition of the DAT at high concentrations (Drucker et al., 1990). A confluence of experimental evidence regarding the role of these harmine substrates in cocaine dependence is emerging, however no studies to date have specifically investigated its potential as a pharmacotherapeutic agent for

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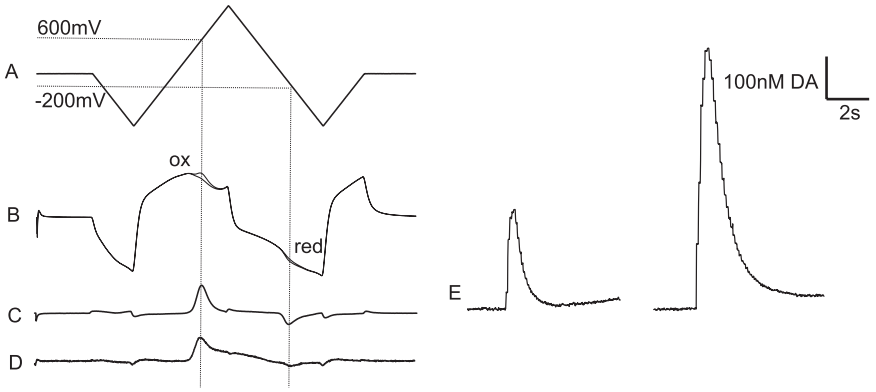

Figure 1. Voltammetry input voltage waveform, current at carbon electrode and subtracted voltammograms showing dopamine (DA) oxidation and reduction peaks.

(A) Input voltage waveform to carbon electrode. The voltage scan goes from 0 to –1 to +1.4 to –1 and back to 0 V at 480 V/s. The whole scan takes 20 ms. (B) The current at the carbon electrode after applying the input voltage in artificial cerebrospinal fluid (aCSF) and in the presence of 10 μM DA. The two scans are superimposed except for a small increase at around 600 mV (where DA oxidises giving off 2 electrons) and at –200 mV where DA is reduced. (C) The voltammogram is derived from (B) and obtained by subtracting the current at the electrode in aCSF from the current at the electrode in the presence of DA, leaving only the Faradaic current from DA oxidation and reduction. Note the oxidation peak at 600 mV. (D) The voltammogram obtained from subtracting the electrode current just prior to electrical stimulation from the current just after electrical stimulation of the nucleus accumbens (NAc) shell (10 pulses at 20 Hz). This voltammogram (D) is taken in the presence of 300 nM harmine and shows that we are measuring DA and that at this concentration harmine does not affect the voltammetric signal (other than by increasing DA efflux). In each case (A)–(D) we have shown 20 ms of data. (C) and (D) are not to scale but (D) is enlarged to aid visualising the oxidation and reduction peaks. The DA oxidation peak in (D) corresponds to about 300 nM DA. (E) Representative stimulated DA efflux events showing change in current sampled at DA oxidation peak, recorded in NAc shell at baseline (left) and following treatment with 300 nM harmine (right). Efflux magnitude and reuptake parameters were calculated from peak height and time constant of exponential decay curve fitted to the reuptake phase, respectively.

this disorder. We have recently reviewed the pharmacology of harmine (Brierley and Davidson, 2012). Given the indications of beneficial treatment from the traditional use of *ayahuasca*, and the relatively favourable safety profile from studies of regular users, detailed investigation of this application would seem warranted.

The objective of this study was to investigate the effects of acute administration of harmine on DA efflux and reuptake using fast cyclic voltammetry in the NAc core and shell sub-regions, and any interactions with acute cocaine-induced modulation of DA neurotransmission.

Materials and methods

Animal husbandry

Adolescent male Wistar rats aged eight weeks were used to prepare brain slices for all experiments. Animals were bred in-house and kept on a 12/12 h light/dark cycle, fed and watered ad libitum and housed four per cage. Animals were sacrificed by cervical dislocation without anaesthesia. All procedures were conducted in accordance with regulations under the UK Animals (Scientific Procedures) Act 1986.

Drugs and reagents

Artificial cerebrospinal fluid (aCSF) was prepared as previously described (Toner and Stamford, 1996): NaCl 126 mM; KCl 4 mM; KH_2PO_4 1.4 mM; $MgSO_4 \cdot 7H_2O$ 1.3 mM; $NaHCO_3$ 26 mM; D-glucose 4 mM; $CaCl_2 \cdot 2H_2O$ 2.4 mM in de-ionised water and oxygenated with 95% O_2/5% CO_2 at room temperature. All chemicals and drugs were purchased from Sigma-Aldrich (Poole, UK). Harmine, ketanserin and moclobemide were dissolved in dimethyl sulfoxide (DMSO) stock solutions prior to preparation of working solutions in aCSF (10 mM stock solutions yielding 0.01% DMSO in working solution).

Fast cyclic voltammetry

Fast cyclic voltammetry is an electrochemical technique which utilises the electroactive properties of the catecholamine neurotransmitters to allow sampling of their concentrations and transmission kinetics in 'real-time' (Michael and Wightman, 1999). Briefly, a triangular voltage waveform is applied to a carbon fibre microelectrode which oxidises monoamines at about 600 mV vs Ag/AgCl reference electrode and the resulting current at the electrode surface is recorded. Calibration of this electrode in a known concentration of DA allows the recorded Faradaic current to be converted to the concentration of DA. For this study, voltammetric scans conducted at 8 Hz were performed by a Millar Voltammetric Analyser (PD Systems, West Molesey, UK), with the evoked current of the complete scan at the electrode and signals sampled at the oxidation peak (Figure 1) captured using a CED1401 micro3 analogue-to-digital converter (Cambridge Electronic Design, UK) and Spike2 v7.1 data capture software.

Brain slice preparation

Following cervical dislocation brains were rapidly removed under ice-cold aCSF and blocked along the coronal plane to leave a block containing the NAc. Sections of 400 μm were taken rostro-caudally from approximately +1.0 to +2.2 mm versus bregma using a vibratome. Sections were transferred to a slice saver containing aCSF at 22±1°C in which slices were suspended on a plastic mesh above a continuous source of 95% O_2/5% CO_2, and left to equilibrate for at least 45 min. At the start of an experiment a slice was transferred to a laminar flow recording chamber

supplied with aCSF via gravity feed through an inline peltier heating unit, at a flow rate of 3 mL/min and solution temperature in the recording chamber at 32±0.5°C. Slices were left to equilibrate in this environment for 45 min prior to electrical stimulation. The recording microelectrode was placed in the NAc (core or shell) at the start of the equilibration period to monitor background DA levels as under certain circumstances (such as poor slice health or increased temperature) large spontaneous release of DA can occur (Davidson et al., 2011), in which case the experiment would be terminated (approximately 5% of experiments in this study).

Carbon fibre microelectrode construction

Carbon fibre microelectrodes were fabricated by inserting a single 8 µm diameter carbon fibre into a 10 cm borosilicate glass capillary (2.0 mm o.d. (outer- diameter), 1.16 mm i.d. (inner-diameter); Harvard Apparatus Ltd, Kent, UK) under vacuum suction. The filled capillaries were pulled to a fine tip seal around the carbon fibre (P-30, Sutter Instruments Co., USA), leaving an exposed length of carbon fibre which was cut to a length of 50–100 µm with a scalpel under microscopic guidance. Microelectrodes were backfilled with aCSF before insertion of a connecting wire for connection to the headstage amplifier. A steel wire auxiliary electrode and an Ag/AgCl reference electrode were also connected to the headstage amplifier and sited within the recording chamber away from the slice. Electrodes were calibrated in 10 µM exogenous DA and only those exhibiting good signal-to-noise ratios and high DA sensitivity were used. These electrodes were recalibrated in 1–10 µM DA following experiments to ensure that sensitivity was maintained and to allow conversion of evoked signals into DA concentrations.

Stimulation protocol

DA efflux was evoked by a stimulation protocol designed to replicate the phasic intraburst firing frequency typical of rodent DA neurons (Benoit-Marand et al., 2001; Hyland et al., 2002), and a pulse number which elicits robust but submaximal DA efflux in brain slice voltammetry studies (Zhang et al., 2009). Hence a train of 10 x 0.1 ms 10 mA pulses was applied at a frequency of 20 Hz, with stimulus trains applied every 5 min. Stimulus train parameters were programmed into the Spike 7 data capture program which output the square waveform via TTL pulses of 1 V to a Neurolog NL800A constant current stimulus isolator (Digitimer, Letchworth, UK), set to output the pulses at 10 mA. The output of the stimulus isolator was applied to the accumbens slice using a bipolar tungsten electrode (Plastics One, Virginia, USA), with tips approximately 300 µm apart, and 200 µm from the recording electrode.

Data aquisition and statistical analysis

Changes in Faradaic current sampled at the DA oxidation peak were plotted by the Spike2 software. For each efflux event the peak height and an index of reuptake was calculated by fitting an exponential decay to the reuptake phase using the analysis feature of the Spike 7 software which calculated the time constant of the half-life of the decay curve. This has been previously shown to give an accurate measure of reuptake rate (Yorgason et al., 2011).

Stimulated efflux events were initiated every 5 min and following three stable consecutive baseline events, in which peak heights were within 10% of each other with no directional trend, the test drug was added. All data were expressed as a percentage of the mean baseline data. For all drug administration protocols, each replicate was conducted on a separate day using slices prepared from different animals. Drug peak effects were compared against baseline data using the mean of three consecutive time points. Statistical analyses were conducted using Prism (Graphpad Software, California, USA), using either one- or two-way analysis of variance (ANOVA) with Bonferroni's post-hoc tests. Data are presented as mean±standard error of mean (SEM), with significance set at $p<0.05$.

Drug treatment protocols

Preliminary experiments revealed that harmine had a maximal effect after 70–90 min, whereas cocaine had a maximum effect after 30–45 min (data not shown). Thus, in the primary experiment group, the accumbens slice was perfused with aCSF for approximately 60 min, including the baseline DA efflux period, then either harmine or vehicle (DMSO) was added for 90 min, thereafter cocaine was added with either harmine or vehicle for 45 min.

In the subsequent group of experiments we aimed to investigate whether the substrates MAO-A or 5-HT_{2A} receptors mediated harmine's mechanism of action. The preferential 5-HT_{2A} receptor antagonist ketanserin was initially investigated alone at 1–10 µM to determine the maximal concentration at which the drug could be used without modulating basal evoked DA neurotransmission. This concentration range is lower than used in previously published brain slice studies (Campbell and Walker, 2001; Chen et al., 2003), to minimise any non-specific antagonist effects due to the low affinity ketanserin also has for 5-HT_{2C} receptors. This ketanserin concentration (1 µM) was superfused for 60 min then given in combination with 300 nM harmine for 90 min. Finally, we assessed the purported role of MAO-A inhibition in mediating harmine-induced DA efflux (Iurlo et al., 2001) by superfusing the slice with moclobemide (10 µM) for 90 min.

Results

Electroactivity of harmine

A concentration range for the investigation of harmine was initially set at 0.1–1 µM, based on the in vitro IC_{50} values at the known targets: MAO-A (4.54 nM, (Schwarz et al., 2003)); DYRK1A (80 nM, (Bain et al., 2007)); 5-HT_{2A} receptor (230 nM, (Grella et al., 1998)) and pharmacokinetic data for human users of *ayahuasca* which show mean peak plasma concentrations of harmine are 540 nM (Callaway et al., 1999). During pilot studies using 1 µM harmine, an additional oxidation peak close to that of DA was observed indicating that harmine was an electroactive compound. To maintain normal sensitivity for DA, the concentration of harmine used was 300 nM, which did not affect electrode sensitivity to DA (see Figure 1). Other drugs tested were not electroactive at the concentrations used (data not shown but see Davidson et al., 2000).

Table 1. Baseline dopamine (DA) release and reuptake in the accumbens core and shell.
On electrical stimulation (10 pulses at 20 Hz) there was no difference in peak DA efflux in the core (n=14) versus the shell (n=49, p=0.224, t-test). The reuptake time constant appeared larger in the shell versus the core and this difference was very nearly significant (p=0.065, t-test). Values are means ± standard error of mean (SEM).

Accumbens region	Peak DA efflux (nM)	Reuptake time constant
Core	309±37	0.77±0.07
Shell	359±24	1.08±0.08

Baseline evoked DA efflux does not differ between sub-regions

At baseline, the extracellular concentration of evoked DA in the NAc core (309±37 nM) and shell (359±24 nM) did not differ significantly (p=0.224), as shown in Table 1. The reuptake time constant was somewhat smaller in the core (0.77±0.07) than the shell (1.08±0.08), however this difference was not quite significant (p=0.065).

Harmine increases evoked DA efflux in NAc shell

Harmine (300 nM) augmented DA efflux in the NAc shell to 148±8% of baseline values (Figure 2). Cocaine rapidly augmented efflux, to 179±20%, and the effects of these two drugs combined appeared additive, to 260±35%. Two-way repeated measures ANOVA revealed significant effects of both harmine ($F_{1,9}$=6.57, p=0.03) and cocaine ($F_{1,9}$=20.85, p=0.0014), however no significant interaction was observed ($F_{1,9}$=0.88, p=0.3726) suggesting that the effect of harmine in augmenting DA efflux is additive to that of cocaine's.

Effect of harmine on DA reuptake in NAc shell

Cocaine significantly increased the time constant of reuptake to 189±19% of baseline value ($F_{1,9}$=65.47, p<0.0001). Harmine had no significant effect on reuptake (111±5% of baseline, $F_{1,9}$=0.62, p=0.45), and no significant interaction was observed between the two variables ($F_{1,9}$=0.09, p=0.76). Harmine treatment in combination with cocaine increased the time constant to 205±19% of baseline (Figure 2).

No effect of harmine on DA efflux in NAc core

In contrast to the NAc shell, harmine had no effect on evoked DA efflux in the core (108±3% of baseline; $F_{1,8}$=0.02, p=0.88), however cocaine increased DA efflux to 231±16% ($F_{1,8}$=42.37, p=0.0002). Harmine in combination with cocaine had a peak effect of 220±31% of baseline (Figure 3). There was no significant interaction between the variables ($F_{1,8}$=0.22, p=0.65).

No effect of harmine on DA reuptake in NAc core

Cocaine significantly augmented the time constant of DA reuptake in the NAc core ($F_{1,8}$ = 36.18, p=0.0003), increasing peak values to 195±4% following treatment alone and to 175±17% in combination with harmine (Figure 3). Harmine had no significant effect on reuptake (110±7% ; $F_{1,8}$=2.58, p=0.15) and no significant interaction was observed between the variables ($F_{1,8}$=0.79, p=0.4).

Harmine increases evoked DA efflux in NAc shell but not core

The peak DA efflux data for harmine alone were reanalysed with harmine treatment and NAc sub-region as independent variables (Figure 4, left panel). Significant effects were observed for harmine treatment ($F_{1,18}$=19.62, p<0.0005), accumbens sub-region ($F_{1,18}$=16.47, p<0.001) and the interaction between the variables ($F_{1,18}$=11.23, p<0.05). Bonferroni's post-hoc test demonstrated that the source of interaction was the augmenting effect of harmine on DA efflux in the NAc shell (p<0.0001).

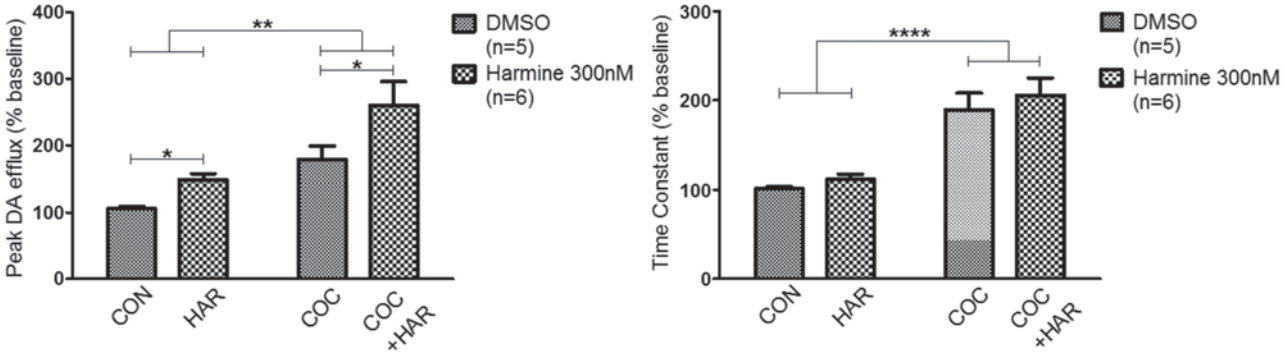

Figure 2. Dopamine (DA) efflux and time constant of reuptake in nucleus accumbens (NAc) shell for control (CON) and harmine (HAR) groups, prior to and following superfusion with 1 μM cocaine (COC).
Left panel: data are mean peak height (±standard error of mean (SEM)) of final three consecutive efflux events within each phase of drug treatment protocol, expressed as percentage of baseline peak height. Right panel: data are mean time constant (±SEM) of final three consecutive efflux events within each phase of drug treatment protocol, expressed as percentage of baseline time constant. Data analysed by two-way repeated measures analysis of variance (ANOVA), *p<0.05, **p<0.01, ***p<0.05 main effect of cocaine, ****p<0.05 main effect of cocaine. DMSO: dimethyl sulfoxide.

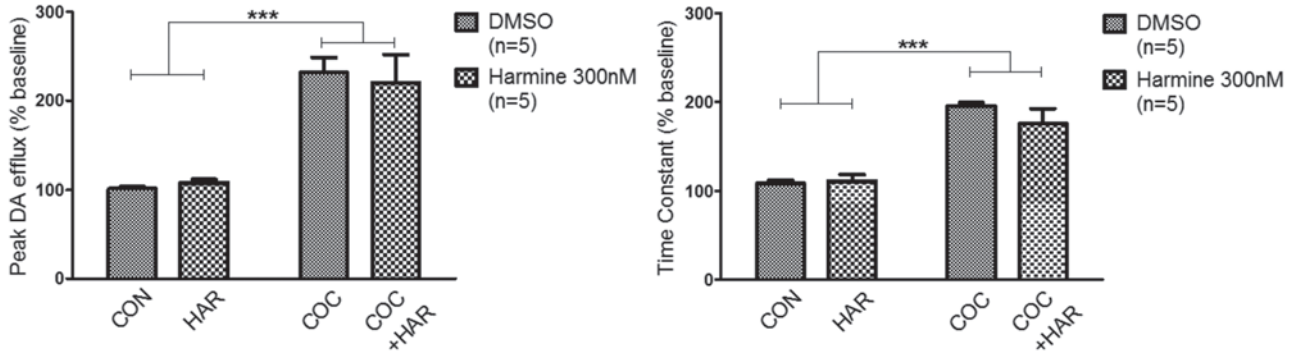

Figure 3. Dopamine (DA) efflux and time constant of reuptake in nucleus accumbens (NAc) core for control (CON) and harmine (HAR) groups, prior to and following superfusion with 1 µM cocaine (COC). All data presented and analysed as in Figure 2. ***p<0.05 main effect of cocaine. DMSO: dimethyl sulfoxide.

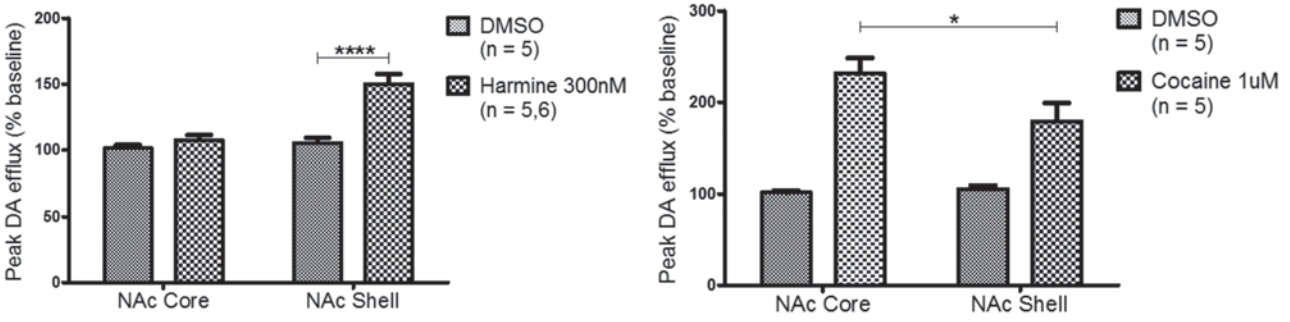

Figure 4. Left panel: sub-region specificity of effect of 300 nM harmine on dopamine (DA) efflux in the nucleus accumbens (NAc). Data analysed by two-way analysis of variance (ANOVA) and Bonferroni's post-hoc tests with harmine treatment and NAc sub-region as independent variables. All groups n=5 except harmine in NAc shell where n=6 (****p<0.0001). Right panel: greater augmentation of DA efflux by cocaine in NAc core than shell. Data analysed by two-way ANOVA and Bonferroni's post-hoc tests with cocaine treatment and NAc sub-region as independent variables. All groups n=5 (*p<0.05). DMSO: dimethyl sulfoxide.

Cocaine increases DA efflux to greater extent in NAc core than shell

As described above for harmine, the peak efflux data for cocaine alone were also reanalysed to investigate any differences between NAc sub-regions (Figure 4, right panel). Significant effects were observed for cocaine treatment only ($F_{1,16}$ = 58.63, p<0.0001), however both sub-region ($F_{1,16}$=3.27, p=0.08) and interaction ($F_{1,16}$=4.47, p=0.0506) were almost significant. Bonferroni's post-hoc test demonstrated a significantly greater augmentation of DA efflux in the NAc core than shell (p<0.05).

No effect of moclobemide in NAc shell

Because harmine modulated DA efflux, but not reuptake, and this effect was only observed in the NAc shell, follow-on experiments were conducted in the shell to assess whether this effect was mediated by either 5-HT_{2A} receptors or inhibition of MAO-A. To examine whether MAO-A inhibition was involved in harmine's effects on DA efflux we tested moclobemide, another reversible inhibitor of MAO-A, against harmine (Figure 5, left panel). Moclobemide (10 µM) had no effect on DA efflux (94±10% of baseline). One-way ANOVA revealed a significant effect ($F_{2,14}$=15.0, p<0.005), with Bonferrroni's post-hoc tests showing significant augmentation of release by harmine compared to both DSMO (p<0.01) and moclobemide (p<0.001).

Effects of ketanserin in NAc shell

To allow investigation of any 5-HT_{2A} receptor-mediated effects of harmine, a preliminary study using the preferential 5-HT_{2A} receptor antagonist ketanserin was conducted to establish a suitable concentration for use in the harmine antagonism experiments (Figure 5, centre panel). Ketanserin was superfused at 1 or 10 µM for 60 min. At 1 µM ketanserin had no significant effect on peak efflux compared to the DMSO control group (105±3% and 102±4% respectively, p>0.05) as analysed by one-way ANOVA and Bonferroni's post-hoc tests. However, at 10 µM ketanserin significantly attenuated DA efflux to 68±2%, ($F_{2,10}$=30.22, p<0.0001) which the post-hoc test revealed was due to significant attenuation compared to both DMSO (p<0.0001) and 1 µM ketanserin (p<0.001).

Attenuation of harmine effects on DA efflux by ketanserin

As 1 µM ketanserin alone had no effect on DA efflux this concentration was used to test whether harmine's effects are mediated via 5-HT_{2A} receptor mechanisms. The augmentation of DA efflux elicited by 300 nM harmine was significantly attenuated by 1 µM ketanserin from 148±8% to 121±3% of baseline (Figure 5, right panel). Two-way ANOVA revealed significant effects for ketanserin ($F_{1,20}$=7.568, p=0.012), harmine ($F_{1,20}$=31.022, p<0.001) and

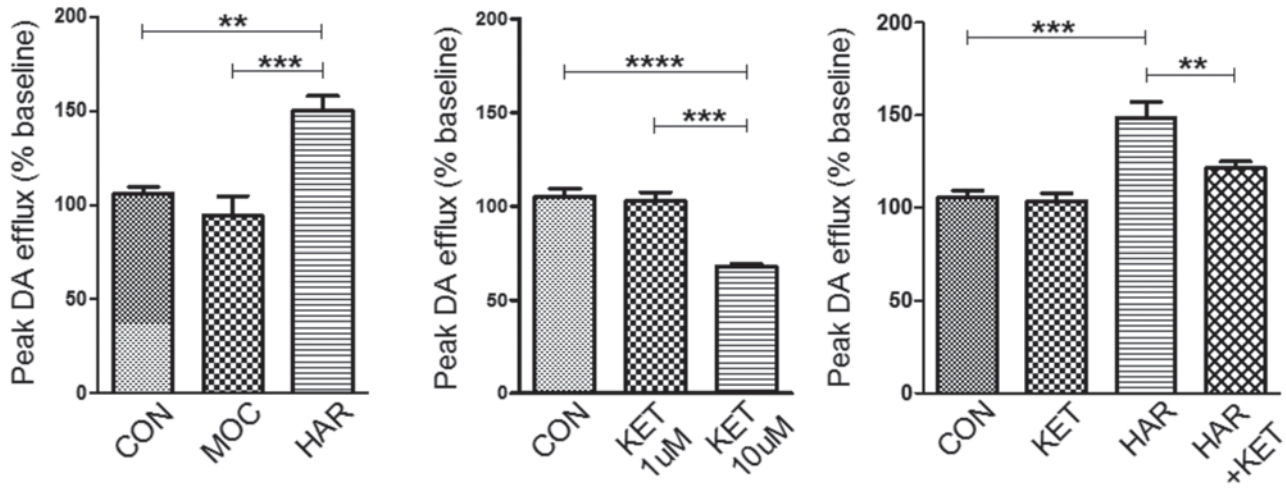

Figure 5. Effects of moclobemide (MOC) and ketanserin (KET) on dopamine (DA) efflux in nucleus accumbens (NAc) shell. Left panel: effect of 10 μM moclobemide on DA efflux, in comparison with 300 nM harmine (HAR) and control (CON) groups, analysed by one-way analysis of variance (ANOVA) with Bonferroni's post-hoc tests. CON and MOC n=5, HAR n=6. Centre panel: effects of 1 or 10 μM ketanserin on DA efflux, analysed by one-way ANOVA with Bonferroni's post-hoc tests. CON n=5, KET both n=4. Right panel: effects of 1 μM ketanserin in combination with 300 nM harmine, analysed by two-way ANOVA with Bonferroni's post-hoc tests. CON n=5, all other groups n=6. **p<0.01, ***p<0.001, ****p<0.0001.

the interaction between treatments ($F_{1,20}$=5.664, p=0.027). Bonferroni's post-hoc tests revealed that the attenuation of harmine's effects on DA efflux by ketanserin was significant (p=0.007) and, although the attenuation was incomplete, the differences between the harmine + ketanserin group and either ketanserin or DMSO control groups were not significant. As previously shown in Figure 2, harmine alone significantly augmented DA efflux compared to the DMSO control group (p<0.001).

Discussion

These data demonstrate several novel findings regarding the modulation of DA efflux by acute harmine in the two NAc sub-regions. These include the observations that evoked DA efflux is augmented by harmine in the shell only, that this augmentation is additive to cocaine-induced augmentation and that it is attenuated by the 5-$HT_{2A/2C}$ receptor antagonist ketanserin. Furthermore, the reversible inhibitor of MAO-A, moclobemide, did not augment DA efflux, suggesting that the canonical MAOI activity of harmine does not play a significant role in presynaptic modulation of DA neurotransmission in the NAc slice preparation.

Effects of cocaine and interactions with harmine

Cocaine was used at a concentration of 1 μM for all experiments to produce a robust but sub-maximal increase in DA efflux, in order that concomitant effects of harmine could be discerned. In the NAc core, cocaine rapidly increased peak DA efflux and increased the reuptake time constant by 230% and 195% respectively. These results are consistent with previously published voltammetry results in the rat NAc core brain slice (Lee et al., 2001). The significant attenuation of DA reuptake rate is consistent with the considerable body of evidence for blockade of the DA transporter by cocaine. Both DA efflux and time constant of reuptake were also significantly augmented by cocaine in the NAc shell by 179% and 189% respectively. That cocaine augments DA efflux to a greater extent in the NAc core than the shell (Figure 4, right panel) is also consistent with previous in vitro voltammetry studies (Budygin et al., 2002).

Harmine had no significant effect on cocaine-induced augmentation of DA efflux or reuptake time parameters in the NAc core, and had no significant effect on either parameter during treatment prior to cocaine application. No studies have been published to date investigating the effects of harmine on cocaine, however a single dose (10 mg/kg i.p.) of the structurally related β-carboline norharman has been shown to attenuate cocaine self-administration in male Wistar rats (Cappendijk et al., 2001). Although these authors did not conduct any investigation into the mechanism of this effect, they speculated that it was likely due to a combined modulation of serotonergic and dopaminergic pathways. These data suggest that at presynaptic terminals, harmine does not modulate the core-specific aspects of cocaine-induced DA efflux. We did not examine the effects of harmine in the ventral tegmental area (VTA) but both 5-HT_{2A} and 5-HT_{2C} receptors are localised to the VTA (Muller and Huston, 2006) and harmine could exert an influence at this site via either a direct interaction with receptors or augmentation of serotonin levels due to inhibition of MAO-A.

Within the NAc shell, harmine significantly augmented evoked DA efflux to 148±8% of baseline levels and, in combination with cocaine, peak efflux was 260±35%. Due to this additive effect on cocaine-induced DA efflux, these data suggest that harmine may not be a viable pharmacotherapeutic agent for the treatment of cocaine dependence. However, antagonism of the hedonic or reinforcing effects of cocaine is only one of several pharmacological approaches to the treatment of cocaine addiction, and one which has not shown efficacy in clinical trials (Grabowski et al., 2004). An alternative approach involves the use of DA-releasing agents as 'agonist therapies' which have shown positive results in preclinical investigations and to a limited degree in clinical trials (Rothman et al., 2008).

Effects of harmine on evoked DA efflux

The effect of harmine is consistent with an in vivo microdialysis study of systemic administration of harmine (0.5 mg/kg i.p.), in which DA efflux peaked at 152% of baseline 60 min after administration (Iurlo et al., 2001). That study also observed a significant attenuation in concentrations of the DA metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid, with the authors concluding that harmine augmented striatal DA efflux via MAO-A inhibition. In their study the dialysis probes were implanted in the lateral striatum and based on the stereotaxic coordinates provided (anterior-posterior (AP)+0.7 mm, medio-lateral (ML)±3.5 mm, dorso-ventral (DV)–7.6 mm), it would appear that they were sampling within the fundus striatum region (Paxinos and Watson, 1997). Two earlier in vivo studies also investigated the effects of harmine on DA-mediated behaviour and neurotransmission, although neither directly demonstrated a mechanistic basis for their results. Harmine, and the structurally related β-carboline harmaline, significantly increased L-3,4-dihydroxyphenylalanine (L-DOPA) induced stereotypical behaviour in a dose-dependent manner in mice (Pimpinella and Palmery, 1995). A neurochemical study investigated the effects of acute harmine on concentrations of DA and L-DOPA in plasma and striata of rats and rabbits following administration of L-DOPA (Menequz et al., 1994). They observed that harmine augmented plasma L-DOPA and striatal DA concentrations in rabbits only, which they interpreted as being indicative that harmine has neuromodulatory effects beyond MAO-A inhibition, however they did not speculate as to the site or mechanism of action.

Effects of harmine on DA reuptake

Previous evidence has demonstrated that harmine acts as a weak competitive inhibitor of the DA transporter (Drucker et al., 1990) with an IC_{50} of around 10 μM. There is also evidence to suggest that harmine may alter DA reuptake rates via internalisation of the DA transporter, due to its potent inhibition of DYRK1A (Gockler et al., 2009). As the concentration of harmine used in this study was limited to 300 nM, it is reasonable to assume that direct inhibition of the transporter protein would not play a significant role in the modulation of DA reuptake, and that this would also be the case for the sub-micromolar physiological concentrations observed following human consumption of *ayahuasca* (Callaway et al., 1999). However, as the experimental concentration of harmine used was ten-fold greater than the in vitro IC_{50} value of 33 nM for inhibition of the DYRK1A kinase, this concentration was expected to elicit a demonstrable response if such inhibition has functional consequences. Furthermore, membrane trafficking of DAT is a rapid process which has been demonstrated to occur within minutes of acute exposure to a variety of ligands (for a review see Schmitt and Reith, 2000). It was therefore somewhat surprising that harmine had no effect on DA reuptake in either the NAc core (110±7%) or shell (109±5%). The mechanism of DYRK1A–mediated internalisation of DA transporters in vivo remains hypothetical, however, with only one piece of evidence demonstrating a significant internalisation in a cell culture model following acute treatment with another inhibitor, (-)-epigallocatechin gallate (Li et al., 2006). Based on the lack of effect on DA reuptake, it is concluded that 300 nM harmine does not modulate DA reuptake, at least acutely, by either DAT inhibition or DYRK1A-mediated internalisation in our studies.

Effect of MAOI on DA efflux in brain slices

The only study investigating the effects of harmine on striatal DA neurotransmission concluded that augmentation of efflux in the lateral striatum occurred due to inhibition of MAO-A (Iurlo et al., 2001). This conclusion was based on the systemic administration of both moclobemide and harmine augmenting DA efflux, although the release time-courses were somewhat different. This finding is inconsistent with several in vivo and in vitro studies investigating the effects of MAOIs on striatal DA release, although differences in sub-region and inhibitor studied must be taken into consideration. An in vivo voltammetry study in anaesthetised rats found only negligible effects on DA efflux in the dorsal striatum following systemic administration of the MAO-B inhibitor pargyline (Garris and Wightman, 1995). An in vivo microdialysis study in awake rats of the irreversible MAO-A inhibitor clorgyline (1 mg/kg, sub-cutaneous (s.c.)) found no effect on DA efflux in the NAc core and, furthermore, no augmentation of cocaine-induced DA efflux (Pepper et al., 2001), although a maximally effective dose of clorgyline (4 mg/kg, s.c.) did augment DA efflux in the prefrontal cortex in another in vivo microdialysis study (Rollema et al., 2011). A brain slice voltammetry study using a comparable model to that presented here found no differences in DA efflux or reuptake in the dorsal striatum of slices from MAO-A knockout mice (Owesson et al., 2002). Two possible explanations can be posited for the differential effects of moclobemide between the data presented by Iurlo et al. (2001) and that presented here, which are not necessarily exclusive. The first is that there may be differential expression, or functional efficacy, of MAO-A within the sub-regions of the striatum. Immunohistochemical studies of enzyme distribution within the rat brain showed low levels of both MAO-A and MAO-B throughout the striatum, in contrast to high levels within the VTA (Willoughby et al., 1988). An alternative explanation is that the functional site of MAO-A inhibition is distal to the striatum and inhibition may be due to an indirect mechanism of modulation via augmented levels of serotonin, the preferential substrate for MAO-A. Augmented concentrations of serotonin have been shown to increase NAc DA efflux via $5\text{-}HT_{2A}$ receptors located in the VTA (Muller and Huston, 2006), an effect which would therefore not be seen in the isolated NAc slice described here. While it is possible that the MAO-A inhibition of harmine may have some functional effect on DA efflux in the striatum in vivo, the evidence presented here indicates that such activity does not modulate DA efflux at presynaptic terminals within the NAc shell. This novel finding that harmine augments NAc DA efflux apparently independently of MAO-A inhibition is of relevance given the canonical classification of harmine as an inhibitor of MAO-A (Herraiz et al., 2010).

Significance of sub-region specificity of harmine

Our data reveal anatomical specificity for the effects of harmine on DA efflux with augmentation seen only in the NAc shell. This novel finding has relevance to the development of pharmacological interventions for cocaine dependence. As DA efflux in the two NAc sub-regions differentially encodes discrete aspects of motivational salience (Meredith et al., 2008) and phases in the development of the neuropathology of drug dependence (Barrot et al., 2002), pharmacological compounds which are able to augment

DA efflux with sub-regional selectivity may offer a more targeted approach for agonist therapies.

This sub-region specific effect on DA efflux also provides an important clue about the mechanism by which harmine elicits this specific response, based on evidence for differential expression of pharmacological targets. Such differential expression profiles have been demonstrated for three relevant proteins, the 5-HT_{2A} receptor (Mijnster et al., 1997), the 5-HT_{2C} receptor (Clemett et al., 2000) and the calcium binding protein calbindin D-28k (Barrot et al., 2000). In the light of the 5-HT_{2A} receptor binding affinity demonstrated for harmine (Glennon et al., 2000; Grella et al., 1998), and the calcium-dependent nature of reserve vesicle pool DA efflux (Venton et al., 2006), the sub-region specificity of harmine effects could be attributed to a mechanism involving these proteins.

To investigate the 5-HT_{2A} receptor mechanism further we used ketanserin. Although widely used as a selective 5-HT_{2A} antagonist (Chen et al., 2003; De Paula et al., 2012; Yadav et al., 2011), ketanserin also exhibits a weaker affinity for the 5-HT_{2C} receptor (Alex and Pehek, 2007), which has been demonstrated as having an inhibitory effect on DA efflux in the NAc shell in vivo (Di Matteo et al., 2000). Unlike the phasic excitatory effect of 5-HT_{2A} receptors (Navailles and De Deurwaerdere, 2011), the 5-HT_{2C} subtype receptors exert both a tonic and phasic inhibitory control over DA efflux in the NAc shell with high constitutive activity (Navailles et al., 2006). Although 60 min treatment with 1 μM ketanserin had no effect on DA efflux, a significant attenuation of efflux was observed following 10 μM treatment. This finding is inconsistent with studies in which the selective 5-HT_{2C} inverse agonist SB 206553, or antagonist SB 242084, injected into the NAc shell elicited significant increases in DA efflux via antagonism of constitutive and phasic activation of 5-HT_{2C} receptors, respectively (De Deurwaerdere et al., 2004; Navailles et al., 2006). The significant attenuation of basal efflux by 10 μM ketanserin presented here is therefore unlikely to be resultant from non-selective antagonism of 5-HT_{2C} receptors, but is indicative of antagonist activity at 5-HT_{2A} receptors, which have functionally opposite effects on DA release. That antagonism of this phasic excitatory activity was observed to attenuate basal DA efflux was unexpected, but can be explained by the nature of the electrical stimulation used to evoke DA release within the brain slice. Application of the stimulus train evokes neurotransmitter efflux from all axon terminals within the stimulation field, including serotonin from raphe afferents (Muller and Huston, 2006). As such, during each evoked DA efflux event, serotonin will also be released into the extrasynaptic matrix surrounding the DA terminals, some of which will bind to presynaptic 5-HT_{2A} receptors. The duration of the stimulus train (451 ms) was derived from a stimulation protocol designed to replicate the typical intraburst firing frequency of rodent DA neurons and pulse number which elicits robust but submaximal DA efflux in brain slice voltammetry studies. This stimulation train is of sufficient duration to allow some modulation of DA efflux during the release event by presynaptic autoreceptors which in the case of D2/3 receptors has been shown to occur with a latency of 200 ms and peak at 700–1000 ms (Lee et al., 2002; Phillips et al., 2002). The magnitude of evoked DA is therefore likely to include an element of 5-HT_{2A} receptor mediated augmentation due to local serotonin efflux. Antagonism of this augmentation effect would appear negligible upon treatment with 1 μM ketanserin, however at 10 μM the concentration may be sufficient to attenuate this serotonergic component of DA efflux.

The most plausible interpretation, therefore, is that these data provide evidence in support of the hypothesis that harmine acts as a 5-HT_{2A} agonist to augment the efflux of DA in the NAc shell. However, further studies are needed to confirm this receptor as the mediator of the observed effects of harmine using more selective 5-HT_{2A} and 5-HT_{2C} inhibitors.

The sub-region specificity of the augmented efflux can be explained by two mechanisms, both of which are consistent with the 5-HT_{2A} agonist activity indicated by these results. The NAc shell has been shown to have higher expression of 5-HT_{2A} receptor mRNA than the core, (Mijnster et al., 1997). Such differential expression levels may be sufficient to mediate the regional specificity of harmine, provided the concentration used in this study is below the threshold for receptor occupancy necessary to elicit any observable response in the core but is high enough to augment efflux to 148% of baseline values in the shell. An alternative and intriguing explanation could be that harmine exhibits functional selectivity for one of the three currently known effector pathways downstream of 5-HT_{2A} receptors (mediated by inositol triphosphate, arachidonic acid or 2-arachindonylglycerol, see Urban et al., 2007 for review).

Limitations

The major limitation of this study is in determining the exact mechanism of action of harmine. As we have described above, we feel that the simplest explanation is that, under these conditions, harmine is acting as an agonist at 5-HT_{2A} receptors. However, we feel it is important to bear in mind that both harmine and ketanserin have multiple receptor targets that should not be dismissed. With respect to ketanserin, it is an antagonist at 5-HT_{2A} and to a lesser extent at 5-HT_{2C} receptors and also it has a strong affinity for the vesicular monoamine transporter ($vMAT_2$; Leysen et al., 1988). This is potentially an important target as a drug which blocked the $vMAT_2$ might be expected to alter basal and/or evoked DA levels. Leysen et al. found that ketanserin bound to the tetrabenazine binding site of the $vMAT_2$ with nanomolar affinity (K_D=12.4 nM) and that 1 μM ketanserin caused a small increase in DA efflux from pre-loaded rat striatal slices, attributed to release of a cytoplasmic pool of amine. Other studies have also found ketanserin to bind to the $vMAT_2$ (Darchen et al., 1988; Henry et al., 1991; Sievert and Ruoho, 1997) but functional data is lacking. It is possible that ketanserin binds to the $vMAT_2$ but has little effect. If ketanserin did have a DA-releasing effect via the $vMAT_2$ then one might expect ketanserin to increase DA levels in microdialysis studies but this is not the case (Bonhomme et al., 1995; Nash 1990). Further, using ketanserin at 1 μM, we did not see any changes in basal or evoked DA efflux in the current study suggesting that under these conditions ketanserin does not cause release of DA through $vMAT_2$ mechanisms. With respect to harmine, we have recently reviewed its pharmacology (Brierley and Davidson, 2012) which not only includes the 5-HT_2 receptor but also the DAT, MAO, imidazoline receptor and DYRK1A. In addition, it is likely that harmine has affinity at other sites, some which are emerging and others as yet unknown. For example, several recent in vitro and pre-clinical studies have shown harmine has anticancer and anti-malarial activities, indicating further pharmacological targets for this compound. Harmine inhibits breast cancer

resistance protein in a breast cancer cell line (Ma and Wink, 2010), has anti-metastatic properties in a mouse lung metastasis model (Hamsa and Kuttan, 2011) and is an inhibitor of the haspin kinase, an emerging therapeutic target in cancer that phosphorylates histone H3 in mitosis (Cuny et al., 2012). Harmine is also a potent antimalarial, with specific affinity for *Plasmodium falciparum* heat shock protein 90, acting synergistically with chloroquine and artemisinin in in vitro and rodent models (Shahinas et al., 2012).

Conclusions

Human consumption of *ayahuasca* has been associated with abstinence from drug and alcohol dependence, and anecdotal evidence suggests that *ayahuasca* may have efficacy as a treatment for cocaine dependence. It is possible that the other main constituent of *ayahuasca*, N,N- dimethyltryptamine (DMT), contributes to the therapeutic effect but discussion of this is beyond the scope of this manuscript. This study focussed on the role of harmine in acutely modulating baseline and cocaine-induced DA neurotransmission in the NAc core and shell, key brain regions involved in the neuropathology of drug dependence. Harmine interacts in vitro with multiple pharmacological targets with established or hypothetical roles in modulating DA neurotransmission. However, only a limited number of in vivo studies have investigated the effects of this compound on the DA system, none of which have directly demonstrated a pharmacological mechanism. The data presented here show that harmine specifically augments evoked DA efflux in the NAc shell, that this effect is additive to that of cocaine, and it is attenuated by ketanserin. Furthermore, the canonical activity of harmine as an inhibitor of MAO-A does not appear to be important in this model. No evidence was found for the hypothetical role of harmine in modulating DA reuptake via DYRK1A dependent internalisation of DA transporters. On the basis of these data it is proposed that harmine acts as a 5-HT_{2A} receptor agonist, with possible functional selectivity at this receptor in the NAc shell, resulting in augmented DA efflux. Such a mechanism may be efficacious as an agonist therapy for cocaine dependence. Further studies are required to confirm the proposed pharmacological mechanism of harmine and determine efficacy in animal models of cocaine dependence.

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Conflict of interests

The authors declare that there are no conflict of interest.

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