Journal of Psychopharmacology

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Proof - Dec 17, 2010

What is This?

The role of nucleus accumbens shell GABA receptors on ventral tegmental area intracranial self-stimulation and a potential role for the 5-HT$_{2C}$ receptor

Dave J Hayes[1,2], John Hoang[1] and Andrew J Greenshaw[1,3]

Abstract

Brain γ-aminobutyric acid (GABA) and 5-hydroxytryptamine (5-HT)$_{2C}$ receptors are implicated in the neuronal regulation of reward- and aversion-related behaviour. Within the mesocorticolimbic pathways of the brain, relationships between GABA containing neurons and 5-HT$_{2C}$ receptor activity may be important in this context. The primary aim of this study was to investigate the role of NAc shell GABA receptors on ventral tegmental area intracranial self-stimulation (ICSS) and to examine the systemic effects of GABAergic ligands in this context. The second aim was to investigate the relationship between GABA receptor- and 5-HT$_{2C}$ receptor-related ICSS behaviour, using systemic administration of the selective agonist WAY 161503. Locomotor activity was assessed to compare the potential motor effects of drugs; feeding behaviour and intra-NAc injections of amphetamine (1.0 μg/side) were used as positive controls. When administered systemically the GABA$_A$ receptor agonist muscimol and antagonist picrotoxin did not selectively change ICSS reward thresholds, although the 5-HT$_{2C}$ receptor agonist WAY 161503 (1.0 mg/kg) decreased reward measures. Intra-NAc shell administration of muscimol (225 ng/side) and picrotoxin (125 ng/side), respectively, decreased and increased measures of reward. Intra-NAc shell baclofen (0-225 ng/side; GABA$_B$ receptor agonist) did not affect any ICSS measures although it increased feeding. Combining picrotoxin and WAY 161503 attenuated the effects of each. These results suggest that a 5-HT$_{2C}$ and GABA$_A$ receptor-mediated neuronal relationship in the NAc shell may be relevant for the regulation of brain reward pathways.

Keywords

5-HT$_{2C}$ receptor, GABA$_A$ receptor, GABA$_B$ receptor, intracranial self-stimulation (ICSS), locomotor activity, nucleus accumbens (NAc), reward, rats, ventral tegmental area (VTA)

Introduction

Understanding the biological basis of reward and aversion is necessary for the development of further treatments for psychiatric disorders such as depression, schizophrenia, and drug addiction (Chau et al., 2004; Diekhof et al., 2008). While the mesocorticolimbic dopamine system is likely necessary in the regulation of reward- and aversion-related behaviours (Berridge, 2007; Schultz, 2007; Wise, 2008), it has become increasingly clear that other neurotransmitters, such as serotonin (5-HT) and γ-aminobutyric acid (GABA), play an integral role (Ikemoto and Wise, 2004; Tzschentke, 2007). GABA and 5-HT are known to regulate neuronal activity through their actions at multiple receptor subtypes (Fink and Gothert, 2007). The GABA$_A$, GABA$_B$, and 5-HT$_{2C}$ receptors have garnered attention regarding their roles in reward- and aversion-related processes particularly because of their effects in animal models of reinforcement, their ability to modulate mesolimbic dopamine release (Alex et al., 2005; Rahman and McBride, 2002), and their putative connection to many psychiatric disorders (Berg et al., 2008; Filip and Frankowska, 2008; Sen and Sanacora, 2008).

GABA receptors are found throughout the brain (Olsen and Sieghart, 2009) and both GABA$_A$ (Ikemoto et al., 1998; Liu and Ikemoto, 2007) and GABA$_B$ (Backes and Hemby, 2008; Sahraei et al., 2009) receptors play a role in reward-related behaviours. The 5-HT$_{2C}$ receptor has been identified on GABAergic cells in the ventral tegmental

[1]Centre for Neuroscience, University of Alberta, Edmonton, AB, Canada.
[2]Mind, Brain Imaging and Neuroethics, Institute of Mental Health Research, Royal Ottawa Health Care Group, University of Ottawa, Ottawa, ON, Canada.
[3]W.G. Dewhurst Laboratory, Neurochemical Research Unit, Department of Psychiatry, University of Alberta, Edmonton, AB, Canada.

Corresponding author:
Dave J Hayes, Mind, Brain Imaging and Neuroethics, Institute of Mental Health Research, Royal Ottawa Health Care Group, University of Ottawa, 1145 Carling Avenue, Room 6441, Ottawa, ON K1Z 7K4, Canada
Email: david.hayes@rohcg.on.ca

area (VTA), prefrontal cortex and raphe nuclei (Bubar and Cunningham, 2007; Liu et al., 2007; Serrats et al., 2005) and also plays a role in regulating reward-related behaviours (Fletcher et al., 2002; Grottick et al., 2001; Hayes et al., 2009a; Mosher et al., 2006; although see Hayes et al., 2009b and Mosher et al., 2005), although the mechanisms involved are unclear.

Electrical stimulation of the VTA supports intracranial self-stimulation (ICSS) in rats and, when used with the psychophysical curve-shift method, provides a unique quantitative method for studying reward-related behaviour. VTA ICSS is stable over many months, often showing no satiation or sensitization effects and results from the direct activation of circuitry that is also associated with natural and drug reinforcers (Carlezon and Chartoff, 2007; Wise, 2002). It results in dopamine release in nucleus accumbens septi (NAc) (Fiorino et al., 1993) and most drugs of abuse potentiate its effects on reward (Kenny, 2007).

Many cells within the VTA, and the majority within the NAc, are GABAergic (Meredith, 1999; Van Bockstaele and Pickel, 1995; Walaas and Fonnum, 1980) and there is evidence that these cells may be involved in mediating ICSS behaviour (Lassen et al., 2007; Steffensen et al., 2001). A recent paper by Carlezon and Thomas (2009) hypothesized that rewarding and aversive states are encoded by the activities of GABA cells within the NAc. Specifically, the authors suggested that an increase in NAc GABA cell activity encodes decreased reward while a decrease in GABA cell activity increased reward.

To date, no ICSS studies have investigated the effects of GABAergic compounds within the NAc shell, a region associated with reward-related inhibitions more so than the core (e.g. Carlezon et al., 1998; Cheer et al., 2007). The primary aim of this study was to pharmacologically test Carlezon and Thomas' (2009) NAc Activity Hypothesis in the context of VTA ICSS, using NAc microinjections of the $GABA_A$ receptor agonist muscimol (0–225 ng/side) and antagonist picrotoxin (125 ng/side), and the $GABA_B$ receptor agonist baclofen (0–225 ng/side). As intra-NAc shell microinjections of GABAergic agonists have been shown to increase feeding behaviour, and intra-NAc shell amphetamine (1.0 μg/side) is known to have reward-enhancing effects on ICSS (Colle and Wise, 1988; Schaefer and Michael, 1988), these were used as positive controls. The systemic effects of muscimol (0–4.0 mg/kg), picrotoxin (0–1.0 mg/kg) and baclofen (0–2.5 mg/kg) were also tested and compared with the results involving NAc microinjections using reward-sensitive rate-frequency threshold ICSS measures.

The second aim was to investigate the relationship between GABA and $5\text{-}HT_{2C}$ receptors using ICSS behaviour. The selective $5\text{-}HT_{2C}$ receptor agonist WAY 161503 (1.0 mg/kg) was used as it has been shown to increase ICSS rate-frequency thresholds (indicating a decrease in reward) without affecting measures of motor performance; although direct stimulation of $5\text{-}HT_{2C}$ receptors in the NAc shell had no effect (Hayes et al., 2009a). The effects of all systemically administered compounds were also assessed in locomotor activity to compare the potential motor effects of drugs. Although the ICSS procedures used in this study do provide a rate-independent measure of reward, the authors acknowledge

that additional data on locomotor activity changes is of value in interpreting potentially reward-selective effects.

Materials and methods

Subjects

Eighty-two male Sprague–Dawley rats (Health Sciences Laboratory Animal Services, University of Alberta, Canada) weighing 200–250 g were housed individually in standard Plexiglas laboratory cages at 20°C and 50% humidity, with a 12-h light/dark cycle (lights from 07:00 to 19:00) with food and water freely available. All testing took place in the dark (ICSS) or under red light (locomotor activity/feeding) during the light phase of the light/dark cycle. All apparatus were cleaned between animals with diluted (1:6) ammonia-based window cleaner (No Name® Glass Cleaner with ammonia). The care and use of animals were in accordance with guidelines of the University of Alberta Health Sciences Animal Welfare Committee and the Canadian Council on Animal Care.

Drugs

The $5\text{-}HT_{2C}$ receptor agonist WAY 161503 : HCl [8,9-dichloro-2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2-*a*]quinoxalin-5(6*H*)-one hydrochloride], the $GABA_A$ receptor agonist muscimol [5-aminomethyl-3-hydroxyisoxazole] and the antagonist picrotoxin [1 : 1 mixture of picrotoxinin and picrotin], and the $GABA_B$ receptor agonist (R)-baclofen [(R)-4-Amino-3-(4-chlorophenyl)butanoic acid] (baclofen) were purchased from Tocris Cookson Inc. (Ellisville, MO, USA). (+)-α-Methylphenethylamine (amphetamine) sulphate was purchased from SmithKlineBeecham Pharmaceuticals (Mississauga, Ontario, Canada). All systemically administered compounds were dissolved in double-distilled water (ddH_2O) and injected subcutaneously 10 min prior to testing, while baclofen was injected intraperitoneally 20 min prior to testing, in a volume of 1.0 ml/kg. Artificial cerebrospinal fluid was freshly prepared (Elliott and Lewis, 1950) and drug solutions made daily (pH 6.0–7.0). All drug doses are expressed as free-base.

Intracranial self-stimulation

Surgery and histology. Using a previously described procedure (Greenshaw, 1993), each animal (10 for each dose–response experiment; 10 for muscimol × picrotoxin; 8 for baclofen × WAY 161503, 8 for each intracranial microinjection experiment) was implanted with a stainless steel, monopolar, stimulating electrode (E363/2; tip diameter 200 μm; Plastics One Ltd., Roanoke, VA) directed to the VTA. A large silver indifferent electrode in the skull served as the relative ground. Animals used for microinjection were also implanted with bilateral cannulae (22 gauge) directed to the rostral shell of the NAc. Stereotaxic coordinates were [mm]: VTA – AP + 2.6, L + 0.5, V + 1.8; NAc shell – AP + 11.0, L + 0.4, V + 2.8, from inter-aural zero, with the incisor bar set at 2.4 mm below the inter-aural line (Paxinos and Watson, 1998). These coordinates were interpolated from

the target site for an angle of $20°$ lateral and anterior for the VTA and $16°$ lateral for the NAc shell (Greenshaw, 1997). Dummy cannulae and injectors aimed at the NAc shell protruded 1 mm below the guide cannulae. Electrode and cannulae placements were verified at the end of the experiment by microscopic inspection of flash-frozen coronal brain sections (40 μm); flash-freezing was achieved using isopentane cooled on dry ice. Only animals with VTA and NAc shell placements were included in the analysis.

Apparatus and procedure.

Monopolar stimulation of the VTA was provided from constant current DC stimulators (cathodal monophasic pulse width of 200 μs; initial training frequency of 100 Hz; train length of 1 s) connected to each animal via a gold-track slip ring. Between pulses, the active electrode and indifferent electrode were connected through a resistor to cancel any effects of electrode polarisation (Greenshaw, 1986). The apparatus and rate-frequency analysis were as described by Ivanová et al. (1997). With this procedure, M50 is the threshold frequency at which half-maximal response rates occur; RMAX is the maximal rate of responding in a session. While M50 is a measure of reward sensitivity (which is dissociable from non-specific changes in behaviour), RMAX is a measure of response performance (see Gallistel and Karras, 1984; and also Fouriezos et al., 1990; Greenshaw and Wishart, 1987; Miliaressis et al., 1986). Animals received a randomized counterbalanced sequence of treatments with 3 days of baseline frequency testing between each treatment. To minimize the use of animals, eight animals from the muscimol and picrotoxin dose–response ICSS experiments were subsequently used in the muscimol × picrotoxin experiment; eight animals from the baclofen dose–response ICSS experiment were subsequently used in the baclofen × WAY 161503 experiment; all animals used in locomotor dose response experiments were used subsequently for interaction experiments.

Microinjection of drugs.

Rats with bilateral cannulae in the NAc shell received randomly assigned, counterbalanced treatments, separated by at least 3 days between each microinjection. Depending on the experiment, treatments included intra-NAc shell microinjections of artificial cerebrospinal fluid (CSF), muscimol (0-225 ng/side), picrotoxin (125 ng/side), baclofen (0-225 ng/side), and amphetamine (1.0 μg/side) administered in a total volume of 0.5 μL at a pump-controlled rate of 0.2 μL per minute (Beehive controller, Bioanalytical Systems, Inc.); the injection cannulae remained in place for a further minute to allow for drug absorption. Immediately following each set of microinjections, each animal was tested for VTA ICSS.

Food intake

Adapted from previously described procedures (Reynolds and Berridge, 2001; Stratford and Kelley, 1997), non-food-deprived animals were placed in standard Plexiglas laboratory cages (free from wood shavings) immediately following the VTA ICSS session. A pre-weighed amount of food in a container (conditions identical to those in their home cage) was made available along with water 25 min after the initiation of each VTA ICSS session (each session was a maximum of 25 min). At the end of a 30 min session (55 min post injection), food intake was calculated by subtracting the initial weight of the food and container from the final weight. Animals were habituated for 3 days prior to the beginning of microinjection treatments. This procedure was subsequently performed on each microinjection treatment day to determine total food intake (measured in grams) in a 30 min session following intra-NAc muscimol and baclofen (0–225 ng/side).

Spontaneous locomotor activity

Apparatus.

Spontaneous locomotor activity was measured using computer-monitored photobeam boxes (I. Halvorsen System Design, Phoenix, AZ, USA). The locomotor apparatus consisted of a clear Plexiglas test cage (43 cm L × 43 cm W × 30 cm H) with a 12 × 12 photobeam grid located 2.5 cm above the floor. These beams measured horizontal activity (measured by the number of infrared beams broken) as well as consecutive beam breaks (repeat activity). Vertical activity (or rearing activity, measured by infrared beams broken following rears on the hind legs) was measured using 12 additional photobeams located 12 cm above the floor.

Procedure.

Animals ($n = 8$/experiment) were habituated to the locomotor activity boxes for 2 consecutive days (60 min/day). They subsequently received randomized and counterbalanced injections with 3 drug-free days between injections. All locomotor activity was monitored over 30 min.

Statistical analysis

Experimental effects for ICSS, spontaneous locomotor activity, and food intake were determined using repeated measures (RM) analysis of variance (ANOVA). Dose–response effects for ICSS and feeding were assessed by a one-way RM ANOVA, and for locomotor activity by a two-way (time-× dose) RM ANOVA. Potential interactions between the systemic effects of baclofen and WAY 161503 on ICSS were assessed by a 3 × 3 RM ANOVA; muscimol and picrotoxin by a 2 × 3 RM ANOVA. The studies investigating the potential interaction between intra-NAc muscimol or picrotoxin and systemic WAY 161503 were assessed by a 2 × 2 RM ANOVA. Investigation of the potential interaction between the locomotor effects of muscimol and picrotoxin and baclofen and WAY 161503 was assessed using 2 × 3 and 2 × 4 RM ANOVAs, respectively. It is important to note that the use of the term 'interact' is used in a statistical sense here, as determined by the ANOVA.

A significant F ratio ($p \le 0.05$) was followed by Newman–Keuls *post hoc* tests ($\alpha = 0.05$) where appropriate. Only horizontal locomotor activity in the studies involving muscimol, and horizontal and rearing (vertical) activity in the study involving baclofen, are reported as the other measures (repeat/consecutive; rearing/vertical) paralleled those for horizontal locomotor activity. All ICSS data are presented

as a percentage of average baseline performance of each animal. Greenhouse–Geisser corrected degrees of freedom are used as a conservative estimate of the F-ratio. Statistical analyses for all experiments were completed using statistical software (SPSS Inc., Chicago, IL, USA).

Results

Effects of intra-NAc shell muscimol and baclofen on ICSS

Intra-NAc shell muscimol (25, 75, 225 ng/side) produced a significant increase in M50 thresholds (indicating a decrease in reward) (Figure 1A, $F(1.89, 13.21) = 8.63$, $p < 0.05$) without effects on RMAX values (indicating no effect on motor performance) (Figure 1B, $F(2, 14) = 1.39$, $p > 0.05$); see the 'Materials and methods' section for definitions of M50 and RMAX and for reference to specific statistical tests. Newman–Keuls *post hoc* tests revealed that the highest dose of muscimol (225 ng/side) produced an increase in M50 thresholds (Figure 1A). The positive control amphetamine (1.0 µg/side) significantly decreased M50 values (Figure 1A, $F(1, 7) = 9.43$, $p < 0.05$) without affecting RMAX values (Figure 1B, $F(1, 7) = 3.42$, $p > 0.05$).

Intra-NAc shell baclofen (25, 75, 225 ng/side) did not significantly affect M50 thresholds (Figure 1C, $F(1.38, 9.63) = 1.60$, $p > 0.05$) or RMAX values (Figure 1D, $F(1.20, 8.42) = 1.42$, $p > 0.05$). This is in contrast to the positive control amphetamine (1.0 µg/side) which significantly decreased

M50 values (Figure 1C, $F(1, 7) = 8.41$, $p < 0.05$) without affecting RMAX (Figure 1D).

Effects of intra-NAc shell muscimol and baclofen on food intake

Intra-NAc muscimol (0–225 ng/side) did not alter feeding (Table 1, $F(1.47, 10.31) = 1.95$, $p > 0.05$), while baclofen (0–225 ng/side) significantly increased feeding (Table 1, $F(2.45, 17.14) = 4.16$, $p < 0.05$). Further analysis using Newman–Keuls *post hoc* tests ($\alpha = 0.05$) revealed that the highest dose of baclofen tested (225 ng/side) significantly increased food intake.

Effects of systemic GABA_A and GABA_B receptor ligands on ICSS

As summarized in Table 2A–D, systemic administration of muscimol (0–4.0 mg/kg) revealed main effects for both M50 ($F(3.24, 29.18) = 7.13$, $p < 0.05$) and RMAX values ($F(2.77, 24.95) = 53.39$, $p < 0.05$) (Table 2A). Newman–Keuls *post hoc* tests revealed that only the highest dose (4.0 mg/kg) produced a significant increase in M50 and RMAX values compared to control. A main effect of picrotoxin (0–1.0 mg/kg) was also seen with M50 ($F(2.04, 18.36) = 5.75$, $p < 0.05$) and RMAX values ($F(1.54, 13.88) = 6.47$, $p < 0.05$), although *post hoc* tests revealed that the highest dose tested produced a
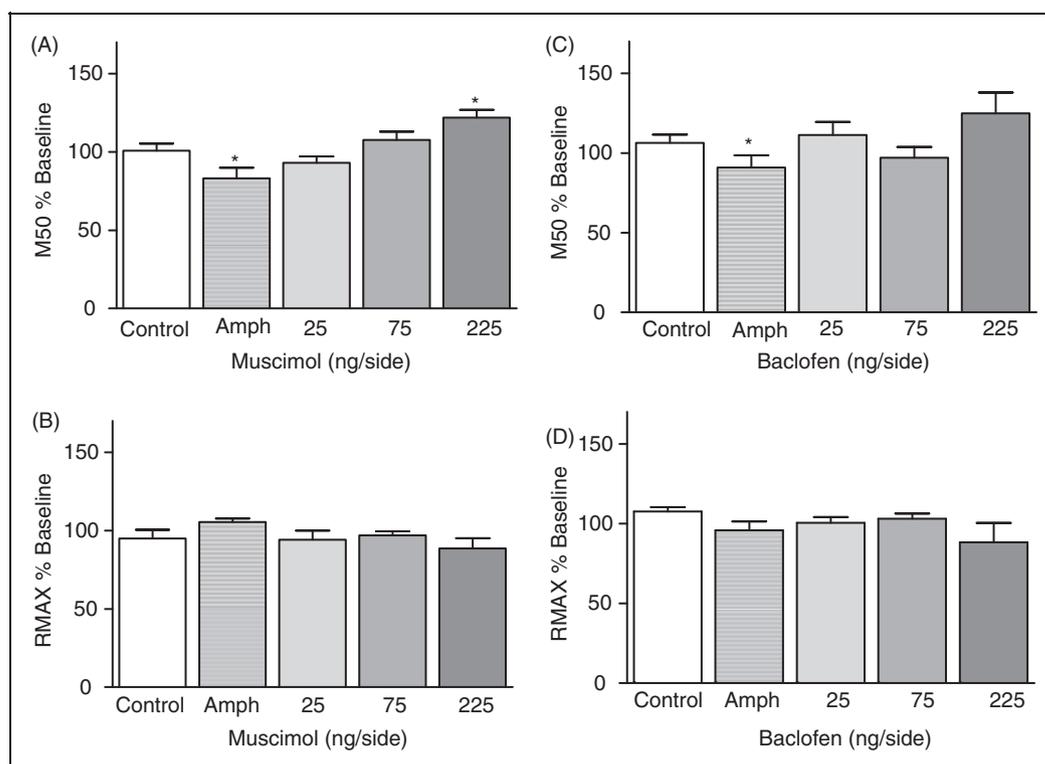

Figure 1. Effects of intra-NAc shell (A, B) muscimol and (C, D) baclofen (0–225 ng/side; $n = 8$ for each experiment), and amphetamine (Amph; 1.0 µg/side), on rate-frequency thresholds (M50 values) and maximal response rates (RMAX values) for VTA ICSS. Data shown are means ± SEM expressed as a percentage of baseline performance. *Significant from control at $p < 0.05$ following Newman–Keuls *post hoc* tests.

significant increase in only M50 thresholds (Table 2B). No interaction was noted for muscimol (4.0 mg/kg) in combination with subthreshold doses of picrotoxin (0.25, 0.5 mg/kg) ($F(1.80, 12.57) = 1.25$, $p > 0.05$; $F(1.66, 11.61) = 0.67$, $p > 0.05$), although there was a main effect of muscimol for M50 ($F(1, 7) = 8.54$, $p < 0.05$) and main effects for both muscimol and picrotoxin for RMAX values ($F(1, 7) = 84.30$, $p < 0.05$; $F(1.99, 13.90) = 5.47$, $p < 0.05$) (Table 2C). The authors did not note any within-session effects following the administration of active drug doses, although the short ICSS sessions make time course analysis difficult. To eliminate the possibility that lower doses of muscimol may be more active following a time delay, the effects of 1.0 mg/kg (a behaviourally active dose) were studied following a delay of up to 1 h; no time course effects were noted in this regard (results not shown).

Analysis following systemic administration of baclofen (0–2.5 mg/kg) revealed main effects for M50 thresholds ($F(2.69, 24.21) = 16.04$, $p < 0.05$) and RMAX values ($F(1.32, 11.86) = 12.31$, $p < 0.05$). Newman–Keuls *post hoc* tests ($\alpha = 0.05$) revealed that the 1.25 and 2.5 mg/kg doses produced a significant increase in M50 compared to control; the highest dose (2.5 mg/kg) decreased RMAX values (Table 2D).

Effects of the systemic baclofen and WAY 161503 on ICSS

Systemic administration of baclofen (0.625, 1.25 mg/kg) and WAY 161503 (0.3, 1.0 mg/kg) revealed main effects of baclofen for M50 thresholds (Figure 2A, $F(1.60, 11.20) = 25.14$,

Table 1. Effects of intra-NAc shell muscimol and baclofen (0–225 ng/side; $n = 8$ per dose response) on food intake (measured in grams) over 30 min subsequent to the ICSS session

	Dose (ng/side)			
	0	25	75	225
Muscimol	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	1.0 ± 0.6
Baclofen	1.3 ± 0.5	1.6 ± 0.5	3.2 ± 0.6	3.4 ± 0.7*

Data shown are means ± SEM. *Significant from control at $p < 0.05$ following Newman–Keuls *post hoc* tests.

Table 2. Frequency thresholds (M50) and maximal response rates (RMAX) (expressed as percent of baseline) ± SEM following systemic administration of muscimol (A; $n = 10$), picrotoxin (B; $n = 10$), muscimol + picrotoxin (C; $n = 10$) and baclofen (D; $n = 10$)

2A.

	Muscimol (mg/kg)							
	0	0.10	0.25	0.50	0.75	1.0	2.0	4.0
M50	100.6 ± 3.9	105.0 ± 5.1	104.3 ± 5.3	97.5 ± 3.9	115.8 ± 6.4	100.3 ± 5.5	119.8 ± 5.7	146.7 ± 11.9*
RMAX	99.5 ± 2.4	102.5 ± 1.7	97.0 ± 2.0	101.0 ± 2.9	98.5 ± 3.1	101.8 ± 1.7	86.0 ± 4.8	45.8 ± 1.9*

2B.

	Picrotoxin (mg/kg)			
	0	0.25	0.50	1.0
M50	100.2 ± 7.1	110.9 ± 7.2	111.4 ± 8.9	145.1 ± 9.2*
RMAX	103.9 ± 2.6	100.4 ± 2.7	95.8 ± 3.2	76.6 ± 7.7

2C.

	Muscimol (M) + Picrotoxin (P) (mg/kg)					
	0	M 4.0	P 0.25	P 0.50	M 4.0 + P 0.25	M 4.0 + P 0.50
M50	91.2 ± 6.3	144.9 ± 15.7*	104.7 ± 3.5	114.3 ± 12.1	128.1 ± 5.2*	150.2 ± 17.6*
RMAX	103.4 ± 2.1	59.3 ± 7.6*	102.2 ± 1.1	97.9 ± 2.1	58.1 ± 6.7*	46.0 ± 2.8*

2D.

	Baclofen (mg/kg)			
	0	0.625	1.25	2.5
M50	99.8 ± 4.0	108.7 ± 5.4	136.6 ± 8.6*	150.4 ± 5.9*
RMAX	103.8 ± 1.8	101.9 ± 1.5	93.5 ± 3.4	69.3 ± 9.0*

Data shown are means ± SEM expressed as a percentage of baseline performance. *Significant from control at $p < 0.05$ following Newman–Keuls *post hoc* tests.
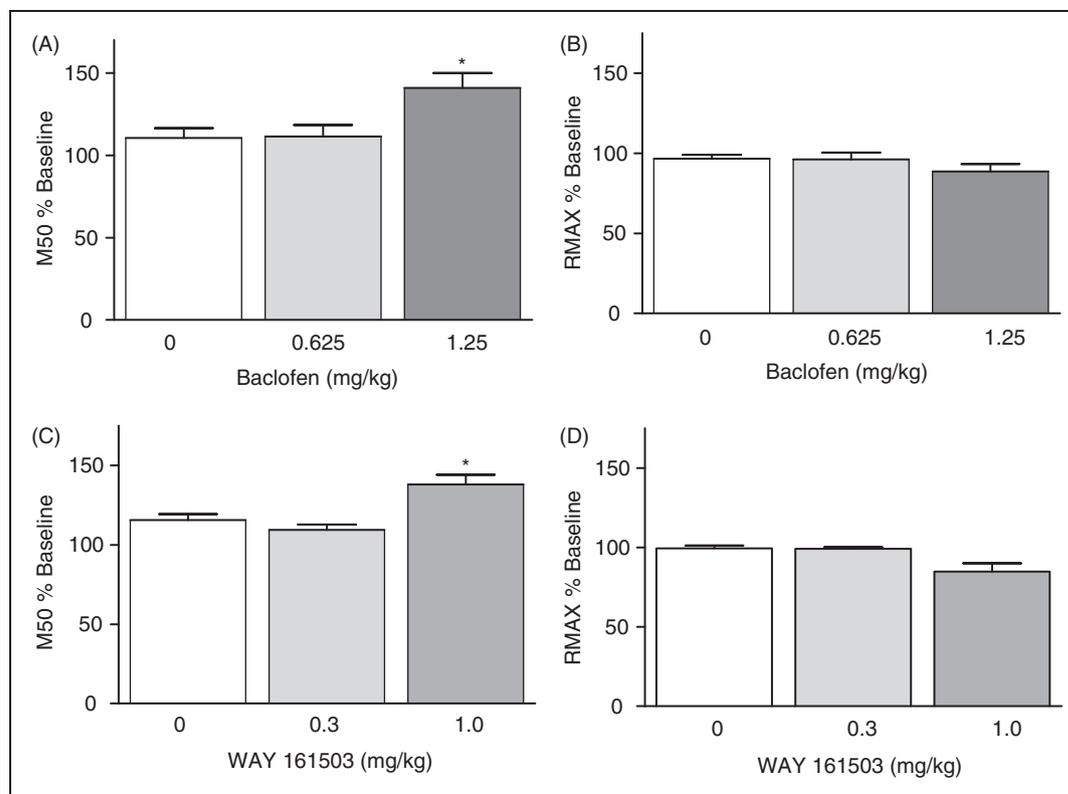
Figure 2. The main effects of baclofen (0.625, 1.25 mg/kg) and WAY 161503 (0.3, 1.0 mg/kg) on (A, C) rate-frequency thresholds (M50) and (B, D) maximal response rates (RMAX), respectively, for VTA ICSS ($n = 8$). Data shown are means ± SEM expressed as a percentage of baseline performance. *Significant from control at $p < 0.05$ following Newman–Keuls *post hoc* tests.

$p < 0.05$) but not for RMAX values (Figure 2B), and for WAY 161503 for M50 (Figure 2C, $F(1.83, 12.78) = 18.22$, $p < 0.05$) and RMAX values (Figure 2D, $F(1.26, 8.82) = 10.19$, $p < 0.05$). WAY 161503 and baclofen did not interact on any measure. Further analysis of baclofen, using Newman–Keuls *post hoc* tests ($\alpha = 0.05$), following the collapse of data across WAY 161503, revealed that the 1.25 mg/kg dose of baclofen was significant from control for M50 (Figure 2A); analysis of WAY 161503, following the collapse of data across baclofen, revealed that the 1.0 mg/kg dose was significant from control for M50 (Figure 2C). No differences were noted for RMAX values (Figure 2B and D).

Effects of intra-NAc shell muscimol and picrotoxin in combination with systemic WAY 161503 on ICSS

Intra-NAc shell muscimol (225 ng/side), once again, produced a significant increase in M50 without having an effect on RMAX values (Figure 3A, $F(1, 7) = 5.92$, $p < 0.05$; Figure 3B, $F(1, 7) = 0.67$, $p > 0.05$), while WAY 161503 (1.0 mg/kg) showed similar effects (Figure 3A, $F(1, 7) = 6.84$, $p < 0.05$; Figure 3B, $F(1,7) = 3.88$, $p > 0.05$). The combination of WAY 161503 and muscimol did not result in an interaction for M50 or RMAX values ($F(1, 7) = 2.00$, $p > 0.05$; $F(1, 7) = 0.66$, $p > 0.05$).

Intra-NAc shell picrotoxin (125 ng/side) produced a significant decrease in M50 without having an effect on RMAX values (Figure 3C, $F(1, 7) = 13.58$, $p < 0.05$; Figure 3D, $F(1, 7) = 0.58$, $p > 0.05$), while WAY 161503 (1.0 mg/kg) significantly increased M50 and decreased RMAX values (Figure 3C, $F(1, 7) = 24.05$, $p < 0.05$; Figure 3D, $F(1,7) = 11.47$, $p < 0.05$). WAY 161503 and picrotoxin did not interact for M50 or RMAX values ($F(1, 7) = 0.01$, $p > 0.05$; $F(1, 7) = 0.66$, $p > 0.05$).

Representative photomicrographs of VTA stimulation sites and NAc shell microinjection sites (Figures 4A and C, respectively) and histological locations (Figures 4B and D, respectively) are displayed for reference.

Effects of systemic GABA receptor ligands and WAY 161503 on locomotor activity

As outlined in Table 3A, muscimol (0–0.75 mg/kg) decreased locomotor activity at all doses tested, as revealed by Newman–Keuls *post hoc* tests ($\alpha = 0.05$) following repeated measured ANOVA ($F(2.96, 20.75) = 5.39$, $p < 0.05$). A main effect of picrotoxin ($F(2.09, 14.64) = 11.82$, $p < 0.05$) was also found; the two highest doses tested (0.5, 1.0 mg/kg) decreased locomotor activity. There was a significant interaction between muscimol (0.10 mg/kg) and picrotoxin (0.25, 0.50 mg/kg) ($F(1.79, 12.54) = 4.87$, $p < 0.05$). *Post hoc* tests
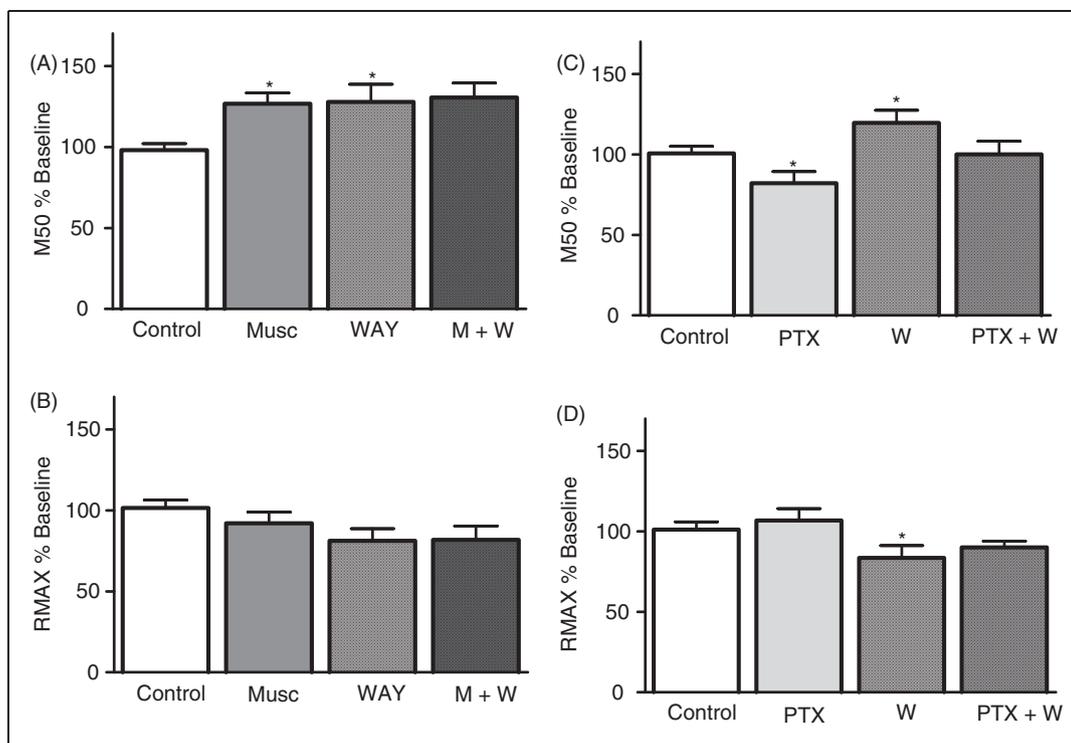
Figure 3. Effects of intra-NAc shell (A, B) muscimol (225 ng/side) and systemic WAY 161503 (1.0 mg/kg; $n = 8$), and (C, D) picrotoxin (125 ng/side) and systemic WAY 161503 (1.0 mg/kg; $n = 8$) on rate-frequency thresholds (M50 values) and maximal response rates (RMAX values) for VTA ICSS. Data shown are means $\pm$ SEM expressed as a percentage of baseline performance. *Main effect at $p < 0.05$.

revealed that muscimol (0.10 mg/kg) and picrotoxin (0.50 mg/kg) reduced locomotor activity while combinations of muscimol and picrotoxin were not significant from baseline. No interaction of drug dose × time was noted (for each of the dose–response curves), therefore further investigation of time course effects was not warranted.

When investigated separately, while the 5-HT$_{2C}$ receptor agonist WAY 161503 significantly decreased locomotor activity (comparable to those data noted below), as revealed by Newman–Keuls *post hoc* tests following RM ANOVA ($F(2.01, 14.07) = 26.12$, $p < 0.05$), there was no main effect of a sub-threshold dose of picrotoxin (0.25 mg/kg; $F(1, 7) = 0.01$, $p > 0.05$) and no interaction (data not shown; WAY × picrotoxin; $F(3.65, 25.54) = 2.30$, $p > 0.05$).

As outlined in Table 3B, repeated measures ANOVA revealed that WAY 161503 (0–1.0 mg/kg) decreased horizontal locomotor activity ($F(2.04, 14.25) = 20.20$, $p < 0.05$) and rearing (vertical) locomotor activity ($F(2.18, 15.26) = 18.79$, $p < 0.05$). A main effect of baclofen (1.25 mg/kg) was also found in both instances ($F(1, 7) = 61.01$; $F(2.65, 18.57) = 4.89$, $p < 0.05$). There was no interaction between WAY 161503 and baclofen, however, their effects appear to be additive given that locomotor activity was substantially lower following the administration of both drugs (as compared with either alone). Further analysis of WAY 161503, following the collapse of data across baclofen, revealed that the dose of 1.0 mg/kg decreased horizontal activity; the 0.3 and 1.0 mg/kg doses decreased rearing activity.

Discussion

The primary aim of this study, to the best of the authors' knowledge the first of its kind, was to investigate the role of GABA receptors, particularly those in the NAc shell, on VTA ICSS, and to compare these effects with those following systemic administration of GABAergic compounds. In general, GABA$_A$, but not GABA$_B$, receptor activation in the NAc shell was found to affect VTA ICSS behaviour. GABA$_A$ receptor activation resulted in decreases in reward-related ICSS behaviour and antagonism resulted in the opposite effect. While the systemic effects of GABAergic compounds are difficult to interpret (as discussed in the following), GABA$_B$ receptor activation appears to decrease reward-related behaviour as indicated by the systemic administration of baclofen; in agreement with prior studies, GABA$_A$ and GABA$_B$ receptors may also be involved in regulating locomotor activity (Agmo and Giordano, 1985; Mukhopadhyay and Poddar, 1995).

Systemic GABAergic compounds on ICSS and locomotor activity

It is important to note that while the systemic effects of GABAergic compounds in ICSS have been investigated, studies have often been hampered by reward-insensitive measures and/or lacked measurements of motor performance
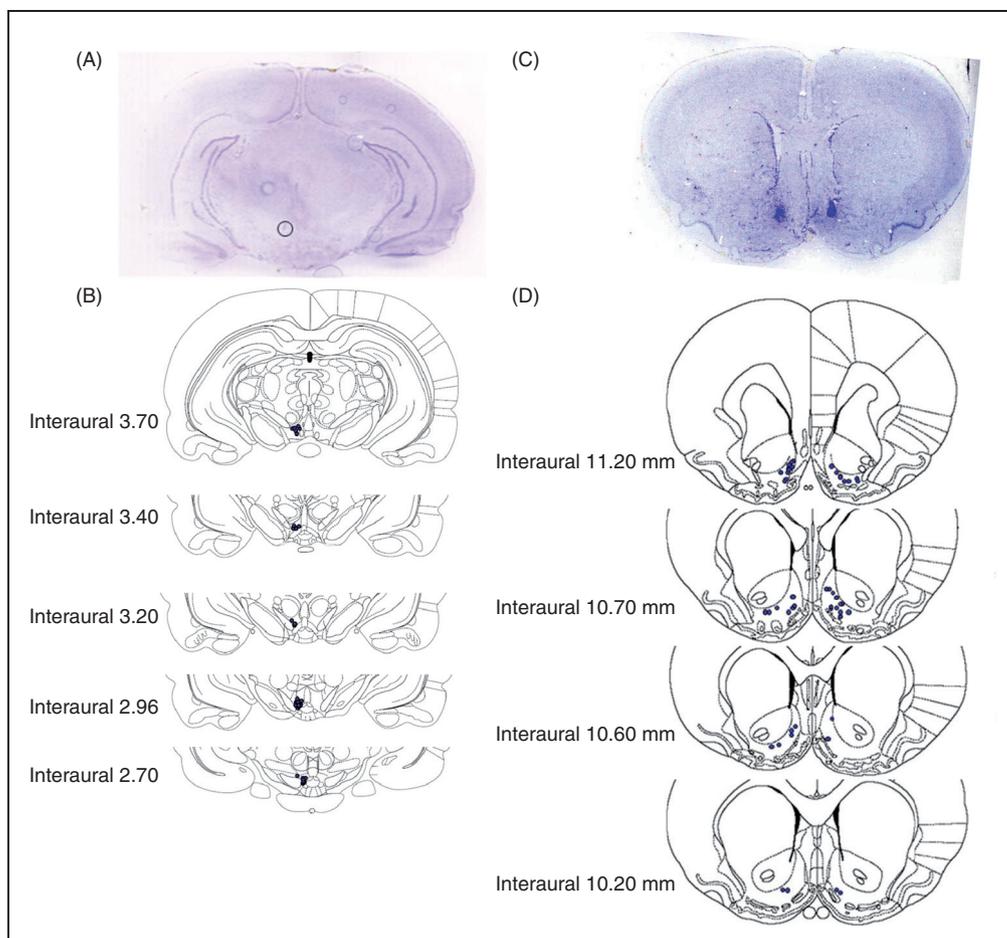
Figure 4. Histological verification of VTA and NAc shell sites. (A) Representative photomicrograph (circle identifies VTA electrode terminal) and (B) histological locations of VTA stimulation sites. (C) Representative photomicrograph and (D) histological locations of NAc shell microinjection sites. (Brain diagrams reproduced from Paxinos G and Watson C (1998) *The Rat Brain in Strereotaxic Coordinates,* 4th *edition* with permission from Elsevier).

Table 3. Effects of systemic GABA receptor ligands on spontaneous locomotor activity, alone and in combination with the 5-HT$_{2C}$ receptor agonist WAY 161503, measured over a 30 min time course. Horizontal locomotor activity (measured in beam breaks ± SEM) following systemic administration of muscimol (0–0.75 mg/kg) and picrotoxin (0–1.0 mg/kg) alone and in combination (A; $n = 8$) and baclofen (1.25 mg/kg) + WAY 161503 (0–1.0 mg/kg) (B; horizontal and vertical/rearing activity; $n = 8$)

3A.

		Muscimol (mg/kg)				
		0	0.10	0.25	0.5	0.75
Picrotoxin (mg/kg)	0	2607 ± 100	1981 ± 103*	2225 ± 90*	1693 ± 75*	1950 ± 82*
	0.25	2407 ± 95	2546 ± 106	–	–	–
	0.50	1839 ± 79*	2329 ± 87	–	–	–
	1.0	997 ± 70*	–	–	–	–

3B.

		WAY 161503 (mg/kg)				
		0	0.1	0.3	1.0	
Baclofen (mg/kg)	0	2367 ± 92	2179 ± 92	1773 ± 79	1109 ± 37*	Horizontal locomotor activity
	1.25	1553 ± 67*	1244 ± 60*	1219 ± 70*	459 ± 26*	
	0	243 ± 13	199 ± 14	126 ± 8*	19 ± 2*	Vertical/rearing locomotor activity
	1.25	134 ± 16*	143 ± 17*	48 ± 5*	3 ± 0.5*	

Data shown are means ± SEM. *Significant from control at $p < 0.05$ following Newman–Keuls *post hoc* tests.

(for example, see Porrino and Coons, 1980; Willick and Kokkinidis, 1995; Zarevics and Setler, 1981). This study is the first to use VTA ICSS with reward-sensitive, rate-frequency threshold, measures (i.e. M50) and measures of motor performance (i.e. RMAX and locomotor activity; for a more detailed discussion of the ICSS procedure, the reader is referred to Carlezon and Chartoff (2007), Konkle et al. (2001) and Miliaressis et al. (1986)).

Systemic administration of the $GABA_A$ receptor agonist muscimol (0–4.0 mg/kg), antagonist picrotoxin (0–1.0 mg/kg), and $GABA_B$ receptor agonist baclofen (0–2.5 mg/kg) had effects on ICSS measures (Table 2A–D). Although all showed increases in M50 thresholds (suggesting decreases in reward-related behaviour) at the higher doses, these results must be interpreted with caution for numerous reasons. For instance, muscimol (4.0 mg/kg) also produced a substantial decrease in RMAX (indicating impaired motor performance) at a dose well above that required to selectively decrease loco-motor activity (Table 3A) and these effects were not attenuated by picrotoxin (Table 2C). Picrotoxin (1.0 mg/kg) and baclofen (1.25 mg/kg) did not affect RMAX (Table 2B and D), however, both doses significantly reduced locomotor activity (Table 3A and B) and picrotoxin may produce increased anxiety and freezing behaviour (Dalvi and Rodgers, 1996; Sienkiewicz-Jarosz et al., 2003) which is not observed at lower doses (Dalvi and Rodgers, 2001; Dombrowski et al., 2006).

Taken together, these results underscore the difficulty of interpreting data from studies using systemically administered GABAergic compounds. This should not be surprising given the ubiquity of GABA receptors and the fact that it is the major inhibitory neurotransmitter. Nonetheless, these results are consistent with reports indicating that systemically administered $GABA_B$ receptor agonists increase rate-current ICSS thresholds (Slattery et al., 2005; Macey et al., 2001) and attenuate the effects of many drugs of abuse (for a review, see Filip and Frankowska, 2008).

Centrally injected GABAergic compounds

Prior studies have shown that $GABA_A$ and $GABA_B$ receptor compounds injected centrally into numerous brain regions affect reward-related behaviours such as self-administration, place conditioning and feeding (Backes and Hemby, 2008; Bechtholt and Cunningham, 2005; Ikemoto et al., 1998; Liu and Ikemoto, 2007; Laviolette and van der Kooy, 2001; Sahraei et al., 2009). Specifically regarding the NAc shell, $GABA_A$ receptor agonists injected into the rostral portion increased feeding behaviour, the hedonic response to sucrose and induced conditioned place preference (Lopes et al., 2007; Reynolds and Berridge, 2001, 2002; Stratford and Kelley, 1997) while the $GABA_B$ receptor agonist baclofen increased feeding (Lopes et al., 2007; Stratford and Kelley, 1997; Ward et al., 2000). In prior studies of ICSS, intra-VTA $GABA_A$ or $GABA_B$ receptor activation during ventral pallidal ICSS produced selective decreases in measures of reward (Panagis and Kastellakis, 2002), while activation in many limbic regions have been largely ineffective (Simmons et al., 2007; Waraczynski, 2007, 2008).

Prior studies underscore the need to investigate the GABAergic system using a central approach, and demonstrate that the role of GABA receptors in reward- and aversion-related behaviours is largely specific to the brain area under investigation. These studies also suggest that rostral NAc shell $GABA_A$ (and possibly $GABA_B$) receptor activation will result in increases in reward-related behaviour: a hypothesis further explored by the present study.

Intra-NAc shell $GABA_A$ and $GABA_B$ receptor compounds on ICSS

The present results suggest that the cells of the rostral NAc shell are involved in the regulation of ICSS behaviour and may be under the tonic control of $GABA_A$ receptors. Activation (muscimol; 225 ng/side; Figure 1A and B) and antagonism (picrotoxin; 125 ng/side; Figure 3C and D) of rostral NAc $GABA_A$ receptors increased and decreased, respectively, VTA ICSS M50 thresholds without affecting RMAX (indicating selective changes in reward-related behaviour). $GABA_B$ receptor activation via baclofen (0-225 ng/side) had no clear affect on VTA ICSS behaviour (Figure 1C and D). These results appear to be in opposition to the NAc Activity Hypothesis put forth by Carlezon and Thomas (2009), and the results noted above following GABA receptor activation in the rostral NAc shell (e.g. Reynolds and Berridge, 2002), which stated that an increase in NAc GABA cell activity would correspond to decreases in reward while cell inhibitions would correspond to increased reward. This is discussed further in the following.

It is unlikely that these results are related to misdirected cannulae as the histologically verified rostral NAc shell cannulae placements (Figure 4C and D) are similar to those in other reward-related studies (Lopes et al., 2007; Reynolds and Berridge, 2001, 2002; Stratford and Kelley, 1997) and intra-NAc amphetamine (1.0 μg/side; used as a positive control) produced well established reward-enhancing effects in all subjects (Colle and Wise, 1988). In addition, while the additional positive control of intra-rostral NAc muscimol did not significantly increase feeding (while baclofen did; Table 1), the present results are consistent with the longer-lasting, and more robust, effects of baclofen over muscimol beyond 30 min (Lopes et al., 2007), which is relevant given that feeding took place from 25–55 min post-injection (and post-ICSS testing). Finally, the rapid onset of behavioural effects in ICSS, slow injection rates (0.2 μL/min), and small injection volumes (0.5 μL/side) helped to minimize the spread of drug and suggest site specificity.

Together, these data suggest that $GABA_A$ receptors in the rostral NAc shell play an inhibitory role in regulating VTA ICSS behaviour, while $GABA_B$ receptors are not of primary importance under the present experimental conditions, although they may be more involved in regulating reward-related behaviours in other brain areas such as the VTA (Willick and Kokkinidis, 1995; Zhou et al., 2005). This is in contrast to their proposed role in feeding and other reward-related behaviours, mentioned above, but is not inconsistent with a differential role for receptors across reward-related

behaviours (see, for example, Backes and Hemby, 2008; Hayes et al, 2009c; Martin-Fardon et al., 2007).

Is the NAc Activation Hypothesis incorrect?

As cells in the NAc are almost entirely medium spiny GABAergic neurons (Meredith, 1999), the aversive effects of inhibitory $GABA_A$ receptor stimulation on VTA ICSS seen in this study are likely due to the inhibition of these cells. Conversely, antagonism of the $GABA_A$ receptor may result in increased ICSS reward through the disinhibition of NAc GABAergic cells, likely increasing GABA cell activity in the NAc. Interestingly, Steffensen et al. (2001) showed that VTA GABA cells increase their activity (and presumably their output of GABA into the NAc) in response to ICSS of the medial forebrain bundle, while Cheer et al. (2005) found that the $GABA_A$ receptor antagonist bicuculline inhibited an ICSS-associated decrease in NAc cell firing. These results, consistent with Carlezon and Thomas' NAc Activity Hypothesis, further support that a decrease in NAc GABA cell activity is related to an increase in reward-related behaviour, although they are difficult to reconcile with the robust results from the present study.

However, these data are consistent with the NAc Activity Hypothesis if one considers the possibility that local ICSS-associated inhibitions within the NAc may be the result of decreased interneuron activity. For instance, the reward-related properties of benzodiazepines have recently been associated with their actions as functional agonists at $GABA_A$ receptors on VTA interneurons (Tan et al., 2010), and there is good evidence for $GABA_A$ receptor-mediated lateral inhibition between GABA interneurons in the NAc (Taverna et al., 2004). In addition, fast-spiking interneurons within the NAc are thought to be entrained by high-frequency oscillations during reward-related behaviours (van der Meer and Redish, 2009). However, given their numbers alone, it cannot be ruled out that these results may also reflect the selective inhibition of a group of GABAergic projection neurons.

As pointed out by Carlezon and Thomas, electrophysiological data suggest that inhibition of NAc cells or local lesions do not produce reward-related effects as might be expected by the NAc Activity Hypothesis (e.g. Yun et al., 2004). However, it is possible that reward- and aversion-related behaviour is not coded by absolute changes in NAc cell activity but by changes relative to baseline (also referred to as intrinsic or resting state) activity. This idea would be well in line with the mounting data implicating the functional role of the brain's so-called resting state activity in determining psychological states (Northoff et al., 2010). In addition, this idea helps to reconcile the present data with both the results supported by the NAc Activation Hypothesis as well as those seen in brain imaging experiments. The latter have demonstrated increased negative blood oxygenated level-dependent activations in the NAc during reward-loss/aversion (de Greck et al., 2008), and these types of activations may be related to increased GABA concentrations (Northoff et al., 2007).

Taken together, these results suggest that while the NAc Activity Hypothesis may help to predict many behavioural outcomes, it may nonetheless be too simplistic at the cellular/circuit level. Numerous possible explanations may help reconcile the array of seemingly contradictory data, for example, the existence of reward- or aversion-specific cells within the NAc as is the case in the anterior cingulate cortex (e.g. Kawasaki et al., 2005), or the precise impact of local circuitry on reward- and aversion-related processing (Taverna et al., 2004). Nonetheless, the present data support a role for $GABA_A$ receptors in the rostral NAc shell in tonically inhibiting VTA ICSS reward and/or aversion-related processing.

Exploring the relationship between 5-HT$_{2C}$ and GABA receptors

The secondary aim of this study was the investigation of the potential relationship between 5-HT$_{2C}$ and GABA receptors in ICSS, given that 5-HT$_{2C}$ receptors are found on mesolimbic GABAergic cells and stimulation of these receptors results in changes in ICSS and locomotor activity similar to those found with GABAergic compounds.

Localization studies suggest that 5-HT$_{2C}$ receptors are primarily found postsynaptically on non-dopaminergic cells (Clemett et al., 2000; Pasqualetti et al., 1999), and have been identified on GABAergic cells of the dorsal raphe (Serrats et al., 2005), prefrontal cortex (Liu et al., 2007) and VTA (Bubar and Cunningham, 2007). Their activation inhibits the release of mesolimbic dopamine (Di Giovanni et al., 2000; Di Matteo et al., 1999) which may be related to GABAergic activity (Boothman et al., 2006; Di Giovanni et al., 2001; Serrats et al., 2005; Stanford and Lacey, 1996). Electrophysiological studies have supported this notion by demonstrating that 5-HT$_{2C}$ receptor activation excites GABAergic cells in the VTA, substantia nigra and raphe nuclei (Di Giovanni et al., 2001; Invernizzi et al., 2007; Liu et al., 2000). Clinically, the 5-HT$_{2C}$, $GABA_A$, and $GABA_B$ receptors have garnered increasing interest regarding their putative roles in the pathophysiology of psychiatric disorders such as depression, schizophrenia, and addiction (for example, see Berg et al., 2008; Filip and Frankowska, 2008; Sen and Sanacora, 2008).

Taken together, these studies warranted an exploration of the potential relationship between the 5-HT$_{2C}$ receptor and the GABAergic system in ICSS and locomotor activity. Given the negative results noted above, interactions between the selective 5-HT$_{2C}$ receptor agonist WAY 161503 and systemic muscimol, or intra-NAc baclofen, in ICSS were not considered.

5-HT$_{2C}$ and GABA$_B$ receptor compounds on ICSS and locomotor activity

The data did not support an interaction between $GABA_B$ and 5-HT$_{2C}$ receptors in the present study, although they have independent effects on ICSS (Figure 2A–D) and locomotor behaviour (Table 3). The investigation of the selective 5-HT$_{2C}$ receptor agonist WAY 161503 on VTA ICSS (Figure 2C and

D; 0.3, 1.0 mg/kg) and locomotor activity (Table 3B; 0.1, 0.3, 1.0 mg/kg) replicated the reward- and locomotor-decreasing effects seen previously with this ligand (Hayes et al., 2009a, 2009c), and other 5-HT$_{2C}$ receptor ligands (Higgins et al., 2001; Kennett et al., 1997; Lucki et al., 1989; Martin et al., 2002) and provided evidence that its effects are not interactive with those of the GABA$_B$ receptor agonist baclofen. It is important to note again that WAY 161503 was selected because of its high selectivity at the 5-HT$_{2C}$ receptor (Rosenzweig-Lipson et al., 2006) and because its behavioural and pharmacological effects have been blocked by highly selective 5-HT$_{2C}$ receptor antagonists (Boothman et al., 2006; Hayes et al., 2009c).

While 5-HT$_{2C}$ receptor activation may inhibit ICSS and locomotor activity by stimulating GABAergic cells (although effects on other cells cannot be excluded), it is unlikely that these effects are GABA$_B$ receptor-dependent under the present experimental conditions. Nonetheless, it is important to note that experiments with a broader range of doses and comparisons between additional selective ligands would help to clarify these tentative conclusions.

5-HT$_{2C}$ and GABA$_A$ receptor compounds on ICSS and locomotor activity

Although inconclusive, the possibility that the reward-related effects of 5-HT$_{2C}$ receptor activity on ICSS are mediated, in part, by subsequent GABA$_A$ receptor activation in the NAc shell remains open. The increase in M50 seen with WAY 161503 (1.0 mg/kg) is comparable to that seen with intra-NAc muscimol (225 ng/side) (Figure 3A), without effects on RMAX (Figure 3B). The effects of WAY 161503 were attenuated by the GABA$_A$ receptor antagonist picrotoxin (125 ng/side) (Figure 3C), results consistent with *in vivo* data by Boothman et al. (2006) showing picrotoxin's ability to attenuate WAY 161503-related reduced 5-HT cell firing. Picrotoxin decreased M50 when administered alone; these effects are likely due to a specific increase in reward as picrotoxin did not affect RMAX (Figure 3D) and the dose used was subconvulsive and does not affect locomotor activity (Bast et al., 2001; Plaznik et al., 1990; Swerdlow et al., 1990).

It is unlikely that the similar effects of WAY 161503, muscimol and their combination on M50 thresholds are due to a ceiling effect as these treatments produced an approximate 30% shift in M50; larger shifts are possible and have been noted by others (Hayes et al., 2009b; Morissette and Boye, 2008; Sonnenschein and Franklin, 2008; Vlachou et al., 2005). While this result could be explained by noting that 5-HT$_{2C}$ receptor activation (e.g. in the VTA) increases GABA cell activity and release in the NAc (which may then activate GABA$_A$ receptors), numerous other possibilities cannot be excluded through this approach. Future studies should, for instance, consider subthreshold doses of these compounds and/or intra-VTA injection of 5-HT$_{2C}$ receptor ligands in ICSS. It should also be noted that the authors believe that the decrease in RMAX by WAY 161503 (Figure 3D) is an artefact given that prior replications (published and unpublished observations) have shown this same dose to be ineffective (Hayes et al., 2009a).

This study was the first, to the best of the authors' knowledge, to explore the potential relationship between 5-HT$_{2C}$ and GABA receptors in reward-related behaviour. Together, these results are consistent with human and animal data suggesting that some GABAergic transmission in the NAc may inhibit reward signalling (de Greck et al., 2008; Northoff et al., 2007; Rahman and McBride, 2002; Yan, 1999). They are also consistent with data investigating the effects of intra-NAc GABA ligands on locomotor activity (Austin and Kalivas, 1989; Morgenstern et al., 1984; Plaznik et al., 1990; Pycock and Horton, 1979). One important limitation of the present study is that because WAY 161503 was systemically administered, we cannot comment directly on the location of 5-HT$_{2C}$ receptors which affect VTA ICSS. As noted above, future studies will be able to answer this question, perhaps by combining intracranial microinjections of 5-HT$_{2C}$ receptor compounds with microdialysis or electrophysiological techniques. In addition, while not addressed by the present experiments, the impact of dopamine signalling on GABAergic function (particularly in the NAc) should not be understated. Because, as noted above, dopamine is released into the (mostly GABAergic) NAc following VTA ICSS, future studies should also focus on understanding GABA–dopamine interactions in these two regions, particularly given that electrical stimulation of the VTA activates NAc neurons both anti- and ortho-dromically, and vice versa (Wolske et al., 1993; Yim and Mogenson, 1980). (For some studies related to this topic, see Cheer et al. (2005) and Lassen et al. (2007)).

Summary and conclusion

These data suggest that NAc shell GABA$_A$ receptors are important in the regulation of VTA ICSS. While the precise role of GABA$_B$ receptors is less clear, they do not appear to be as important in the regulation of ICSS. In addition, this report reflects an early step in investigating a potential 5-HT$_{2C}$–GABA$_A$ receptor relationship in behaviour. While the present location(s) of the 5-HT$_{2C}$ receptors which are key to regulating ICSS behaviour is currently unknown (although those in the NAc shell are likely not involved) (Hayes et al., 2009a), the present data do not exclude the possibility that 5-HT$_{2C}$ receptor-related changes in ICSS behaviour are due to downstream release of GABA in the NAc shell and subsequent effects at GABA$_A$ receptors. These results are in line with evidence underscoring the GABAergic system as integral in regulating ICSS behaviour (Cheer et al., 2005; Ishida et al., 2001; Lassen et al., 2007; Steffensen et al., 2001), and with studies showing that reduced activation in the NAc can reduce some reward-related behaviours (de Greck et al., 2008; Knapp et al., 2009; Vassoler et al., 2008).

Finally, these results underscore the caveat of using the general terms 'reward' or 'aversion' at the biological level given that they may reflect a number of related and/or overlapping processes, which may be reflected to varying degrees across reward-related behaviours (Berridge and Robinson, 2003; Salamone, 2006; Salamone et al., 2005). Evidence of this comes from the fact that the NAc has been identified as

responding to both reward and aversion (Lowe et al., 2007; Roitman et al., 2005; Wheeler et al., 2008). In this context, the Carlezon and Thomas (2009) NAc Activity Hypothesis may predict the outcome of many reward- and aversion-related behaviours under a broad range of conditions, although this may not be the case for VTA ICSS.

Funding

Conflict of interest

The authors have no conflicts of interest to declare.

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