Serotonin-3 Receptors in the Posterior Ventral Tegmental Area Regulate Ethanol Self-Administration of Alcohol-Preferring (P) Rats

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Abstract

Several studies indicated the involvement of serotonin-3 (5-HT$_3$) receptors in regulating alcohol-drinking behavior. The objective of this study was to determine the involvement of 5-HT$_3$ receptors within the ventral tegmental area (VTA) in regulating ethanol self-administration by alcohol-preferring (P) rats. Standard two-lever operant chambers were used to examine the effects of 7 consecutive bilateral micro-infusions of ICS205-930 (ICS), a 5-HT$_3$ receptor antagonist, directly into the posterior VTA on the acquisition and maintenance of 15% (v/v) ethanol self-administration. P rats readily acquired ethanol self-administration by the 4th session. The three highest doses (0.125, 0.25 and 1.25 ug) of ICS prevented acquisition of ethanol self-administration. During the acquisition post-injection period, all rats treated with ICS demonstrated higher responding on the ethanol lever, with the highest dose producing the greatest effect. In contrast, during the maintenance phase, the 3 highest doses (0.75, 1.0 and 1.25 ug) of ICS significantly increased responding on the ethanol lever; following the 7-day dosing regimen, responding on the ethanol lever returned to control levels. Micro-infusion of ICS into the posterior VTA did not alter the low responding on the water lever, and did not alter saccharin (0.0125% w/v) self-administration.. Micro-infusion of ICS into the anterior VTA did not alter ethanol self-administration. Overall, the results of this study suggest that 5-HT$_3$ receptors in the posterior VTA of the P rat may be involved in regulating ethanol self-administration. In addition, chronic operant ethanol self-administration, and/or repeated treatments with a 5-HT$_3$ receptor antagonist may alter neuronal circuitry within the posterior VTA.

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Introduction

A role for serotonin-type 3 (5-HT$_3$) receptors in mediating the actions of ethanol is suggested by several studies. Ethanol enhanced 5-HT$_3$ receptor-mediated ion currents (Lovinger and White 1991; Sung et al., 2000), and 5-HT$_3$ receptor antagonists inhibited the increase in extracellular dopamine (DA) levels in the nucleus accumbens elicited by ethanol when the antagonist was given systemically (Carboni et al., 1989; Wozniak et al., 1990) or locally (Campbell et al. 1996). In addition, systemic administration of 5-HT$_3$ receptor antagonists suppressed voluntary ethanol consumption of rats under 24-hr free-choice conditions (Fadda et al., 1991; Knapp and Pohorecky 1992; McKinzie et al., 1998; Rodd-Henricks et al., 2000). Furthermore, an in vivo microdialysis study (Campbell et al., 1996) demonstrated that local administration of a 5-HT$_3$ receptor antagonist inhibited 5-HT$_3$ agonist- and ethanol - stimulated somatodendritic DA release in the ventral tegmental area (VTA), supporting a role for 5-HT$_3$ receptors in regulating basal and ethanol -stimulated activity of VTA DA neurons.

In contrast to the effects of 5-HT$_3$ receptor antagonists to reduce 24-hr ethanol intakes (Fadda et al., 1991; Knapp and Pohorecky 1992; McKinzie et al., 1998; Rodd-Henricks et al., 2000), under scheduled access conditions, several studies indicated that systemic injections of 5-HT$_3$ receptor antagonists (at doses that were effective under 24-hr free-choice alcohol drinking) had little effect on ethanol drinking or operant self-administration (Knapp and Pohorecky 1992; McKinzie et al., 1998, 2000; Svennsson et al., 1993; Beardsley et al., 1994), suggesting that conditioning factors may play a role in reducing the effectiveness of the antagonists. However, there are two studies (Hodge et al., 1993b; Tomkins et al., 1995) that reported a reduction in ethanol intake, under scheduled access conditions, following systemic administration of 5-HT$_3$ antagonists. The apparent disagreement between these latter two studies and those cited above may be related to only very high doses being effective in one study (Hodge et al., 1993b), or the use of ondansetron in the other study (Tomkins et al., 1995), which did not produce a typical dose-response and had only small effects on ethanol intake.

Studies with the intracranial self-administration technique demonstrated that ethanol was self-infused into the posterior VTA but not the anterior VTA of alcohol-preferring (P) and Wistar rats (Rodd-Henricks et al., 2000; Rodd et al., 2004), suggesting that the posterior VTA is a neuroanatomical site supporting the reinforcing effects of ethanol . Additional studies indicated that co-administration of 5-HT$_3$ receptor antagonists blocked the self-infusions of ethanol into the posterior VTA of P and Wistar rats (Rodd-Henricks et al., 2003; Rodd et al., 2005b), suggesting that the reinforcing effects of ethanol within the posterior VTA are mediated through activation of local 5-HT$_3$ receptors.

There is evidence that the mesolimbic DA system is involved in regulating alcohol drinking (reviewed in Koob et al., 1998). Several microinjection studies indicated that the VTA is involved in regulating alcohol drinking (Hodge et al., 1993a, 1996; Katner et al., 1997; Nowak et al., 1998, 2000). Moreover, the studies of Hodge et al., (1993a) and Nowak et al., (2000) indicated that microinfusion of quinpirole, a DA D_2 receptor agonist, reduced ethanol intake, suggesting that activation of VTA DA neurons is involved in maintaining ethanol drinking. Therefore, the objective of the present study was to examine the effects of micro-injecting a 5-HT$_3$ receptor antagonist into the VTA of P rats to test the hypothesis that 5-HT$_3$ receptors in the posterior VTA are involved in regulating ethanol self-administration.

Materials and methods

Animals

Naïve, female P rats from the 55th -57th generations weighed 250–320 g at the start of the study. Female rats were used mainly because adult female body weights and head size remain more constant than male rats, which yields better consistency and accuracy in stereotaxic placements (Ikemoto et al., 1997a, 1998; Rodd-Henricks et al., 2000). Female rats have been used in previous studies involving microinjection techniques that examined the VTA (Gatto et al., 1994; Rodd-Henricks et al., 2000, 2003; Rodd et al., 2005a, b). There has been no evidence that estrous cycle has an effect on operant behaviors (e.g., Rodd-Henricks et al., 2000, 2002), as indicated by no major cyclical individual fluctuations in operant behavior in rats given access to ethanol over several weeks after responding was established; the usual average has been less than 10% variation across days.

Rats were double-housed upon arrival in the vivarium and were maintained on a 12-hr reverse light-dark cycle (lights off at 0900 hr) for at least two weeks before beginning experimental procedures. Food and fresh tap water were always available in the home cages. The vivaria and facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. The final number of animals indicated for each experiment represents 90–95% of the total number that underwent surgery; 5–10% of the rats were excluded, mainly due to the loss of the guide cannula before completion of all experimental sessions. Any data accrued for these animals were not used because their injection sites could not be verified.

Surgical procedure

Prior to surgery, all rats were weighed and handled for at least 20 min daily over one week. All rats were stereotaxically implanted with bilateral 22-gauge guide cannula (Plastic One) under iosflurane anesthesia. The stereotaxic coordinates (Paxinos and Watson, 1998) targeted placement of the cannula tip 1.0 mm above the VTA region of interest. Placements for the posterior VTA were aimed at -5.4 to –5.8 mm bregma, 8.5 mm ventral from the surface of the skull, and each cannula was 2.1 mm lateral in the right or left hemisphere at a 10-degree angle from vertical such that the cannula tip terminated above and slightly lateral to the midline posterior VTA. Coordinates for bilateral placements aimed above the anterior VTA were –4.6 to –4.8 mm bregma, 8.5 mm ventral from the surface of the skull and 2.1

mm lateral from the midline at a 10-degree angle from vertical. Except for when microinjections were being conducted, a 28-gauge capped stylet was threaded in place to seal each guide cannula. The stylet extended 0.5 mm beyond the tip of the guide. Rats were individually housed after surgery and allowed 7 days to recover before experimental procedures began. Animals were handled daily following surgery day. Body weights and general behavior were monitored to ensure full recovery prior to commencing experimental sessions.

Ethanol Self-Administration

The rats were experimentally naive and were not acclimated to the operant chamber before commencement of operant sessions and data collection. Ethanol self-administration experiments were conducted in standard two-lever operant chambers (Coulbourn Instruments, Allentown, PA) contained within ventilated, sound-attenuated enclosures. In each chamber, the two operant levers were located on the same wall, 15 cm above the grid floor and 13 cm apart. Directly beneath each lever was a trough through which a dipper cup (0.1 ml) rose to deliver response-contingent fluid. Upon a reinforced response, a small cue light was illuminated within the drinking trough during the 4 sec access to the dipper cup. One lever activated a dipper that delivered water and the other activated a lever that delivered the ethanol solution. The assignment of the water and ethanol levers in the left or right position was counterbalanced among subjects, but the levers remained in the same position throughout for each rat. Operant chambers were illuminated by dim lights during test sessions. All sessions were 60 min in duration and were conducted daily during the dark period.

For testing, subjects were brought to a microinjection area, the stylet was removed, and an injection cannula was screwed into place. When secured, the microinjection cannula extended 1.0 mm beyond the tip of the guide. During the microinjection procedure, rats were housed in a glass cylinder (diameter – 24 cm). The right hemisphere VTA was always infused first. The rate of infusions was regulated by a Harvard microinjection pump at a steady rate of 0.5 μl of infusate/30 sec/side. The microinjector cannula remained inserted for an additional 30 sec to allow the infusate to diffuse away from the cannula tip. Following the initial injection into the right VTA, the left side was injected with the same infusate. Again, a 30 sec post-injection period was allowed. Immediately following the left hemisphere microinjection, rats were transported into the adjacent operant testing room and placed into their assigned chambers. The 5-HT$_3$ antagonist, ICS 205,930 (ICS; Sigma), was dissolved in artificial cerebrospinal fluid (aCSF); the solution was adjusted to pH 7.4 ± 0.1 with 0.1 M HCl or 0.1 M NaOH. The 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.

Micro-infusion of ICS during Acquisition of Ethanol Self-Administration

For the acquisition experiment, female P rats (n = 46) were randomly assigned to one of six groups (n = 6–9/group). To evaluate whether the microinjection procedure might alter the acquisition of oral operant ethanol self-administration, a no-injection control was included in the experimental design. The no-injection control group was handled exactly as all other microinjected groups, except that the injector was not screwed into the dummy cannulae

(animals were placed into glass cylinders and the injection pump was activated). A vehicle group received infusions of aCSF into both hemispheres for seven consecutive sessions. The other groups were given 0.075, 0.125, 0.25, or 1.25 µg ICS/side for all seven sessions. The ICS compound was chosen because this antagonist is readily soluble in aCSF and has been used in previous micro-infusions (Rodd-Henricks et al., 2003; Rodd et al., 2005a,b) and microdialysis (Campbell et al., 1996) experiments.

Following the surgery recovery period, rats were given two days of mock injections, i.e., they were taken into the microinjection room and were treated as if they were to be injected (stylets removed, injectors attached, 30 sec post-injection period), except that no injection was given. On the day after the second mock injection, rats were microinjected with their assigned infusate and were immediately transferred to the operant testing room. Rats had never entered the operant testing room prior to this first operant test session. Both the EtOH (15% v/v) and water levers were maintained on a continuous (fixed-ratio; FR 1) reinforcement schedule. Rats received bilateral microinjections into the posterior VTA daily prior to each of the 7 acquisition sessions. Following this 7-day microinjection phase, all rats were given 6 more daily operant sessions without microinjections.

Micro-infusion of ICS during Maintenance of Ethanol Self-Administration

Naïve female P rats (n = 37) were placed into the operant chamber with access to the ethanol solution and water on a FR1 schedule of reinforcement. Rats were allowed to acquire ethanol self-administration on the FR1 schedule for 4 weeks. The reinforcement schedule on the ethanol lever was then increased to FR3 in weeks 5 – 7 and to FR5 in weeks 8–10. The response requirement was increased to demonstrate that the ethanol solution was more reinforcing than water, and to have a baseline level that would permit measurement of drug effects that yielded either increases or decreases in responding. Water was maintained on an FR1 schedule throughout. During 10 weeks of ethanol self-administration, between-session, within-subject variation in responding averaged less than 10% of total responding for all rats.

For all maintenance experiments, cannula implants were performed after long-term maintained responding was well established. Surgeries were performed on the 71st or 72nd day of operant exposure to ethanol, and were performed on all groups equally for both days. After recovery, operant sessions were re-initiated. Rats were randomly assigned to one of six groups (n = 6–7/group). A vehicle group received infusions of aCSF in both hemispheres for seven consecutive sessions. The other groups were given 0.25, 0.5, 0.75, 1.0 or 1.25 µg ICS/side for all seven sessions. Higher doses of ICS were tested in the maintenance than acquisition experiment because initial results indicated that the 0.25 µg dose had little effect on responding during the maintenance phase. On the 5th and 6th day following surgery, all rats were processed through the mock injection procedure outlined in the acquisition experiment, and in subsequent sessions the rats were given bilateral microinjections immediately before the 7 operant sessions. Following the microinjection phase of the experiment, all rats were tested in operant sessions for 7 additional daily sessions without further microinjections.

Micro-infusion of ICS during Maintenance of Saccharin (SAC) Self-Administration

P rats acquire saccharin self-administration at a slower, less uniform rate than EtOH (Toalston et al., 2008). The mean number of daily 1 hr sessions required for a P rat to acquire saccharin (0.0125% w/v) self-administration is about 10–13 (Toalston et al., 2008; current data set), while 95% of P rats will acquire operant oral self-administration of 15% EtOH in 4 sessions (Rodd-Henricks et al., 2002; Toalston et al., 2008, current data). In contrast, it takes approximately 19 daily sessions for 85% of P rats to acquire consistent saccharin self-administration (Toalston et al., 2008; current data). Therefore, it is impractical to examine the effects of compounds on the acquisition of saccharin self-administration in P rats.

Similar to the experiment examining the effects of ICS on the maintenance of ethanol operant self-administration, naïve female P rats (n = 15) were placed into the operant chamber with access to a SAC (0.0125 % w/v) solution in one dipper and water in the other dipper, with both dippers on a FR1 schedule of reinforcement. The reinforcement schedule on the SAC lever was increased to FR3 in weeks 5 – 7 and to FR5 in weeks 8–10. Water was maintained on an FR1 schedule of reinforcement. All other handling, surgery, and experimental protocols were as described in the ethanol maintenance experiment. However, rats were only assigned to two groups, an aCSF vehicle (n = 7) or 1.25 μg ICS/side (n = 8) group. The 1.25- μg dose was selected based upon the effects of this dose on ethanol self-administration. Rats received bilateral microinjections of the assigned infusate immediately prior to 7 consecutive daily sessions, and then were allowed to respond for saccharin for 7 additional daily sessions.

Micro-infusion of ICS into the Anterior VTA during Maintenance of Ethanol Self-Administration

Rats were treated identically to animals tested in the posterior VTA maintenance study. Rats were randomly assigned to one of three groups (n = 5–7/group; n = 17 total). A vehicle group received infusions of aCSF in both hemispheres for seven consecutive sessions. The other groups were given 0.75 or 1.25 μg ICS/side for all seven sessions. On the 5th and 6th day following surgery, all rats were processed through the mock injection procedure. Following the microinjection phase of the experiment, all rats were allowed to self-administer ethanol for 7 more daily sessions without further microinjections.

Histology

All rats that completed the experiments received a 1% bromophenol blue solution infusion into the cannula sites after CO_2 euthanasia. Brains were removed and immediately frozen at –70° C. To perform histology, frozen brains were equilibrated at –15° C in a cryostat microtome, and then sliced into 40 um sections. The sections were 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 Analyses

Analyses for the acquisition data consisted of a mixed factor ANOVA with a between-subject variable of 'group' and a repeated measure of 'session.' Lever discrimination was

examined by contrasting ethanol and water lever responses within each group. Data analyses for the maintenance experiment consisted of a mixed factor ANOVA with a between-subject variable of 'group' and a repeated measure of 'session.' In all experiments, data generated during the post-injection period were analyzed with a similar 'group' by 'session' mixed factor ANOVA.

Results

The posterior VTA was defined as the VTA region at the level of the interpeduncular nucleus, coronal sections at –5.3 to –6.0 mm from bregma. The anterior VTA was defined as the VTA between –4.8 to –5.2 mm from bregma (Fig. 1). Cannula placements outside the VTA included injection sites located in the substantia nigra, interpeduncular nucleus, red nucleus, and caudal linear nucleus of the raphe. These rats were excluded from the study (n = 3).

Micro-infusion of ICS into the Posterior VTA during Acquisition of Ethanol Self-Administration

In agreement with previous studies (Rodd-Henricks et al., 2002), P rats readily learned to distinguish the ethanol lever from the water lever within 4 sessions, without any prior training or shaping experience (Fig. 2). Lever discrimination, preference for responding on one lever compared to another lever, was evident in the no-injection control group during the 3rd microinjection session ($F_{1,5} = 11.85$; $p = 0.02$) and in the aCSF group during the 4th session. By the 4th session, responding on the ethanol lever increased to 30–35 responses/session, whereas responses on the water lever decreased to less than 10 responses/session. During the 4th–7th microinjection sessions, the no-injection, aCSF, and 0.075 μg ICS/side groups all showed a preference for responding on the ethanol lever compared to the water lever (F values > 14.96; p values < 0.006).

The analysis revealed that there was a significant effect of administration of ICS into the posterior VTA on the acquisition of ethanol self-administration (Fig. 2; group - $F_{5,40} = 6.8$; $p < 0.001$; session - $F_{6,35} = 2.48$; $p = 0.041$) and that this effect varied across injection session (group x session – $F_{30,195} = 1.7$; $p = 0.018$). The significant interaction term justified decomposing the data by performing individual ANOVAs on each session with the dependent variable of group. During the 1st and 2nd session, bilateral microinjection of ICS had no effect on either ethanol or water lever responding (F values $_{5,40} < 2.0$; p values > 0.102). On the 3rd microinjection session, there was a significant effect of group ($F_{5,40} = 5.8$; $p < 0.0001$), with post-hoc comparisons (Tukey's b) indicating that the no-injection control, aCSF, and 0.075 μg ICS/side responded significantly higher on the ethanol lever than did rats administered 0.125, 0.25, and 1.25 μg ICS/side. During the 4th-7th microinjection sessions, bilateral microinjection of 0.125, 0.25, or 1.25 μg ICS/side reduced responding on the ethanol lever (group F values $_{5,40} > 3.2$; p values < 0.016). Post-hoc comparisons performed on these sessions indicated that ethanol lever responding in the 0.125and 1.25 μg ICS/side groups was significantly lower than in the no-injection, aCSF, and 0.075 μg ICS groups during sessions 4–7, while the 0.25 μg ICS groups was reduced in sessions 4, 5, and 7.

At no point during the microinjection phase of the experiment did ICS impact the amount of responding on the water lever (Fig. 3; group - $F_{5,40} = 6.8$; $p < 0.001$; group x session – $F_{30,195} = 0.93$; $p = 0.577$). There was a significant effect of 'session' on water responding ($F_{6,35} = 4.6$; $p = 0.002$). This effect was based upon a gradual reduction in water responding from the 1st session (total average 15.7 ± 1.7) to the 7th session (total average 9.4 ± 1.2).

Examining ethanol responding during the post-injection phase following 7 consecutive bilateral microinjections of ICS revealed that there was a significant carryover (sessions 8– 13) effect (Fig. 2; group – $F_{5,40} = 4.2$; $p = 0.004$, session - $F_{5,36} = 36.0$; $p < 0.0001$) and that this carryover effect varied across injection session (group x session – $F_{25,200} = 2.5$; $p < 0.001$). The 0.125, 0.25, and 1.25 µg ICS groups readily acquired ethanol self-administration when ICS microinjections were terminated, as indicated by the increased responding on the ethanol lever without any change in responding on the water lever during the post-injection phase. Isolating the interaction term by performing separate ANOVAs on each session revealed that, during the 1st and 2nd post-injection session, there were no group differences (F values $_{5,40} < 0.4$; p values > 0.94). There were significant group differences during the 3rd – 6th post-injection sessions (F values $_{5,40} > 3.6$; p values < 0.009). Post-hoc comparisons indicated that, during the 3rd post-injection session, rats in the 1.25 µg ICS group responded more than all other groups, and rats in the 0.075 µg ICS group responded more on the ethanol lever than no-injection and aCSF groups. During the 4th post-injection session, rats administered the highest concentration of ICS (1.25 µg ICS) responded more than all other groups. All groups that received microinjections of ICS during the microinjection period responded more than the no-injection and aCSF groups during the 4th – 6th post-injection session.

Although all ICS groups significantly increased responding on the ethanol lever during the post-injection period, responses on the water lever did not change during this period (Fig. 3; group - $F_{5,40} = 1.8$; $p = 0.12$, session - $F_{5,36} = 1.4$; $p = 0.24$; group x session – $F_{25,200} = 0.953$; $p = 0.533$).

Micro-infusion of ICS into the Posterior VTA during Maintenance of Ethanol Self-Administration

For the maintenance experiments, a concurrent FR5-FR1 schedule of reinforcement was used to increase the work requirement for ethanol while maintaining the low requirement for water. This schedule has been used previously to assess the reinforcing effects of EtOH (Rodd-Henricks et al., 2002). With this procedure, responses on the water lever remained low (average responses for all microinjection sessions was 12 responses/session, with a range of 10 – 14 responses/session), and were not altered by any of the treatments with ICS (data not shown; all p values > 0.67).

An ANOVA performed on the last 3 sessions prior to surgery revealed that all groups responded similarly on the EtOH lever ($F_{5,36} = 0.4$; $p = 0.83$). Additionally, a mixed factor ANOVA performed on recovery days 3–5 indicated that responding on the ethanol lever was not different among the groups (Fig. 4; group - $F_{5,31} = 0.1$; $p = 0.991$; session - $F_{2,62} = 0.95$; $p = 0.393$; group x session - $F_{10,62} = 0.79$; $p = 0.643$). The average water responses prior to surgery were 12 responses/session for all groups, with no significant differences between

groups (range of 11 – 14 responses/session). Following surgery, water lever responding was maintained at a low level for all groups, with no group differences (average of 11 responses/ session; range of 9 – 13 responses/session).

The analysis revealed that there was a significant effect of administration of ICS into the posterior VTA on the maintenance of ethanol self-administration (Fig. 4; group - $F_{5,31} = 4.3$; p = 0.004; session - $F_{6,186} = 3.3$; p = 0.004), and that this effect varied across injection sessions (group x session - $F_{30,186} = 2.7$; p < 0.001). Individual ANOVAs performed on each microinjection session revealed a significant effect of group for all sessions (F values $_{5,31}$ > 2.9; p values < 0.028). Post-hoc comparisons (Tukey's b) revealed that on the 1st microinjection session, the group given 1.25 μg ICS/side responded more on the ethanol lever than did rats given aCSF. During the 2nd microinjection session, rats administered 0.75 and 1.25 μg ICS/side responded significantly more on the ethanol lever than rats given aCSF, or 0.25 or 0.50 μg ICS/side. During the 3rd and 4th microinjection sessions, rats given the three highest doses of ICS (0.75, 1.0 and 1.25 μg ICS/side) responded more on the ethanol lever than all other groups. During the 5th–7th microinjection sessions, rats administered 0.75 and 1.0 μg ICS/side responded more on the EtOH lever than all other groups.

A mixed-factor ANOVA conducted on the initial 7 post-injections sessions revealed a carryover effect of ICS microinjections (Fig. 4; session $F_{6,186} = 10.8$; p < 0.001; group x session - $F_{30,186} = 4.1$; p < 0.001). On the 1st post-injection session, rats administered 0.75 and 1.0 μg ICS/side responded more on the ethanol lever than all other groups ($F_{5,31} = 4.2$; p = 0.005; Tukey's b p < 0.05). During the 2nd and 3rd post-injection sessions, rats previously administered the three highest levels of ICS (0.75, 1.0 and 1.25 μg ICS/side) responded more on the ethanol lever than all other groups (F values 5, 31 > 3.7; p values > 0.011). There was no significant effect of group on any other post-injection session (p values > 0.176). Responses on the water lever were not elevated during the post-injection sessions, and did not differ between groups (p values > 0.56).

Micro-infusion of ICS into the Posterior VTA during Maintenance of SAC Self-Administration

P rats readily learn to self-administer 0.0125% (w/v) SAC without any prior training or shaping procedures. In a 2-lever operant paradigm, P rats readily discriminated the SAC from the water lever. With a concurrent FR5-FR1 schedule of reinforcement for SAC versus water, P rats responded significantly more on the SAC than water lever. Responses on the water lever with saccharin concurrently available were low (data not shown), and similar to responses on the water lever when ethanol was concurrently available (Fig. 3).

An ANOVA performed on the average of the last 3 sessions prior to surgery revealed that both groups responded similarly on the SAC lever (Fig. 5; $F_{1,13} = 0.07$; p = 0.80). Additionally, a mixed factor ANOVA performed on recovery days 3–5 indicated that recovery from surgery was equivalent in both groups (Fig. 5; all p values > 0.449). The overall analysis revealed that administration of 1.25 μg ICS/side into the posterior VTA had no effect on the maintenance of SAC self-administration (Fig. 5; group - $F_{1,13} = 0.05$; p = 0.83; session - $F_{6,78} = 1.05$; p = 0.4; group x session - $F_{6,78} = 1.02$; p = 0.42). Additionally,

there was no carryover effect of bilateral administration of ICS directly into the posterior VTA on SAC responding following the termination of ICS injections (all p values > 0.69).

Micro-infusion of ICS into the Anterior VTA during Maintenance of Ethanol Self-Administration

Overall, the analysis revealed that microinjections of ICS into the anterior VTA had no effect on ethanol self-administration. An ANOVA performed on the last 3 sessions prior to surgery revealed that all groups responded similarly on the ethanol lever ($F_{2,16} = 0.7$; p = 0.53). The analysis revealed that there was no significant effect of administration of ICS into the anterior VTA on the maintenance of operant ethanol self-administration (Fig. 6; group - $F_{2,16} = 0.95$; p = 0.41; session - $F_{6,96} = 1.4$; p = 0.211; group x session – $F_{12,96} = 1.5$; p = 0.11).

The average responses on the water lever prior to surgery were 14 responses/session for all groups, with no significant differences between groups (range of 12 – 15 responses/session). Following surgery, responding on the water lever was maintained at a low level for all groups, with no group differences (average of 12 responses/session; range of 11 – 14 responses/session).

Discussion

The major findings of this study were that local administration of the 5-HT$_3$ receptor antagonist ICS into the posterior VTA had divergent effects on ethanol self-administration depending on ethanol drinking experience; decreasing ethanol self-administration during acquisition (Fig. 2) and enhancing ethanol self-administration during maintenance (Fig. 4). Furthermore, ICS microinjections during the acquisition period appeared to produce alterations within the posterior VTA that resulted in markedly increased ethanol self-administration during the post-injection phase (Fig. 2). Overall, the results of this study support the hypothesis that 5-HT$_3$ receptors within the posterior VTA are involved in regulating ethanol intake. In contrast to its effects of enhancing ethanol self-administration during maintenance, the ICS compound had no effect on responses on the water lever or on saccharin self-administration under these conditions (Figs. 3 and 5). In addition, the regulation of ethanol self-administration by 5-HT$_3$ receptors within the VTA appears to be localized to the posterior rather than the anterior portion (Fig. 6). The posterior VTA receives significant 5-HT input (Herve et al., 1987) and 5-HT terminals synapse on mesolimbic DA neurons (Van Bockstaele et al., 1994). However, the localization of 5-HT$_3$ receptors within the anterior VTA has not been established.

With regard to acquisition, the three highest doses of ICS prevented acquisition of ethanol self-administration when microinfused prior to each of the first 7 sessions (Fig. 2). These results suggest that activation of 5-HT$_3$ receptors within the posterior VTA may be needed for acquisition of oral ethanol self-administration. Apparently, these doses may be high enough to block the activating (Campbell et al., 1996; Lovenger and White, 1991; Sung et al., 2000) and reinforcing (Rodd-Henricks et al., 2003; Rodd et al., 2005b) effects of ethanol at 5-HT$_3$ receptors in the VTA. Under acquisition conditions, the reinforcing effects of ethanol may be blocked by ICS, and, therefore, the P rats do not increase responding on the

ethanol lever and demonstrate lever discrimination. Alternatively, it is possible that ICS may be interfering with learning the operant task, resulting in lower responses on the ethanol lever. However, this latter possibility is not compatible with the higher responding that was observed for the ICS treated group during the post-injection sessions. If the ICS compound was interfering with learning then responses on the ethanol lever during the post-injection sessions should be similar for the control and ICS groups. In support of the idea that local microinjection of the ICS compound was not interfering with learning the operant task, a previous study (Rodd et al., 2005b) indicated that local co-administration of 200 μM ICS into the posterior VTA did not interfere with acquisition of acetaldehyde self-infusions.

During the post-injection acquisition sessions, the 0.125 and 0.25 μg ICS treated groups acquired ethanol self-administration behavior within 3 sessions, and all 4 groups previously microinjected with ICS responded significantly more on the ethanol lever than did the two control groups (Fig. 2), suggesting that the ICS pretreatment regimen may have produced changes within the posterior VTA. A pharmacological effect on 5-HT$_3$ receptors as opposed to a behavioral contrast effect during the post-injection period is suggested by the findings that the 0.125 and 1.25 μg doses produced the same effects in reducing ethanol responding during the initial acquisition sessions, whereas the 1.25 μg-dose-group had significantly higher responding on the ethanol lever than all other groups during the post-injection phase (Fig. 2). A solely behavioral contrast or compensatory response during the post-injection phase would have been suggested if both the 0.125 and 1.25 μg doses produced similar effects during the post-injection phase. In addition, it is not clear if a similar post-injection increase in responding would have occurred if saccharin had been used instead of ethanol. The prediction would be that, since the reinforcing effects of saccharin are not mediated through activation of 5-HT$_3$ receptors in the posterior VTA (Fig. 5), then no markedly enhanced responding for saccharin should be observed in the post-injection period.

In general, chronic administration of an antagonist would be expected to produce up-regulation of the receptor. Because local administration of a 5-HT$_3$ receptor agonist increases DA neuronal activity, as indicated by increased somatodendritic release of DA (Campbell et al., 1996), the expectation is that the pretreatment regimen with local ICS administration up regulates 5-HT$_3$ receptors and increases the overall net excitability of VTA DA neurons. The up regulation of 5-HT$_3$ receptors in the posterior VTA increases the excitatory effects of ethanol on VTA DA neurons and, thereby, increases the reinforcing effects of ethanol. On the other hand, evidence indicates that repeated systemic injections of 5-HT$_3$ receptor antagonists decreased the number of spontaneously active VTA DA cells in rodents (Minabe et al., 1991; Prisco et al., 1992; Rasmussen et al., 1991), and reduced DA neurotransmission in the nucleus accumbens (Liu et al., 2006). However, with systemic injections, the 5-HT$_3$ receptor antagonists are producing effects at multiple CNS sites and pathways, which may have the end result of decreasing VTA DA neuronal activity, whereas local administration of the antagonist is having a direct effect within the VTA, which could produce up-regulation of 5-HT$_3$ receptors in this region.

In contrast to the effects of ICS during acquisition of ethanol self-administration (Fig. 2), microinjection of ICS during maintenance had no effect at a dose that blocked acquisition; moreover, at higher doses, ICS significantly increased ethanol self-administration (Fig. 4).

Perhaps the best illustration of this divergent effect of ICS on oral ethanol self-administration is the 1.25 μg ICS group (reduction during acquisition, increase during maintenance). These results suggest that different mechanisms within the posterior VTA may be involved in acquisition and maintenance of ethanol self-administration. The concept that different mechanisms may underlie acquisition and maintenance of alcohol intake is consistent with published findings (Ford et al., 2008; Ikemoto et al., 1997b). The development of mechanisms maintaining ethanol self-administration may reflect the impact of a combination of factors, e.g., chronic intermittent ethanol exposure, the reinforcing effects of ethanol, learning and maintaining high responding on the appropriate lever, and conditioning factors associated with the operant chamber. The increased responding on the ethanol lever by the 0.75, 1.0 and 1.25 μg ICS groups, compared to the control group (Fig. 4), suggests that alterations in local 5-HT$_3$ receptors and/or local circuits may have developed with chronic ethanol self-administration. One possibility is that daily ethanol self-administration has produced up-regulation of 5-HT$_3$ receptors in the posterior VTA, and the 3 highest doses of ICS only partially block the 5-HT$_3$ receptors. Under conditions of partial blockade of the receptor, the P rats increase their responding on the ethanol lever in an attempt to overcome this partial inhibition. An alternative explanation is that a history of ethanol self-administration has produced alterations in neural circuits within the posterior VTA, so that now 5-HT$_3$ receptors regulate the upper limit for ethanol self-administration; blocking these receptors removes this upper limit, resulting in the P rats self-administering more ethanol. In addition, at the highest dose used in the maintenance phase, the ICS compound, with repeated microinjections, may be diffusing to adjacent sites and/or having an effect at the GABA$_A$ receptor (Klein et al., 1994), in addition to its effect on local 5-HT$_3$ receptors, to produce mixed effects on ethanol self-administration.

Based upon the general findings in the literature, the expected results of micro-injecting a 5-HT$_3$ receptor antagonist into the VTA would have been to reduce ethanol self-administration. Although a reduction during the acquisition phase was observed (Fig. 2), an increase or no effect of ICS was found during the maintenance phase (Fig. 4). First, the differences observed with ICS between the acquisition and maintenance phases suggest that ethanol self-administration may have produced alterations in the posterior VTA neuronal circuits. Chronic alcohol drinking (Rodd et al., 2005c,d) or repeated local ethanol administration (Ding et al., 2009b) produced alterations in the posterior VTA, which increased the sensitivity of this region to the stimulating and reinforcing effects of ethanol. Therefore, changes within the posterior VTA neuronal circuitry may have resulted in ICS activating circuits and enhancing the reinforcing effects of ethanol.

The finding that local microinjection of ICS into the posterior VTA increased ethanol self-administration is in apparent disagreement with several studies reporting that 5-HT$_3$ receptor antagonists reduced ethanol intake of rats under 24-hr free-choice conditions (Fadda et al., 1991; Knapp and Pohorecky 1992; McKinzie et al., 1998, 2000; Rodd-Henricks et al., 2000). Two factors need to be considered to explain this apparent disagreement. First, systemic injections were used in the alcohol drinking studies, and the 5-HT$_3$ receptor antagonists are acting in multiple regions; these combined effects could be contributing to reducing 24-hr free-choice drinking. Second, doses of 5-HT$_3$ receptor antagonists that reduce 24-hr free-choice drinking are not effective in reducing ethanol intake under

scheduled access conditions (Beardsley et al., 1994; Knapp and Pohorecky, 1992; McKinzie et al., 1998, 2000; Svennsson et al., 1993), possibly due to conditioning factors having a role in altering the activity of the 5-HT system and reducing the effectiveness of the 5-HT$_3$ receptor antagonists. It is possible that conditioning factors may be altering the activity of 5-HT and other pathways to the posterior VTA resulting in ICS producing a local effect, which enhances on-going ethanol self-administration.

The lack of effect of ICS injected into the anterior VTA on ethanol self-administration (Fig. 6) may be a result of fewer 5-HT$_3$ receptors being present in this region, and/or the anterior VTA is not involved in mediating alcohol drinking behavior. A microdialysis study (Liu et al., 2006b) suggested that there was a heterogeneous distribution of functional 5-HT$_3$ receptors within the VTA with higher numbers in the posterior than anterior VTA. There is also evidence for a heterogeneous distribution within the VTA for the reinforcing effects of ethanol (Rodd- Henricks et al., 2000) and the reinforcing effects of activation of 5-HT$_3$ receptors (Rodd et al., 2007), with the posterior VTA supporting reinforcement, and the anterior VTA not supporting reinforcement processes. Therefore, it is possible that the lack of effect of ICS in the anterior VTA on ethanol self-administration may be due to fewer functional 5-HT$_3$ receptors in this region, and/or the lack of involvement of the anterior VTA in mediating ethanol reinforcement.

Studies using microinjection techniques to examine effects on behavior have two major problems to address, i.e., site specificity and pharmacological selectivity. There will always be diffusion of the injected compound away from the site of injection. A concern is whether the observed effect is due to the target site, a site adjacent to the target, or a combination of both. The finding that the highest dose of ICS infused into the anterior VTA did not have an effect on ethanol self-administration suggests that there is a limited effective diffusion range for the ICS compound, since injection sites in the anterior VTA were 0.4 to 0.6 mm from the posterior VTA. The most prominent structure adjacent to the posterior VTA is the substantia nigra (SN). Effects in this region would be expected to alter general motor performance. However, since ICS did not alter saccharin responding (Fig. 5), this would suggest that ICS is not diffusing to the SN in sufficient quantities to alter motor performance. The possibility that diffusion to other adjacent sites (e.g., the red nucleus) might be influencing the results cannot be ruled out.

The ICS compound has also been reported to act at GABA$_A$ receptors, producing a bi-phasic response, depending upon the concentration of the ICS and the subunit composition of the GABA$_A$ receptor, with the higher μM concentrations of ICS generally inhibiting GABA- mediated Cl$^-$ currents (Klein et al., 1994). There is microdialysis evidence that local administration of a GABA$_A$ receptor antagonist into the posterior VTA has little effect on extracellular levels of DA in the nucleus accumbens, suggesting that DA neurons in this region are not under tonic GABA$_A$ receptor mediated inhibition (Ding et al., 2009a), whereas DA neurons in the anterior VTA appear to be under tonic GABA$_A$ receptor mediated inhibition (Ikemoto et al., 1997c). If increases in DA neuronal activity are associated with the reinforcing effects of ethanol and ethanol drinking, then the inhibitory effects of ICS on acquisition of ethanol self-administration may not be a result of its actions at GABA$_A$ receptors. However, since ICS had an opposite effect on ethanol self-

administration under maintenance conditions, suggesting that ethanol self-administration may have altered neuronal circuitry within the posterior VTA, it is possible that the effects of ICS in enhancing on-going ethanol self- administration may be due in part through an action at GABA$_A$ receptors.

The saccharin experiment was conducted to ensure that the microinjection of ICS into the posterior VTA did not have a general effect on disrupting operant behavior. Since the highest dose of ICS did not alter saccharin responding (Fig. 5), these results suggest that the enhanced maintenance responding for ethanol produced by ICS (Fig. 4) may be a result of a history of ethanol self-administration by the P rats. In addition, the observation that the highest dose of ICS did not alter saccharin responding suggests that the reduced responding on the ethanol lever produced by ICS during the acquisition experiment was not due to impaired motor control.

In conclusion, the results of the present study indicate that 5-HT$_3$ receptors within the posterior VTA are involved in regulating ethanol intake and reinforcement. A history of ethanol self-administration or pretreatment with local administration of a 5-HT$_3$ receptor antagonist may alter 5-HT$_3$ receptor function and/or neural circuits within the posterior VTA.

Acknowledgments

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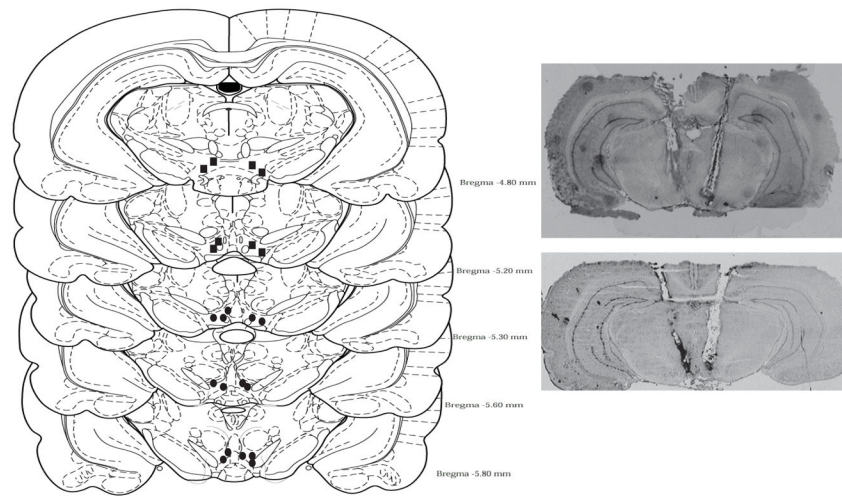
Fig. 1.
Representative injection sites in the anterior and posterior VTA of P rats. Overlapping injection sites are not indicated. Closed circles represent injection sites within the posterior VTA (defined as –5.3 to –6.0 mm Bregma), and closed squares represent injection sites within the anterior VTA (defined as –4.8 to –5.2 mm Bregma). On the right hand side are photomicrographs which indicate an anterior (top) and posterior (bottom) VTA dual placement.
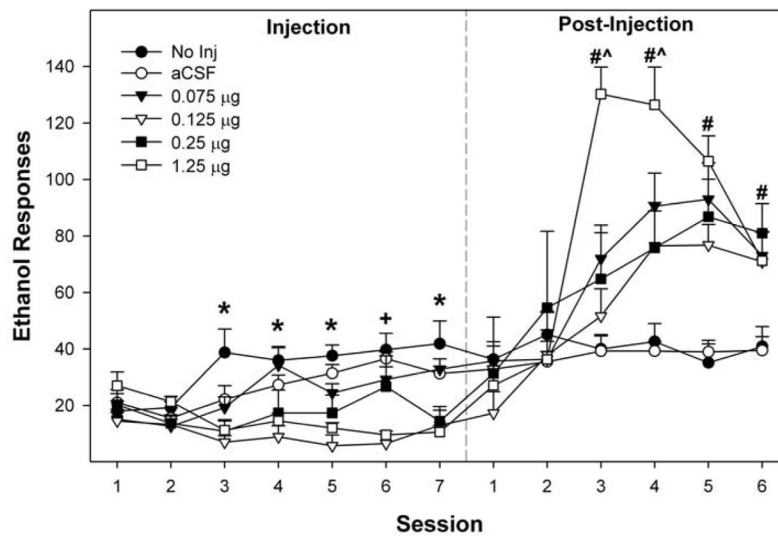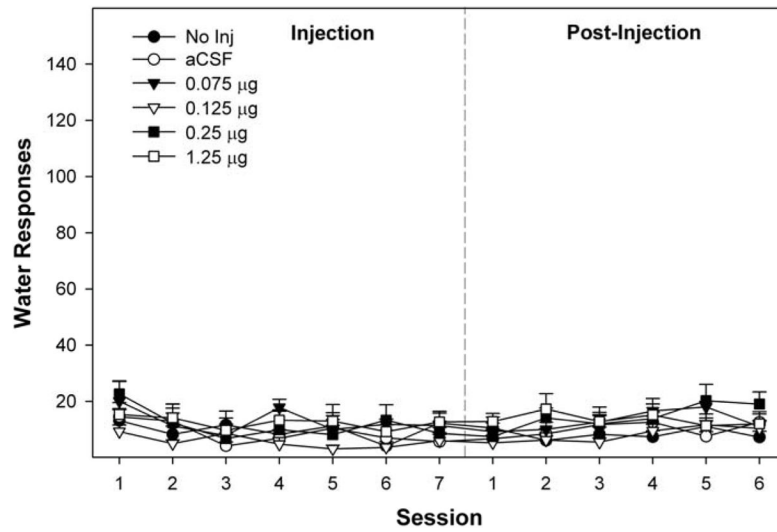
Fig. 2.
Number of EtOH responses (FR1) ± SEM following microinjections of ICS205-930 into the posterior VTA during acquisition of ethanol self-administration. Asterisks (*) indicate that rats bilaterally infused with 0.125, 0.25 or 1.25 μg/side of ICS responded significantly less than all other groups. Plus (+) symbol indicates that rats bilaterally infused with 0.125 or 1.25 μg/side of ICS responded significantly less than all other groups. Pound (#) symbols indicate that rats that received ICS during the injection period responded more for ethanol than no injection and aCSF controls.

Fig. 3.
Number of water responses (FR1) ± SEM following microinjections of ICS into the posterior VTA during acquisition of ethanol self-administration. ICS did not alter water responding.
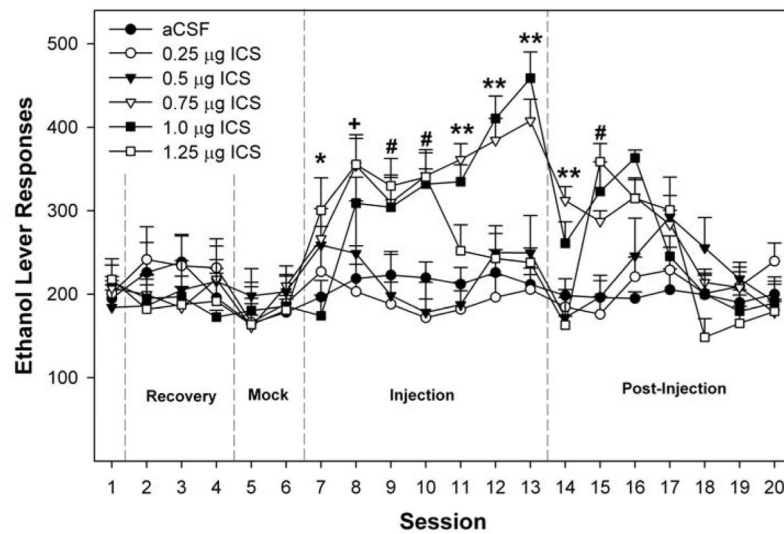
Fig. 4.

Number of ethanol responses (FR5) ± SEM following microinjections of ICS205-930 into the posterior VTA during maintenance of ethanol self-administration. Single asterisk indicates that rats bilaterally infused with 1.25 μg/side of ICS responded significantly more than the aCSF group. Plus symbols indicate that rats that received bilaterally microinjections of 1.0 and 1.25 μg/side of ICS responded more for ethanol than aCSF controls and 0.25, 0.5 μg/side of ICS groups. Pound symbols indicate that rats that received 0.75, 1.0 and 1.25 μg/side of ICS responded more for ethanol than all other groups. Double Asterisks indicate that rats that received 0.75 and 1.0 μg/side of ICS responded more for ethanol than all other groups.
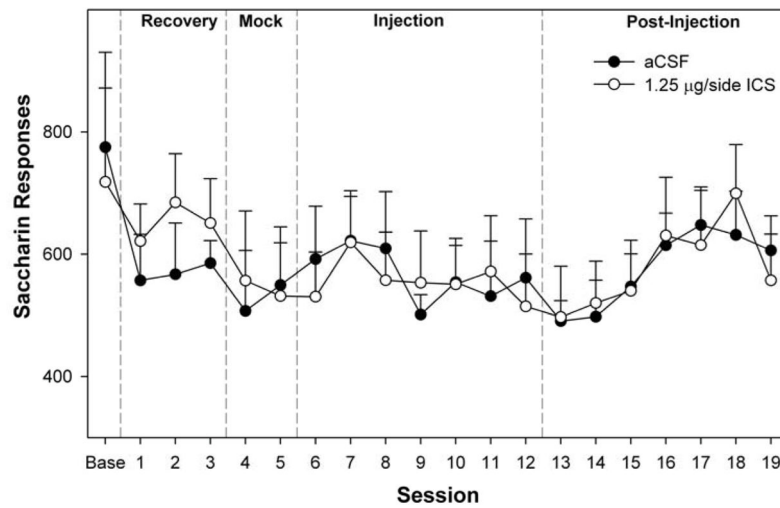
Fig. 5.
Number of saccharin responses (FR5) ± SEM following microinjections of ICS205-930 into the posterior VTA during maintenance of Saccharin self-administration. Microinjections of ICS into the posterior VTA did not alter saccharin self-administration.
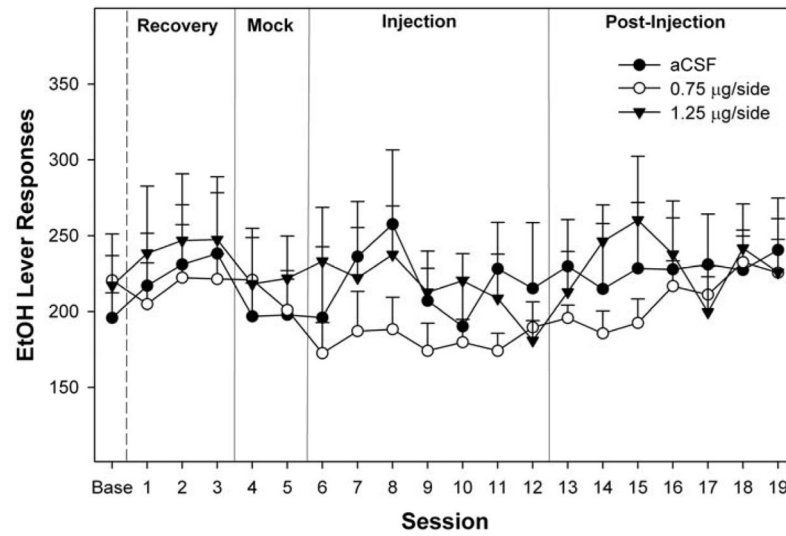
Fig. 6.
Number of ethanol responses (FR5) ± SEM following microinjections of ICS205-930 into the anterior VTA during maintenance of ethanol self-administration. Microinjections of ICS into the anterior VTA did not alter ethanol self-administration.