The Reinforcing Actions of a Serotonin-3 Receptor Agonist within the Ventral Tegmental Area: Evidence for Subregional and Genetic Differences, and Involvement of Dopamine Neurons

Zachary A. Rodd, Victoria E. Gryszowka, Jamie E. Toalston, Scott M. Oster, Dong Ji, Richard L. Bell, William J. McBride

Department of Psychiatry, Institute of Psychiatric Research, Indiana University School of Medicine, Indianapolis, IN 46202 (ZAR, VEG, DJ, RLB, WJB), and Department of Psychology, Purdue School of Science, Indiana University-Purdue University at Indianapolis, Indianapolis, IN 46202 (JET, SMO).
Running Title: Reward Properties of CPBG

Address Correspondence to:

Dr. Zachary A. Rodd, Indiana University School of Medicine, Institute of Psychiatric Research, 791 Union Drive, Indianapolis, IN 46202-4887 USA
Phone: 317-278-3003; Fax: 317-274-1365; E-mail: zrodd@iupui.edu

Number of Text Pages: 35
Number of Tables: 0
Number of Figures: 8
Number of References: 39
Number of Words in Abstract: 248
Number of Words in Introduction: 867
Number of Words in Discussion: 1698
List of Nonstandard Abbreviations: ICSA – intracranial self-administration; CPBG - 1-(m-chlorophenyl)-biguanide; P rat – alcohol preferring (P) rat
Recommended Section: Neuropharmacology
ABSTRACT

Rationale: Studies from our laboratory indicated that local perfusion of the ventral tegmental area (VTA) with a serotonin-3 (5-HT₃) receptor agonist increased dopamine (DA) neuronal activity, and that the self-infusion of ethanol (EtOH) and cocaine into the posterior VTA could be inhibited with co-administration of a 5-HT₃ receptor antagonist. Objectives: The study tested the hypothesis that activating 5-HT₃ receptors within the VTA produces reinforcing effects. The study also examined whether there were differences between Wistar rats and a line of rats selectively bred for high alcohol consumption with regard to the self-infusion of a 5-HT₃ receptor agonist within the VTA. Methods: Adult female alcohol-preferring (P) and Wistar rats were allowed to self-infuse the 5-HT₃ receptor agonist, 1-(m-chlorophenyl)-biguanide (CPBG), into the posterior or anterior VTA. Additionally, experiments examined the effects of co-infusion of the 5-HT₃ antagonist ICS 205,930 (ICS), and the DA D₂,₃ agonist quinpirole on the self-infusion of CPBG. Results: Both Wistar and P rats readily self-administered CPBG into the posterior, but not anterior, VTA. P rats self-infused lower concentrations of CPBG (0.10 µM) than did Wistar rats (1.0 µM). Co-infusion of either ICS or quinpirole reduced CPBG self-infusion into the posterior VTA. Conclusions: The results of this study suggest that activation of 5-HT₃ receptors within the posterior VTA produces reinforcing effects, and that these reinforcing effects are mediated through activation of DA neurons. Additionally, the data suggest that selective breeding for alcohol-preferece results in the posterior VTA being more sensitive to the reinforcing effects of CPBG.
INTRODUCTION

An in vivo microdialysis study (Campbell et al. 1996) demonstrated that local administration of a serotonin-3 (5-HT\textsubscript{3}) receptor agonist increased somatodendritic dopamine (DA) release in the ventral tegmental area (VTA), suggesting that 5-HT\textsubscript{3} receptors are involved in regulating DA neuronal activity within the VTA. In support of this interpretation, electrophysiological studies indicated that systemic administration of 5-HT\textsubscript{3} receptor antagonists decreased the number of spontaneously active VTA DA neurons in rodents (Minabe et al. 1991; Rasmussen et al. 1991).

The results of several studies suggested a role for the involvement of 5-HT\textsubscript{3} receptors in mediating the excitatory actions of ethanol (EtOH). EtOH can enhance 5-HT\textsubscript{3} receptor-mediated ion currents (Lovinger and White 1991); 5-HT\textsubscript{3} receptor antagonists inhibited the increase in extracellular DA levels in the nucleus accumbens elicited by EtOH when the antagonist was given systemically (Carboni et al. 1989; Wozniak et al. 1990) or locally (Campbell and McBride 1995). Moreover, the in vivo microdialysis study of Campbell et al. (1996) indicated that local perfusion with a 5-HT\textsubscript{3} receptor antagonist blocked EtOH-stimulated somatodendritic DA release within the VTA, suggesting that the activating effects of EtOH on VTA DA neurons were mediated in part through 5-HT\textsubscript{3} receptors.

The intracranial self-administration (ICSA) technique (Bozarth and Wise 1980; Goeders and Smith 1987; McBride et al. 1999; Wise and Hoffman 1992) has been used to study the reinforcing effects of EtOH within the VTA (Gatto et al. 1994; Rodd-Henricks et al. 2000; Rodd et al. 2004a,b, 2005b). The results of these studies suggested that selective...
breeding for alcohol preference might increase the sensitivity of the VTA to the reinforcing effects of EtOH (Gatto et al. 1994; Rodd et al. 2004a), and that the posterior VTA supported the self-infusions of EtOH, whereas the anterior VTA did not (Rodd-Henricks et al. 2000; Rodd et al. 2005b). In addition, studies, using co-infusion of a D_{2,3} agonist (quinirole) to activate cell body D_{2} autoreceptors and reduce DA neuronal activity, indicated that the self-infusion of EtOH into the posterior VTA required activation of DA neurons (Rodd et al. 2004b, 2005b).

Because of the evidence indicating that local administration of a 5-HT_{3} receptor antagonist in the VTA inhibited EtOH-stimulated somatodendritic DA release (Campbell et al. 1996), our laboratory undertook a study to examine the involvement of 5-HT_{3} receptors in the reinforcing effects of EtOH within the posterior VTA of Wistar rats (Rodd-Henricks et al. 2003). The results of this study indicated that co-infusion of 5-HT_{3} receptor antagonists blocked the self-infusion of EtOH into the posterior VTA, suggesting that activation of 5-HT_{3} receptors is involved in mediating the reinforcing effects of EtOH in this region. In a subsequent study, Rodd et al. (2005b) reported that ICS 205-930 (ICS) also reduced the self-infusion of EtOH into the posterior VTA of alcohol-preferring (P) rats, but that this antagonist was significantly less effective in the P rat than Wistar rat in reducing EtOH self-infusions. This difference in the effectiveness of the antagonist to reduce EtOH self-infusions between the P and Wistar rats may be a result of differences in the 5-HT_{3} receptors and/or local circuits mediating the actions of the 5-HT_{3} receptors within the VTA.

Numerous reports have indicated that the VTA is not a homogenous structure. Arnt and Scheel-Kruger (1979) demonstrated functional differences between the anterior and
posterior VTA in the locomotor activating effects of GABA_A agonists and antagonists.

Ethanol and acetaldehyde are self-administered into the posterior, but not anterior, VTA (Rodd-Henricks et al., 2000, 2002; Rodd et al., 2004b, c). In mice, research has shown that injections of a GABA_B agonist into the posterior, but not anterior, VTA increased locomotor activity (Boehm et al., 2002). Cocaine was self-infused into the posterior, but not anterior, VTA of Wistar rats and that this self-infusion could be blocked by co-infusion of quinpirole or a 5-HT_3 receptor antagonist (Rodd et al., 2005a). The sub-regional differences in self-administration of cocaine are likely related to the differences in neuronal circuitry between the anterior and posterior VTA.

Immunohistochemical studies have indicated that the posterior VTA has a greater density of µ-opioid receptors in the posterior than the anterior VTA (Mansour et al., 1995). Over-expression of an AMPA receptor subunit (GluR1) in the posterior and anterior VTA produced differential consequences on morphine conditioned place preference (Carlezon et al., 2000). A recent series of experiments revealed that the posterior and anterior VTA have distinct innervation patterns (posterior VTA heavily innervates the Acb shell) and that altering cAMP response element binding protein levels in the anterior and posterior VTA produced contrasting effects on the reinforcing properties of morphine and cocaine (Olson et al., 2005).

Because the findings suggest that activation of 5-HT_3 receptors is involved in mediating the self-infusions of EtOH and cocaine in the posterior VTA of Wistar and P rats, the current study was undertaken to test the hypothesis that activating 5-HT_3 receptors within the posterior VTA will produce reinforcing effects. This hypothesis was tested using
the ICSA technique to study the self-infusion of 1-(m-chlorophenyl)-biguanide (CPBG), a 5-HT$_3$ receptor agonist, into the VTA of Wistar and P rats. CPBG expresses a 100-fold greater affinity for the 5-HT$_3$ receptors than serotonin (Kirpatrick et al., 1990), and CPBG has no significant effects at other serotonin receptors (Walcourt-Ambakederemo and Winlow, 1995).

MATERIALS AND METHODS

Experimentally naïve, female Wistar rats (Harlan, Indianapolis, IN) and P rats from the 51$^{st}$ and 52$^{nd}$ generations weighing 250-320 g at time of surgery were used. Female rats were used in the present study because female rats were used in previous studies involving the ICSA of EtOH, and female P rats maintain their body weights and head size better than male P rats for more accurate stereotaxic placements (Gatto et al. 1994; Rodd et al., 2004a, 2005a,b; Rodd-Henricks et al. 2000). Rats were double-housed upon arrival and maintained on a 12-hr reverse light-dark cycle (lights off at 0900 hr). Although not systematically studied, the estrus cycle did not appear to have a significant effect on ICSA behavior in the present or previous ICSA studies (Rodd et al., 2004a; Rodd-Henricks et al. 2000; Gatto et al. 1994; Ikemoto et al. 1997, 1998), as indicated by no obvious fluctuations in ICSA behavior in rats given similar doses of the same agent for four or more sessions. Food and water were freely available except during testing.

Animals used in this study were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the
National Institute on Drug Abuse, NIH, and the Guide for the Care and Use of Laboratory Animals of the National Research Council, 1996. The number of animals indicated for each experiment represents 95% of the total number that underwent surgery; 5% of the animals were not included for analyses mainly due to the loss of the guide cannula before completion of all experimental sessions. The data for these animals were not used because their injection sites could not be verified.

General Test Condition

While under isoflurane anesthesia, a unilateral 22-gauge guide cannula (Plastic One) was stereotaxically implanted in the right hemisphere of each subject, aimed 1.0 mm above the target region. Coordinates (Paxinos and Watson 1998) for placements into the posterior VTA were 5.4 mm posterior to bregma, 2.1 mm lateral to the midline, and 8.5 mm ventral from the surface of the skull at a 10-degree angle to the vertical. Similar coordinates were used for the female P and Wistar rats in this study. Coordinates (Paxinos and Watson 1998) for placements into the anterior VTA were 4.8 mm posterior to bregma, 2.1 mm lateral to the midline, and 8.5 mm ventral from the surface of the skull at a 10-degree angle to the vertical. In between experimental sessions, a 28-gauge stylet was inserted into the guide cannula and extended 0.5 mm beyond the tip of the guide. Following surgery, all rats were individually housed and allowed to recover 7-10 days. Animals were handled for at least 5 min daily following the fourth recovery day. Subjects were not acclimated to the experimental chamber prior to the commencement of data collection, nor were they trained on any other operant paradigm.
The experimental chambers (30 x 30 x 26 cm; w x h x d) were situated in a sound-attenuating cubicle (64 x 60 x 50 cm, Coulbourn Instruments, Allentown, PA) and illuminated by a dim house light during testing. Two identical levers (3.5 x 1.8 cm) were mounted on a single wall of the experimental chamber, 15 cm above a grid floor, and were separated by 12 cm. Levers were raised to this level to avoid accidental brushing against the lever and to reduce responses as a result of general locomotor activation. Directly above each lever was a row of three different colored cue lights. The light (red) to the far right over the active bar was illuminated during resting conditions. A desktop computer equipped with an operant control system (L2T2 system, Coulbourn Instruments) recorded the data and controlled the delivery of infusate in relation to lever response.

An electrolytic microinfusion transducer (EMIT) system (see Bozarth and Wise, 1980) was used to control microinfusions of drug or vehicle. Briefly, two platinum electrodes were placed in an infusate-filled cylinder container (28 mm in length x 6 mm in diameter) equipped with a 28-gauge injection cannula (Plastics One, Roanoke, VA). The electrodes were connected by a spring-coated cable (Plastic One) and a swivel (Model 205, Mercotac, Carlsbad, CA) to a constant current generator (MNC, Shreveport, LA) that delivered 6 µA of quiescent current or 200 µA of infusion current between the electrodes. Depression of the active lever delivered the infusion current for 5 sec, which led to the rapid generation of H₂ gas (raising the pressure inside the airtight cylinder), and, in turn, forcing 100 nl of the infusate through the injection cannula. During the 5-sec infusion and additional 5-sec timeout period, the house light and right cue light (red) were extinguished and the left cue light (green) over the active lever flashed on and off at 0.5-sec intervals.
The artificial cerebrospinal fluid (aCSF) consisted of (in mM): 120.0 NaCl, 4.8 KCl, 1.2 KH$_2$PO$_4$, 1.2 Mg SO$_4$, 25.0 NaHCO$_3$, 2.5 CaCl$_2$, and 10.0 d-glucose. CPBG, ICS205-930 (ICS), and quinpirole (all purchased from Sigma, St. Louis) were dissolved in the aCSF solution. When necessary, 0.1 M HCl or 0.1 M NaOH was added to the solutions to adjust pH levels to 7.4 ± 0.1.

For testing, rats were brought to the testing room, the stylet was removed, and the injection cannula screwed into place. To avoid trapping air at the tip of the injection cannula, the infusion current was delivered for 5 sec during insertion of the injector, which resulted in a single non-contingent administration of infusate at the beginning of the session. Injection cannulae extended 1.0 mm beyond the tip of the guide. The experimental chamber was equipped with two levers. Depression of the ‘active lever’ (FR1 schedule of reinforcement) caused the delivery of a 100-nl bolus of infusate over 5 sec followed by a 5-sec time-out period. During both the 5-sec infusion period and 5-sec time-out period, responses on the active lever did not produce further infusions. Responses on the ‘inactive lever’ were recorded, but did not result in infusions. The assignment of active and inactive lever with respect to the left or right position was counterbalanced among subjects. However, the active and inactive levers remained the same for each rat throughout the experiment. No shaping technique was used to facilitate the acquisition of lever responses. The number of infusions and responses on the active and inactive lever were recorded. The duration of each test session was 4 hr and sessions occurred every other day.
Self-Administration of CPBG into the VTA of Wistar and P rats

Wistar rats with cannulae placements aimed at the posterior VTA were randomly assigned to one of five groups (n = 42, 7-9/group). A vehicle group received infusions of aCSF for all seven sessions. The other groups were given 0.1, 1, 10, or 50 µM CPBG for the initial four sessions, aCSF for sessions 5 and 6 (extinction), and the original infusate for session 7 (reinstatement). Wistar rats with cannulae placements aimed at the anterior VTA were randomly assigned to one of three groups (n = 24, 7-9/group). A vehicle group received infusions of aCSF for all seven sessions. The other groups were given 10 or 50 µM CPBG for the initial four sessions, aCSF for sessions 5 and 6 (extinction), and the original infusate for session 7 (reinstatement). In addition, there were 8 Wistar rats with cannulae placement outside of the VTA. These rats were not included in the statistical analyses.

P rats with cannulae placements aimed at the posterior VTA were randomly assigned to one of seven groups (n = 61, 8-10/group). A vehicle group received infusions of aCSF for all seven sessions. The other groups were given 0.01, 0.1, 1, 10, 50 or 100 µM CPBG for the initial four sessions, aCSF for sessions 5 and 6 (extinction), and the original infusate for session 7 (reinstatement). P rats with cannulae placements aimed at the anterior VTA were randomly assigned to one of four groups (n = 32, 8/group). A vehicle group received infusions of aCSF for all seven sessions. The other groups were given 1, 10 or 100 µM CPBG for the initial four sessions, aCSF for sessions 5 and 6 (extinction), and the original infusate for session 7 (reinstatement). In addition, there were 6 P rats with cannulae placement outside of the VTA. These rats were not included in the statistical analyses.
Co-infusion of ICS during maintenance of CPBG self-infusions

Wistar rats were randomly assigned to one of three groups (n = 23, 7-8/group). All groups self-infused 10 µM CPBG during the initial 4 sessions, 10 µM CPBG and 10, 100, or 200 µM ICS during sessions 5 and 6, and 10 µM CPBG alone during session 7. A control group given ICS alone was not included in the present study because prior studies indicated that ICS alone, or other 5-HT₃ receptor antagonists, did not impair lever responding. For example, in one study, we examined the effects of co-administration of three 5-HT₃ antagonists on the self-infusion of EtOH into the posterior VTA (Rodd-Henricks et al., 2003). Responses on the active and inactive lever for 100 µM zacopride, LY278-584, or ICS 205,930 were similar to responses on these levers for aCSF self-infusions into the posterior VTA of Wistar rats (Rodd-Henricks et al., 2003), suggesting that these antagonists do not impair operant responding when infused into the posterior VTA. Another ICSA study indicated that higher concentrations of ICS (400 µM) did not affect acetaldehyde self-infusions (> 100 active lever responses/session) into the posterior VTA (Rodd et al., 2005b).

Co-infusion of Quinpirole during maintenance of CPBG self-infusion

Wistar rats were randomly assigned to one of three groups (n = 15, 5/group). All groups self-infused 10 µM CPBG during the initial 4 sessions, 10 µM CPBG and 1, 10, or 100 µM quinpirole during sessions 5 and 6, and 10 µM CPBG alone during session 7. The concentrations of quinpirole used were derived from studies indicating that co-administration of 1 µM did not alter ethanol or acetaldehyde self-administration into the posterior VTA, but 100 µM was effective at reducing self-administration (Rodd et al., 2004b, 2005b). A control group given only quinpirole was not included in the present study.
because prior studies indicated that quinpirole by itself did not alter operant responding when infused into the posterior VTA (Rodd et al., 2004b, 2005b).

**Histology**

At the termination of the experiment, 1% bromophenol blue (0.5 µl) was injected into the infusion site. Subsequently, the animals were given a fatal dose of Nembutal and then decapitated. Brains were removed and immediately frozen at −70°C. Frozen brains were equilibrated at −15°C in a cryostat microtome and then sliced into 40 µm sections. Sections were then stained with cresyl violet and examined under a light microscope for verification of the injection site using the rat brain atlas of Paxinos and Watson (1998).

**Statistical Analysis**

Data analysis consisted of a group x day mixed ANOVA, with a repeated measure of ‘day’ (all 7 sessions) performed on the number of infusions. Additionally, for each individual group, lever discrimination was determined by type (active or inactive) x day mixed ANOVA with a repeated measure of ‘day’. Lever discrimination is a key factor when a stimulant is self-administered (e.g., EtOH, cocaine, amphetamine). Without a detailed analysis of lever discrimination, it is impossible to distinguish between reinforcement-contingent behavior and drug-stimulated locomotor activity. The direct comparison between P and Wistar rats examined the mean number of infusions received during the first 4 ICSA sessions with a line x group between subject ANOVA. To determine the effects of co-administration of ICS or quinpirole on CPBG self-administration two separate repeated measure ANOVAs were performed. The first ANOVA examined the number of infusions between session 4 (last day of CPBG alone self-administration) and sessions 5 and 6 (co-
administration sessions) with group as a between subject factor. The second ANOVA examined the number of infusions between sessions 5 and 6 (co-administration) and session 7 (CPBG reinstatement) with co-administration group as a between subject factor.

RESULTS

The anterior VTA was defined as the VTA region at the level of the mammilary nuclei, coronal sections -4.8 to -5.2 mm relative to bregma (Fig. 1). The posterior VTA was defined as the VTA region at the level of the interpeduncular nucleus, coronal sections –5.3 to –6.04 mm relative to bregma (Fig. 1). Accidental cannula placements surrounding the VTA included injection sites located in the substantia nigra (3 P rats, 4 Wistar rat), red nucleus (2 P rats, 2 Wistar rats), and caudal linear nucleus of the raphe (1 P rat, 2 Wistar rats). Rats with injector tip placements outside the VTA in both P and Wistar rats displayed an overall low level of infusions and active lever responding throughout all sessions (average infusions and active lever responses for initial 4 sessions were 8.2 + 0.7 and 14.6 + 1.2, respectively). For all sessions, the number of infusions of CPBG for placements outside the VTA was not significantly different than the aCSF group with injection sites in the VTA (p values > 0.58). Similarly, examination of the active lever responses revealed that rats administering CPBG into areas outside the VTA displayed equivalent low levels of responding on both the active and inactive levers (p values > 0.68).

Self-infusion of CPBG into the VTA by Wistar and P rats

A select range of CPBG concentrations infused into the posterior VTA supported self-infusions by Wistar rats. For Wistar rats receiving infusions into the posterior VTA, an ANOVA on the average number of infusions (Figure 2, left panel) received during the initial
4 test days (acquisition) revealed a significant effect of Dose (F_{4,37} = 10.8; p < 0.0001). Post-hoc comparisons (Tukey’s b) indicated that rats given 1.0, 10 and 50 µM CPBG received significantly more infusions than rats given aCSF. Additionally, rats given 10 µM CPBG received more infusions than all other groups.

In contrast, in the anterior VTA, 10 and 50 µM CPBG were not significantly self-infused above aCSF levels by Wistar rats (Fig. 2; F_{2,21} = 0.5; p = 0.61).

Throughout the sessions, responses on the active lever for the self-infusion of aCSF into the posterior VTA was low (20 or fewer responses/session) and did not differ with regard to responses on the inactive lever (p = 0.71; Fig. 3, top left panel). For Wistar rats, the 1, 10 and 50 µM CPBG infusate groups (Fig. 3, middle and bottom panels show 10 and 50 µM CPBG) responded significantly more on the active than inactive lever throughout the 4 acquisition sessions (p values < 0.006). When aCSF was substituted for CPBG in sessions 5 and 6, responses on the active lever decreased to the low levels observed for the inactive lever. When CPBG was restored in session 7, responding on the active lever increased and was significantly higher than responses on the inactive lever in rats self-administering 1, 10 and 50 µM CPBG (p values < 0.013). Wistar rats self-administering 0.1 µM CPBG had moderate levels of responding on the active lever (40 responses/session); lever discrimination was observed only during session 4 (data not shown).

In contrast to the posterior VTA data, Wistar rats given the effective concentrations of CPBG to self-infuse into the anterior VTA (Fig. 3, right panels) had low levels of responding on the active lever (~18 responses/session) that did not differ from responses on
the inactive lever or from active lever responses for the aCSF group (20.4 ± 8.2 responses/session).

For P rats with injection sites in the posterior VTA, an ANOVA on the average number of infusions (Figure 4, left panel) received during the initial 4 test days (acquisition) revealed a significant effect of Dose ($F_{6,54} = 36.2; p < 0.0001$). Post-hoc comparisons indicated that P rats given 0.1, 1.0, 10, 50 and 100 µM CPBG received significantly more infusions than rats given only aCSF. Additionally, P rats given 10 µM CPBG received significantly more infusions than all the other groups, whereas P rats given 0.1, 1.0, 10 and 50 µM CPBG received more infusions than rats given 100 µM CPBG.

In contrast, for P rats with placements in the anterior VTA, none of the CPBG concentrations tested were significantly self-infused above aCSF levels (Fig. 4, right panel; p values > 0.57).

Direct comparison between P and Wistar rats with injection sites within the anterior and posterior VTA was possible because the experiments with Wistar and P rats were conducted during the same time period. Analysis of only the same concentrations given to both strains indicated a significant Strain effect and a significant dose x strain interaction (strain: $F_{1,76} = 11.2; p = 0.001$; strain x dose: $F_{4,73} = 35.8; p < 0.0001$). The interaction term was decomposed by holding dose constant and comparing the number of infusions between P and Wistar rats. Individual ANOVAs performed for each concentration of CPBG indicated that P rats received more infusions of 0.1, 1, 10 and 50 µM CPBG than did Wistar rats (p values < 0.023).
P rats self-administering CPBG into the posterior VTA readily discriminated between the active and inactive lever at concentrations ranging from 0.1 – 100 µM CPBG (Fig. 5 shows responses on the active and inactive lever for 0.1, 1.0 and 10 µM CPBG).

Additionally, P rats self-administering 0.01 µM CPBG displayed lever discrimination during sessions 3, 4, and 7 (p values < 0.044; data not shown), and received more self-infusions than aCSF during those sessions (individual ANOVAs performed for each session, indicated by post-hoc tukey’s b).

Responses on the active and inactive levers by P rats self-infusing CPBG with placements in the anterior VTA were comparable to responses when aCSF was given (and to responding by Wistar rats given aCSF in the posterior VTA, or CPBG with placements in the anterior VTA) and did not display lever discrimination (p values > 0.41; data not shown).

Patterns of responding on active lever by Wistar and P rats

The response patterns on the active lever (in 30-min blocks) by Wistar (left) and P (right) rats, which were given 10 µM CPBG, are depicted in Figure 6. For both Wistar and P rats, the self-infusion of CPBG was readily acquired in the first session (approximately 60-90 min; Fig. 6, top panel). During session 4, Wistar and P rats received the highest number of infusions of CPBG within the first 30-60 min and during the last 30-60 min, while maintaining modest levels of infusions during the middle 2-hr period. During the second aCSF substitution session (session 6), the responses on the active lever were generally low throughout the 4-hr session with the highest infusions occurring in the initial 30-min period. When 10 µM CPBG was restored in session 7, rats readily reinstated responding on the active lever. However, the pattern of responding in session 7 and session 4 was not similar;
in session 7, high levels of responding on the active lever were maintained for longer periods initially, the 2-hr stable modest level of responding was not evident, and there were no discernable higher levels of responding during the last 30- to 60-min segments of the session.

**Co-infusion of ICS or quinpirole on CPBG self-infusions**

Throughout the 4 acquisition sessions, rats readily self-infused 10 µM CPBG and responded significantly more on the active than inactive lever (all F values \( > 26.7 \); all p values < 0.001; Fig. 7). Repeated measure ANOVAs performed on the number of active lever presses and infusions obtained from session 4-6 with an independent variable of ICS concentration indicated a significant day x concentration interaction for both dependent variables (F values \( > 8.1 \); p values < 0.0001). Co-administration of 100 or 200 µM ICS in sessions 5 and 6 reduced the number of active lever responses (all F values \( > 47.5 \); all p values < 0.023), whereas 10 µM ICS did not alter CPBG self-administration (p = 0.87). When CPBG alone was given during session 7, responding on the active lever returned to levels observed in session 3 and 4 for the rats that had co-infused 100 or 200 µM ICS. Similar effects were observed for number of infusions/session. Briefly, the number of infusions obtained was significantly lower during session 6 compared to session 4 in rats that co-administered 100 or 200 µM ICS by 68% (47 infusions reduced to 15) or 63% (45 infusions reduced to 17), respectively. In contrast, for rats that self-infused 10 µM CPBG and 10 µM ICS there was a non-significant increase in the number of infusions obtained during session 6 compared to session 4 (54 compared to 49 infusions, respectively).
Repeated measure ANOVAs performed on the number of active lever presses and infusions obtained from session 4-6 with an independent variable of quipirrole concentration indicated a significant day x concentration interaction for both dependent variables (F values $4,24 > 7.2$; p values < 0.0001). Co-infusion of 10 or 100 µM quinpirole in sessions 5 and 6 (Fig. 8) reduced the number of active lever responses (all F values $2,3 > 10.9$; all p values < 0.04), whereas 1 µM quinpirole did not alter CPBG self-administration (p = 0.54). When CPBG alone was given during session 7, responding on the active lever returned to levels observed in session 3 and 4. The number of infusions obtained per session was also reduced by co-administration of either 10 or 100 µM quinpirole. The number of infusions obtained was significantly lower during session 6 compared to session 4 in rats that co-administered 10 or 100 µM quinpirole by 62% (54 infusions reduced to 20) or 72% (48 infusions reduced to 13), respectively. In contrast, for rats that self-infused 10 µM CPBG and 1 µM quinpirole there was a little change in the number of infusions obtained during session 6 compared to session 4 (51 infusions compared to 52, respectively).

**DISCUSSION**

The results of present study indicate that Wistar and P rats self-infuse CPBG directly into the posterior, but not anterior, VTA (Figs. 2 and 4), readily discriminate the active from the inactive lever during acquisition, reduce responding on the active lever when artificial CSF is substituted for CPBG (extinction), and reinstate responding on the active lever when CPBG is restored (Figs. 3 and 5). In addition, the self-infusion of 10 µM CPBG into the posterior VTA is blocked by ICS (Fig. 7), supporting an action of CPBG at the 5-HT3
receptor. Taken together, these results indicate that CPBG is producing reinforcing effects within the posterior VTA, and support our hypothesis that activating 5-HT$_3$ receptors within the VTA is reinforcing. The reinforcing effects of CPBG could occur through activation of 5-HT$_3$ receptors located on DA neurons and/or on terminals of excitatory inputs to these DA neurons, thereby increasing the activity of these neurons (Campbell et al. 1996). Synaptic connectivity of 5-HT terminals with VTA DA neurons has been reported (Van Bockstaele et al. 1994). Also, the present results provide additional support for the idea that the reinforcing effects of EtOH (Rodd-Henricks et al. 2003) and cocaine (Rodd et al. 2005a) within the posterior VTA are mediated at least in part by activation of 5-HT$_3$ receptors.

The reinforcing effects of CPBG could also occur, in part, through activation of 5-HT$_3$ receptors located on GABA neurons. The inhibition of GABA activity in the VTA, which leads to an increase in DA neuronal activity in the VTA, following cocaine administration is mediated through 5HT receptors (Cameron and Williams, 1994). Although the interaction between GABA and 5HT is thought to be primarily mediated through 5HT$_{1B/D}$ and 5HT$_{2AC}$ receptors, there is evidence that some effects of activating 5HT$_3$ receptors are mediated by GABA neurons (Bankson and Yamamoto, 2004; Bonagamba et al., 2000).

For both the Wistar and P rat, CPBG is self-infused into the posterior VTA but not into the anterior VTA (Figs. 2 and 4). These findings are in agreement with several studies (Ikemoto and Wise 2002; Ikemoto et al. 1998; Rodd et al. 2004b, 2005a,b; Rodd-Henricks et al. 2000) indicating the posterior VTA but not the anterior VTA is involved in supporting self-infusion behavior. The differences between the anterior and posterior VTA with regard
to the reinforcing effects of these pharmacological agents may be a result of a combination of neuroanatomical factors, such as (a) more DA neurons in the posterior than anterior VTA projecting to the nucleus accumbens (Olson et al. 2005), (b) a higher proportion of DA to GABA neurons in the posterior than anterior VTA (Olson et al. 2005), (c) the posterior VTA has higher 5-HT innervation (Herve et al. 1987), and (d) the VTA contains DA neurons with topographical afferent and efferent projections (Kalen et al. 1988; Brog et al. 1993; Tan et al. 1995) that may differ between the anterior and posterior sites. Additionally, possible explanations for that lack of self-infusion of CPBG into the anterior VTA are too few 5-HT$_3$ receptors, and/or the cellular distributions of 5-HT$_3$ receptors within anterior VTA do not favor activation of DA neurons. These possibilities could also explain why ethanol (Rodd-Henricks et al. 2000) and cocaine (Rodd et al. 2005a) are not self-infused into the anterior VTA, because their reinforcing effects involve activation of 5-HT$_3$ receptors. Alternatively, it is possible that the anterior VTA may not be involved in mediating local chemically induced reinforcing effects. Limited ICSA studies, thus far, suggest that the posterior VTA rather than the anterior VTA is involved in supporting reinforcement processes (Ikemoto and Wise 2002; Ikemoto et al. 1999; Rodd et al. 2004a, 2005a,b; Rodd-Henricks et al. 2000), although additional studies may indicate involvement of the anterior VTA as well.

The posterior VTA of the P rat appears to be more sensitive and more responsive to the reinforcing effects of CPBG than the posterior VTA of Wistar rats (Figs. 2-5). The P rat self-infused lower concentrations of CPBG (0.01 – 0.10 μM) than did Wistar rats (0.10 – 1.0 μM), and received significantly more self-infusion than did Wistar rats (e.g., self-infusions for P rats at 1.0 and 10.0 μM CPBG were approximately 40 and 80, whereas the number of
self-infusions for the Wistar rats at these two concentrations were approximately 25 and 50). Additionally, the concentration of 5-HT\textsubscript{3} receptors antagonists required to block EtOH self-administration into the posterior VTA is higher (4-fold) in P rats compared to Wistar rats (Rodd et al., 2004). These results suggest that there may be differences between the P and Wistar rat in the function (e.g., affinity) of the 5-HT\textsubscript{3} receptor, number of 5-HT\textsubscript{3} receptors, cellular distribution of 5-HT\textsubscript{3} receptors, and/or neuronal circuitry within the posterior VTA between P and Wistar rats. Differences between the P and Wistar rat, with regard to the 5-HT\textsubscript{3} receptor, may also reflect differences in the excitatory tone of the VTA DA neuronal system, possibly making the posterior VTA of the P rat generally more sensitive than the posterior VTA of Wistar rats to reinforcing effects of a wide range of compounds. However, studies examining such comparisons between P and Wistar rats have only been completed for EtOH (Rodd et al. 2004a) and CPBG; in both cases, the posterior VTA of the P rat was more sensitive. Because the reinforcing effects of ethanol appear to involve activation of 5-HT\textsubscript{3} receptors, the greater sensitivity of the P rat to the reinforcing effects of ethanol may be related to the differences in the posterior VTA 5-HT\textsubscript{3} receptors (Figs. 2 and 4) between the P and Wistar rat.

The dose-response for the self-infusion of CPBG yielded an inverted ‘U-shape’ plot that is most notable in the P rat (because of a more comprehensive dose range; Fig. 4) than Wistar rat (Fig. 2). The inverted ‘U-shape’ plot may be a result of the higher concentrations giving a greater reinforcing effect with each infusion (therefore, fewer infusions are needed as the dose increases beyond 10 µM CPBG) and/or an effect of the higher concentrations of CPBG acting at sites other than 5-HT\textsubscript{3} receptors. For example, the reduction in self-infusions
at the highest concentrations may be a result of CPBG inhibiting DA uptake (Campbell et al. 1995). Inhibiting DA re-uptake would lead to increased extracellular levels of DA, which could act at D₂ autoreceptors and reduce DA neuronal activity. Reducing VTA DA neuronal activity would result in lower CPBG self-infusions. The results with quinpirole indicate that activating D₂ autoreceptors within the posterior VTA reduces CPBG self-infusions (Fig. 8), suggesting that the reinforcing effects of CPBG are dependent upon the activity of DA neurons. The observation that the peak number of infusions occurred at 10 µM CPBG for both the P and Wistar rats could reflect the action of CPBG at the DA transporter at the higher concentrations. Alternatively, the reduced responding on the active lever at the highest concentrations of CPBG could be a result of non-specific effects on general motor performance.

Within the first session, both Wistar (Fig. 3) and P (Fig. 5) rats readily learn to discriminate the active lever from the inactive lever when CPBG was self-administered into the posterior, but not anterior, VTA. Animals used in this study have had no previous experience in the operant chambers. This rapid learning to discriminate the active from the inactive lever within one or two sessions is in agreement with several studies for the self-infusion of EtOH (Gatto et al. 1994; Rodd-Henricks et al. 2000, 2003), cocaine (Rodd et al. 2005a), or opioids (Bozarth and Wise 1981; Devine and Wise 1994) into the VTA. An operant oral self-administration study with P rats indicated that it takes 4 sessions for these rats to learn to discriminate the EtOH-reinforced lever from the water-reinforced lever (Rodd-Henricks et al. 2002), and Wistar rats typically need to undergo extensive shaping techniques to learn to respond for a reinforcer. The mechanisms underlying the learning
processes for readily being able to discriminate the active from the inactive lever within the first session of the ICSA experiments are unknown, but are likely related to direct chemical stimulation of VTA DA systems.

The pattern of responding on the active lever (Fig. 6) indicated that in the first session both the Wistar and P rat were actively rearing up and pressing the active lever throughout the 4-hr session. In the 4th session, the Wistar and P rats appear to ‘load up’ in the first 30 to 60 min, and again in the last 30 to 60 min of the session. During session 6 (the 2nd aCSF session), the pattern of responding is again similar for the Wistar and P rats. The small amount of responding on the active lever that does occur is observed within the first 30 min (Fig. 6); when no reinforcement is received in this initial period, there is almost no responding on the active lever thereafter by either the P or Wistar rat. When CPBG is restored in session 7, high levels of responding on the active lever are evident beyond the initial 30 to 60 min segment, suggesting a ‘deprivation-like effect’, which is more pronounced in the P rat than Wistar rat.

A major concern with the ICSA technique is that diffusion away from the target site may be producing self-infusion behavior. Diffusion is occurring with the present experimental conditions. However, with the small volumes injected diffusion to sites adjacent to the target site does not appear to contribute to the self-infusion behavior. For one, there is a clear delineation between the effects observed in the present study (Figs. 2 and 4) and in previous studies (Ikemoto et al. 1998; Rodd et al. 2004b, 2005a,b) for self-infusions between the anterior and posterior VTA. In addition, the ICSA procedure did not elicit self-administration behavior when placements were located in sites adjacent to the target region.
(Ikemoto et al. 1997; Rodd-Henricks et al. 2000). Therefore, with the present experimental conditions, it does not appear that diffusion of CPBG to sites adjacent to the posterior VTA is contributing to self-infusion behavior.

In summary, the present findings support the hypothesis that activating 5-HT₃ receptors within the posterior VTA produces reinforcing effects, and these effects are mediated by activation of VTA DA neurons.
REFERENCES


FOOTNOTES

Supported in part by research grants AA10721, AA11261 and AA12262, and by center grant AA07611 from NIAAA.
LEGENDS FOR FIGURES

Fig. 1. The figure is an illustration of representative injection sites in the anterior and posterior VTA of Wistar (depicted on left side of diagrams) and P (depicted on right side of diagrams) rats. Overlapping sites are not indicated. On the right side of the illustration, for the P rat, closed circles represent injection sites within the posterior VTA (defined as –5.3 to –6.0 mm Bregma), and closed squares represent injection sites within the anterior VTA (defined as –4.8 to –5.2 mm Bregma). On the left side of the illustration, for the Wistar rat, open circles represent injection sites within the posterior VTA and open squares indicate injection sites within the anterior VTA.

Fig. 2. Average number of infusions by Wistar rats (+ SEM) across the initial 4 sessions (acquisition) as a function of infusate concentration (0 to 50 µM CPBG) and cannula placement (posterior or anterior VTA). Asterisks indicate infusions significantly higher than aCSF. Plus symbol indicates a significantly higher value for 10 µM CPBG compared to all other groups (p < 0.05; Tukey’s b post-hoc; n = 7-9/group).

Fig. 3. The number of active and inactive lever presses (means + SEM) for Wistar rats self-infusing 0, 10 or 50 µM CPBG into the posterior or anterior VTA during sessions 1-4, aCSF for sessions 5 and 6, and original infusate during session 7. Asterisks indicate significantly (p < 0.05; Tukey’s b) higher responding on the active lever versus responding observed for rats self-administering aCSF and versus responses on the inactive lever (p < 0.05) within a
given infusate group (determined by one-way ANOVAs performed on individual sessions contrasting active and inactive lever presses). Plus symbols represent significantly more responding on the active lever compared to all other groups as well as to the inactive lever.

Fig. 4. Average number of infusions by alcohol-preferring (P) rats (+ SEM) across the initial 4 sessions (acquisition) as a function of infusate concentration (0 to 100 µM CPBG) and cannula placement (posterior or anterior VTA). Asterisks indicate infusions significantly higher than aCSF and 0.01 and 100 µM CPBG values. Plus symbol indicates significantly higher value for 10 µM CPBG compared to all other groups, whereas the symbol indicates significantly higher infusions than aCSF (p < 0.05; Tukey’s b post-hoc; n = 8-10/group).

Fig. 5. Number of active and inactive lever presses (means ± SEM) for P rats self-infusing 0.1, 1.0 or 10 µM CPBG into the posterior VTA during sessions 1-4, aCSF in sessions 5 and 6, and CPBG again in session 7. Asterisks indicate significantly (p < 0.05) higher responding on the active lever versus responding observed for rats self-administering aCSF, and versus responses on the inactive lever within a given infusate group (determined by one-way ANOVAs performed on individual sessions contrasting active and inactive lever presses). Plus symbols indicate significantly more responding than all other groups and responses on the inactive lever.

Fig. 6. Patterns of responding on the active lever by Wistar (left panels) and P (right panels) rats self-infusing 10 µM CPBG into the posterior VTA. Overall, both the Wistar and P rat
readily acquired CPBG self-infusion during the 1st operant session, displayed a similar pattern of responding during maintenance (session 4), extinguished responding when aCSF alone was given (session 6), and reinstated responding on the active lever when 10 µM CPBG was returned (session 7).

**Fig. 7.** Effects of co-infusing ICS205-930 on the self-infusion of 10 µM CPBG into the posterior VTA by Wistar rats. For the first 4 sessions, 10 µM CPBG alone was given. In sessions 5 and 6, ICS205-930 (10, 100, or 200 µM) was co-infused with 10 µM CPBG. In session 7, only 10 µM CPBG was given. Data are the means ± SEM; n = 7-8/group. Asterisks indicate responses on the active lever significantly (p < 0.05) higher than responses on the inactive lever. Plus symbols indicate that responses on the active lever in sessions 5 and 6 are significantly (p < 0.05) lower than responses on the active lever in sessions 4.

**Fig. 8.** Effects of co-infusing quinpirole on the self-infusion of 10 µM CPBG into the posterior VTA by Wistar rats. For the first 4 sessions, 10 µM CPBG alone was given. In sessions 5 and 6, quinpirole (1, 10, or 100 µM) was co-infused with 10 µM CPBG. In session 7, only 10 µM CPBG was given. Data are the means ± SEM; n = 5/group. Asterisks indicate responses on the active lever significantly (p < 0.05) higher than responses on the inactive lever. Plus symbols indicate that responses on the active lever in sessions 5 and 6 are significantly (p < 0.05) lower than responses on the active lever in sessions 4.