Published in final edited form as:

Psychopharmacology (Berl). 2006 July ; 187(1): 1–12. doi:10.1007/s00213-006-0389-0.

Reduction in repeated ethanol-withdrawal-induced anxiety-like behavior by site-selective injections of 5-HT_{1A} and 5-HT_{2C} ligands

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Abstract

Rationale—Anxiety-like behavior resulting from repeated withdrawals from chronic ethanol diets is counteracted by systemic administration of a 5-HT_{2C} receptor antagonist or a 5-HT_{1A} receptor partial agonist.

Objectives—This study investigated whether prior treatment with these agents into the amygdala, dorsal raphe nucleus, nucleus accumbens, or paraventricular nucleus during early withdrawals would ameliorate the social interaction deficits observed after a subsequent withdrawal.

Methods—Sprague–Dawley rats were exposed to three cycles of 5 days of forced ethanol diet (4.5%, w/v), with 2 days of control diet after the first and second cycles. Drugs were administered into one of four brain sites 4 h after removal of ethanol on the first and 2nd cycles but not the third.

The social interaction test was performed 5 h after removal of ethanol on the third cycle. Drugs tested included SB-243213, a 5-HT_{2C} receptor inverse agonist; buspirone, a 5-HT_{1A} receptor partial agonist; and Ro 60 1075, a 5-HT_{2C} receptor agonist.

Results—Only SB-243213 (at 3 µg, but not at 1 and 0.3 µg) counteracted the social interaction deficits after injections into the amygdala, while buspirone (at 0.3 and 1 µg but not at 0.1 µg) reduced deficits only when given into the dorsal raphe nucleus. In contrast, the 5-HT_{2C} receptor agonist, Ro 60 1075, accentuated the behavioral deficit after two weekly injections into the amygdala.

Conclusions—These results are consistent with the involvement of 5-HT_{2C} receptors in the amygdala and 5-HT_{1A} autoreceptors in the dorsal raphe nucleus in repeated ethanol withdrawal-induced sensitization of anxiety-like behavior.

Keywords

Anxiety-like behavior; Repeated ethanol exposures and withdrawals; Social interaction test; 5-HT_{1A}, and 5-HT_{2C} receptors; Amygdala; Dorsal raphe nucleus

Introduction

The involvement of serotonergic mechanisms in anxiety-like behavior is well-established, from the generation of anxietylike behavior in animals after the administration of drugs with a serotonergic mode of action (e.g., Bagdy et al. 2001; Bristow et al. 2000; Campbell and Merchant 2003; Gordon and Hen 2004; Yilmazer-Hanke et al. 2003) to the treatment of anxiety disorders with selective serotonin reuptake inhibitors and other agents (e.g., Gordon and Hen 2004; Lanfumey and Hamon 2004; Wood 2003). It is becoming increasingly clear, however, that the effects of serotonergic drugs may depend upon the brain site where the receptors are located. For example, 5-HT_{1A} receptor agonists may have anxiogenic effects after administration into the dorsal hippocampus or amygdala where postsynaptic receptors are located (Andrews et al. 1994; File et al. 1996; Gonzalez et al. 1996; Hogg et al. 1994), but anxiolytic effects after administration into the dorsal raphe nucleus where 5-HT_{1A} autoreceptors are located (Andrews et al. 1994; File et al. 1996; Lanfumey and Hamon 2004).

Another study reported anxiolytic-like effects of 5-HT_{1A} receptor agonists after injection into the ventral hippocampus, but not after injection into the nucleus accumbens (Stefanski et al. 1993). Additionally, there are 5-HT_{1A} receptors in the paraventricular nucleus of the hypothalamus that mediate the effects of 5-HT_{1A} agonists on the hypothalamic–pituitary–adrenal axis (Carrasco and Van de Kar 2003).

There have been few reports on the central effects of 5-HT_{2C} receptor ligands, due in part to the paucity of selective ligands. Thus, while there is evidence for 5-HT_{2C} receptors in the amygdala that mediate the anxiogenic effects of 5-HT_{2C} agonists (Campbell and Merchant 2003), there is essentially no information on the behavioral role of these receptors in other brain sites. It has been reported that 5-HT_{2C} receptors in the nucleus accumbens may mediate the release of dopamine (De Deurwaerdere et al. 2004; Eberle-Wang et al. 1997), but the behavioral consequences of such treatments have not been reported. Likewise, a recent anatomical study has reported that 5-HT_{2C} receptors are located on GABA interneurons in the dorsal raphe nucleus (Serrats et al. 2005), but there is no information about the behavioral implications of this observation. Finally, 5-HT_{2C} receptors in the paraventricular nucleus may mediate the serotonergic influence on stress responses (Bagdy 1996; Jorgensen et al. 2002), but behavioral effects have not been characterized.

There is increasing evidence of the involvement of serotonergic mechanisms in anxiety associated with ethanol and diazepam withdrawal (Andrews and File 1993; File et al. 1993;

Overstreet et al. 2003). Moreover, several studies have reported changes in serotonin (5-HT) receptor subtypes in rats after chronic treatment with ethanol (e.g., Overstreet et al. 1995; Pandey and Pandey 1996; Pandey et al. 1992; Ulrichsen 1991). Recently, acute systemic treatment with both buspirone and a selective 5-HT_{2C} receptor antagonist blocked the reduction in social interaction after withdrawal from ethanol (Knapp et al. 2004; Overstreet et al. 2003).

Based on the assumption that progressive adaptive changes in the brain underlie the anxiety-like behavior in ethanol-withdrawn rats, we examined whether treatment with selective serotonergic agents during early withdrawals would counteract the anxiety seen after the final withdrawal. Specifically, we determined that a 5-HT_{1A} receptor partial agonist and a 5-HT_{2C} receptor antagonist were also effective in counteracting the reduced social interaction when given during the withdrawal periods after the first and second cycles of a three-cycle multiple withdrawal protocol (Overstreet et al. 2003).

Although both compounds effectively counteracted the adaptation associated with repeated ethanol withdrawals, the brain sites containing the 5-HT_{1A} and 5-HT_{2C} receptors mediating these effects have not been identified. Therefore, we predicted that buspirone might counteract anxiety-like behavior after injection into the dorsal raphe nucleus, where there is a high concentration of 5-HT_{1A} autoreceptors (Lanfume and Hamon 2004), while the 5-HT_{2C} receptor inverse agonist might be effective after injection into the amygdala, where there are 5-HT_{2C} receptors mediating anxiety responses (Campbell and Merchant 2003). For the present investigation, rats multiply exposed to and withdrawn from ethanol were injected into the amygdala, dorsal raphe nucleus, nucleus accumbens, or the hypothalamic paraventricular nucleus to test this prediction.

Materials and methods

Animals

Sprague–Dawley rats (Charles River, Raleigh, NC, USA) were obtained at 160–180 g and were maintained at 22°C and 40% humidity with a 12:12 light:dark cycle (lights on from 0700–1900 hours). After adaptation to the laboratory for 5 days, surgeries were performed (see next section). After a 7-day recovery they were placed on a nutritionally complete lactalbumin-dextrose diet for 3 days (Frye et al. 1983; Knapp et al. 1998; Moy et al. 1997; Overstreet et al. 2003). The experimental rats were exposed to a diet containing ethanol (ED;4.5%, w/v), while control rats remained on the control diet (CD) in which additional dextrose replaced the ethanol isocalorically. The CD rats were pair-fed based on the average volume of ED consumed. Rats undergoing this protocol achieved blood ethanol concentrations of about 100 mg% during the first two cycles and up to 200 mg% during the third cycle (Overstreet et al. 2002).

Surgery and histology

Surgery was performed under pentobarbital (50 mg/kg, i.p.) anesthesia. Methyl atropine (2 mg/kg, i.p.) was injected to reduce respiratory secretions. Once a sufficient depth of anesthesia was achieved, the rat was placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA). Holes were drilled in the skull at the appropriate locations and cannulae were inserted so that the tip was approximately 3 mm above the site of interest (see below). Jeweler's screws were implanted into the skull and dental acrylic was applied to attach the cannulae to the skull. Once recovered from anesthesia, the rats were given acetaminophen (children's Q-Pap, cherry flavor, 6 mg/ml) in the drinking water for 48 h. Liquid diets were initiated 1 week after recovery from surgery.

Amygdala injections were bilateral at the following coordinates relative to Bregma: -2.8 AP, ± 4.5 ML, -6.5 DV. Insertion angle was zero. Cannula length was 14.5 mm (6.5 mm inside, 8 mm outside skull). Needle length was 16.0 mm. All injections were 1 μ l per side. Because the dorsal raphe is a midline structure, bilateral injections were not needed. All injections were angled to this midline location from the drill hole on the right side of the skull. Coordinates relative to *Lambda* were +1.2 mm AP, -2.0 mm ML and -3.5 mm DV. Insertion angle was 17° . Cannula length was 11.5 mm (3.5 mm inside skull, 8 mm outside skull). To limit brain damage near the raphe from the relatively larger guide cannula, injection needle length was 14.5 mm, and, therefore, projected 3.0 mm past the cannula tip into the raphe during injections. All injections were 0.5 μ l.

Injections into the nucleus accumbens were bilateral at the following coordinates relative to Bregma: +1.00 AP, ± 2.0 ML, -3.0 DV. Insertion angle was zero. Cannula length was 11.5 mm. Needle length was 16.0. All injections were 1 μ l. Injections into the paraventricular nucleus were unilateral and all from the right side. Coordinates relative to Bregma were -1.88 AP, -3.0 ML, -3.5 DV. Insertion angle was 22.3° . Cannula length was 11.5 mm. Needle length was 16.0 mm. All injections were 0.5 μ l.

All cannulae were made from 26-gauge stainless steel tubing. All injection needles were made from 32-gauge stainless steel tubing. All injections were made over a time period of 1 min with an additional 30 s before removal of the needle from the site. Before and after injections, cannulae were kept closed with stainless steel plugs (32-gauge wire) that were inserted into the cannula to a point 0.5 mm beyond the cannula tip. To prevent damage to the targeted brain regions, the cannulae were placed from 1.5 to 4.5 mm above the injection site, depending on the target, and the smaller gauge injection needles projected past the cannulae tips to the areas of interest.

Within 48 h of the completion of the study, rats were anesthetized with pentobarbital for determination of the locations of the cannulae; 0.5 μ l of dye was injected into the cannulae, using the same injectors used to deliver the drugs, and then animals were killed. The brain was removed and the location of the dye was recorded. Only rats with correct cannulae locations were used in the statistical analyses.

Drugs

Buspirone hydrochloride (RBI-Sigma, St. Louis, MO, USA) was the 5-HT_{1A} partial agonist used in this study. The primary injection concentration of 1 μ g/ μ l was selected from previously published reports (File et al. 1993, 1996; Overstreet et al. 2003); concentrations of 0.3 and 0.1 μ g/ μ l were also used in one experiment. SB-243213 (gift of Glaxo-Smith-Kline, Harrow, UK), the 5-HT_{2C} receptor inverse agonist (Wood et al. 2001), was given at an injection concentration of 3 μ g/ μ l; this dose was based on preliminary studies; concentrations of 1 and 0.3 μ g/ μ l were also used in one experiment. This compound was used because it has functionally similar effects to SB-242084 (Overstreet et al. 2005; Wood et al. 2001). The 5-HT_{2C} receptor agonist Ro 60 1075 (gift of Roche, Basel, Switzerland) is highly selective for the 5-HT_{2C} receptor (Kennett et al. 2000; Overstreet et al. 2003) and was given at an injection concentration of 0.3 μ g/ μ l. All drugs were dissolved or suspended in artificial CSF.

Protocol

The study consisted of seven experiments that tested the efficacy of drugs that interact with 5-HT receptor subtypes, as described above, and an eighth experiment on whether the 5-HT_{2C} agonist would potentiate withdrawal-induced deficits in social interaction. The first, third, fifth, and sixth experiments consisted of 16 rats maintained on control diet (CD) and up to 24 cannulated rats maintained on ethanol diet (ED) for a total of 15 days. Those rats on ED were

withdrawn from ethanol and placed on CD for 2 days after the fifth and tenth days of exposure to ethanol. Rats maintained on ED were injected with the drugs of interest (buspirone at 1 µg/µl and SB-243213 at 3 µg/µl) into relevant brain sites (amygdala, dorsal raphe nucleus, nucleus accumbens, and paraventricular nucleus of the hypothalamus) at 4 h after removal of the fifth and tenth ethanol exposures. At approximately 5 h after the last (15th) ethanol exposure, rats (matched for weight) were placed as pairs in the social interaction test for a 5-min session.

The second and fourth experiments examined the dose-dependent effects of SB-243213 and buspirone after injection into the amygdala and dorsal raphe nucleus, respectively. Rats were cannulated and treated as described above. At 4 h into withdrawal after the first and second cycles of ethanol exposure, rats received either bilateral intra-amygdala injections of SB-243213 (0.3, 1.0, or 3.0 µg/µl) or unilateral intra-raphé injections of buspirone (0.1, 0.3, or 1.0 µg/µl). For both drugs artificial cerebrospinal fluid served as the vehicle. The social interaction test was conducted 5 h after the ethanol in the third cycle was removed.

The seventh experiment was a control experiment designed to determine if buspirone and SB-243213 would alter subsequent behavior in rats maintained on control liquid diet if injected into the dorsal raphe nucleus and amygdala, respectively. After recovery from surgery rats were maintained on CD throughout. After 5 and 10 days, the rats were injected into the amygdala (SB-243213 at 3 µg/µl) or dorsal raphe nucleus (buspirone at 1 µg/µl). After a further 5 days' exposure to CD the rats were placed in the arena for the recording of social interaction behavior.

The design was altered for the 5-HT_{2C} receptor agonist. Three groups ($n=8$) were maintained on control diet (CD) for 13 days. Two groups received surgery beforehand to implant cannulae into the amygdala and the third group was a non-cannulated control. One group was given 0.3 µg Ro 60 1075 and the other vehicle bilaterally into the amygdala on the 8th and 13th days (1 and 6 days before exposure to ethanol) of exposure to the diet. Immediately after the 13th day on the diet, they were exposed to 4.5% ethanol for 5 days, a treatment that does not normally produce a deficit in social interaction behavior (Overstreet et al. 2002). After the 18th day of exposure to the diet (13 days CD; 5 days ED), ethanol was removed and the social interaction test was carried out 5 h later. It was previously shown that systemic treatment with Ro 60 1075 to rats maintained on CD did not affect social interaction behavior (Overstreet et al. 2003).

Social interaction test

The social interaction test was used in these studies because it can detect the anxiolytic and anxiogenic effects of serotonergic agents (e.g., Bagdy et al. 2001; Bristow et al. 2000; Gonzalez et al. 1996; Overstreet et al. 2003). A modification of the standard social interaction test was used to conserve animals. In the present studies, treated cannulated rats were paired with non-cannulated rats maintained on CD and the social interactions initiated by each member of the pair were recorded, thereby requiring fewer rats (e.g., Irvine et al. 2001; Overstreet et al. 2002). In a study of 25 pairs of rats maintained on CD and 25 on ED, the rats exhibited essentially independent behavior, as there was no significant correlation between the pairs in either group (Overstreet et al. 2002). Furthermore, Overstreet et al. (2003) showed that analyses of social interaction behavior provide identical conclusions regardless of whether an individual rat or a pair of rats is used as the unit of analysis. Some rats maintained on CD were paired with more than one cannulated rat, but only their scores on the first interaction were used in the analysis. Unpublished findings indicated that a naive rat's social interaction behavior is unaffected by whether its partner is familiar or unfamiliar with the test arena.

Experienced observers who were blind to the experimental condition carried out the social interaction test in a square open field (60×60 cm, with 16 squares marked out on the floor). The rats were unfamiliar with the open field, and the lighting conditions were low to generate

an intermediate level of social interaction behavior. Rat pairs were matched on the body weights and placed simultaneously in the open field. During the 5-min session, line crosses (by two forepaws) and time spent in social interaction (grooming, sniffing, following, and crawling over or under) were scored individually for each rat (Kampov-Polevoy et al. 2000; Overstreet et al. 2002, 2003).

Data analysis

Statistical analyses were carried out using the GBStat software package. The data were initially analyzed by oneway ANOVAs. If the main effects were statistically significant, post hoc analyses were performed using Tukey's protected *t* tests.

Results

5-HT_{2C} receptor inverse agonist SB-243213 counteracts multiple withdrawal-induced anxiety after amygdala injection, but 5-HT_{1A} partial agonist buspirone does not

The effects of SB-243213, a 5-HT_{2C} receptor inverse agonist, and buspirone, a 5-HT_{1A} receptor partial agonist, on social-interaction behavior after injection into the amygdala are illustrated in Fig. 1. After amygdaloid treatment during the first two withdrawals, but not the third, SB-243213 completely counteracted this anxiety-like behavior (Fig. 1, upper panel). However, buspirone had no comparable effect in this brain region (Fig. 1). One-way ANOVA confirmed the large differences among groups ($F [3,31]=12.17, p < 0.0001$) and post hoc tests established that the group treated with SB-243213 was significantly different from the ED group treated with vehicle, but not from the CD group.

It appeared that multiple exposures to withdrawal from ethanol also reduced line crosses (Fig. 1, lower panel). However, the one-way ANOVA did not reveal a significant group difference ($F [3,31]=2.61, p = 0.07$). Determination of cannulae locations revealed that between 80 and 90% of the placements into the amygdala were correct. Examples of the locations of several representative correct sites are illustrated in Fig. 2, along with a photomicrograph from one animal.

As illustrated in Fig. 3, intra-amygdala injection of SB-243213 dose-dependently counteracted the deficit in social interaction seen in rats multiply withdrawn from ethanol. ANOVA confirmed the significant group differences ($F [4,33]=13.35, p < 0.0001$) and post hoc tests revealed that only the group treated with 3.0 μ g of SB-243213 was significantly different from the group given vehicle. There were no significant differences ($F [4,33]=0.36, p > 0.1$) in the line crosses of the various groups (data not shown). Cannulae locations were accurate in about 90% of the animals and every rat had at least one correct placement. Rats with unilaterally correct placements did not differ from the other members of the group, so all data were included in the analysis.

5-HT_{1A} partial agonist buspirone counteracts multiple withdrawal-induced anxiety after dorsal raphe injection but 5-HT_{2C} receptor inverse agonist SB-243213 does not

The pattern of effects after microinjection of drugs into the dorsal raphe nucleus was opposite of that observed for the amygdala: microinjection of buspirone into the raphe counteracted the deficit in social interaction induced by repeated ethanol withdrawals, but SB-243213 was ineffective (Fig. 4, upper panel). The significant group differences were confirmed by ANOVA ($F [3,39]=17.72, p < 0.0001$) and post hoc tests confirmed that the buspirone-treated group was significantly different from the vehicle-treated ED group but not the CD group.

ANOVA ($F [3,39]=1.91, NS$) indicated no significant differences in the locomotor activity among the groups (Fig. 4, lower panel). About 70–80% of the cannulations into the dorsal

raphe nucleus were correct. Figure 5 illustrates the results for several of the positive placements as well as a photomicrograph of one animal. Only data from these positive placements were used in the statistical analyses.

In the dose–response study, buspirone also significantly counteracted the deficit in social interaction after injection into the dorsal raphe nucleus, as illustrated in Fig. 6. ANOVA confirmed the significant group differences ($F [4,27]=8.72, p <0.0001$) and post hoc tests indicated that both 0.3 and 1.0 μg of buspirone significantly increased social interaction above that exhibited by the vehicle-treated rats. There were no significant effects ($F [4,27]=0.33, \text{NS}$) on line crosses (data not shown). Five animals had cannula placements too far anterior, and their data were not included in the statistical analyses.

Neither buspirone nor SB-243213 counteract multiple withdrawal-induced anxiety after injection into the nucleus accumbens

Unlike the case with the amygdala and the dorsal raphe nucleus, neither buspirone nor SB-243213 was effective in counteracting anxiety after microinjection into the nucleus accumbens (Fig. 7, upper panel). Although the ANOVA revealed a significant group difference ($F [3,44]=10.95, p <0.0001$), Post hoc tests indicated that all of the groups exposed to ED were significantly different from the CD group. Thus, intra-accumbal injection of buspirone or SB-243213 did not counteract ethanol-withdrawal-induced anxiety. Once again, there were no significant differences in locomotor activity (Fig. 7, lower panel), as revealed by the line cross data analysis ($F [3,44]=2.12, \text{NS}$). More than 80% of the cannulation placements into the nucleus accumbens were correct. Because neither of the drugs affected ethanol-withdrawal-induced anxietylike behavior, cannulae placements are not provided.

Neither buspirone nor SB-243213 counteract multiple withdrawal-induced anxiety after injection into the paraventricular nucleus

Injections of buspirone or SB-243213 into the paraventricular nucleus of the hypothalamus were also ineffective in counteracting ethanol-withdrawal-induced anxiety-like behavior (Fig. 8, upper panel). Although there were significant group differences revealed in the ANOVA ($F [3,32]=4.92, p <0.01$), all of the groups exposed to ethanol were significantly different from the group exposed to CD. The pattern for locomotor activity was very similar to that for social interaction (Fig. 8, lower panel). There were significant group differences ($F [3,31]=4.89, p <0.01$), with all groups exposed to ED being significantly less active than the group exposed to CD. Approximately 85% of the cannulations into the paraventricular nucleus were correct. Data on cannula location are not shown because neither of the drugs affected ethanol-withdrawal-induced anxiety-like behavior.

Microinjections of neither SB-243213 nor buspirone alone altered social interaction behavior in control rats

Neither buspirone microinjected into the dorsal raphe nucleus nor SB-243213 microinjected into the amygdala influenced social interaction behavior in rats maintained on control diet (Fig. 9). ANOVA revealed no significant group differences. Thus, the mere injection of SB-243213 into the amygdala or buspirone into the dorsal raphe nucleus does not alter subsequent social interaction behavior. These drug treatments were also without effect on locomotor activity (data not shown).

A 5-HT_{2C} agonist sensitizes withdrawal-induced anxiety after amygdala injection

When the 5-HT_{2C} agonist Ro 60 1075 was microinjected twice into the amygdala of rats on control liquid diet, withdrawal from a single 5-day exposure to ethanol resulted in anxiety-like behavior, as illustrated in Fig. 10. Thus, activation of the 5-HT_{2C} receptors in the amygdala

can substitute for two initial ethanol withdrawals and induce significant reductions in social interaction (Fig. 10).

Rats exposed to 4.5% ethanol diet for 5 days did not exhibit a reduced social interaction, a finding that confirms a previous report (Overstreet et al. 2002). The difference among the groups was highly significant ($F [2,21]=30.21, p < 0.0001$) and Tukey's tests confirmed that the group treated with Ro 60 1075 was significantly different from the other two groups. There were no significant differences in line crosses (data not shown).

Body weight and ethanol intake

There were no significant differences in the body weights and ethanol intakes in most of the different treatment groups that were maintained on CD or ED, as summarized in Table 1. Therefore, any differential behavioral effects of the treatments cannot be explained by differences in these measures. Also included in this table is the number of animals in each group after those with cannulae in the wrong areas were eliminated.

Discussion

The present findings support a role for serotonin in ethanol-withdrawal-sensitized anxiety-like behavior. Buspirone, the 5-HT_{1A} partial agonist, counteracted this anxiety after pretreatment into the raphe nucleus but not into the amygdala, whereas, SB-243213, the 5-HT_{2C} receptor inverse agonist, was effective only after pretreatment into the amygdala. However, neither drug was effective when microinjected into the nucleus accumbens or the paraventricular nucleus. These data clearly point to the selective involvement of specific brain regions in the adaptive change induced by multiple withdrawals. Although these positive and negative results were predictable on the basis of acute anxiogenic and or anxiolytic effects of serotonergic drugs (e.g., Andrews et al. 1994; Campbell and Merchant 2003; File et al. 1996; Hogg et al. 1994; Rioja et al. 2004), it is important to emphasize that the present study utilized a pre-treatment strategy, where the drugs were given during each of the first two withdrawals and the behavior was assessed during the final withdrawal 1 week later. Consequently, it is likely that the drug treatments were disrupting adaptive changes within specific brain regions that occur during chronic repeated ethanol exposures and withdrawals that sensitize anxiety-like behavior during the final withdrawal (Breese et al. 2004; Overstreet et al. 2002).

The counteracting effects of the 5-HT_{2C} receptor inverse agonist but not 5-HT_{1A} receptor partial agonist after pretreatment into the amygdala are consistent with previous reports of others, as suggested above. The 5-HT_{2C} receptor inverse agonist SB-243213 is functionally similar to 5-HT_{2C} receptor antagonists in all behavioral tests (Kennett et al. 1997; Wood et al. 2001), including ethanol-withdrawal-induced anxiety-like behavior (Overstreet et al. 2005). In the present study, the 5-HT_{2C} receptor inverse agonist counteracted the anxiety-like behavior when microinjected into the amygdala during the first and second withdrawals (at a dose of 3 µg).

Furthermore, pretreatment with the 5-HT_{2C} receptor agonist Ro 60 1017 into the amygdala potentiated the anxiety-like behavior in a manner similar to results with systemic administration of the agonist (Overstreet et al. 2003). This finding is consistent with the report of Campbell and Merchant (2003). On the other hand, acute administration of 5-HT_{1A} receptor agonists into the amygdala did not have an anxiolytic effect. Given that an anxiogenic effect can occur upon 5-HT_{1A} receptor agonist administration into the amygdala, (Gonzalez et al. 1996), buspirone would not be expected to counteract ethanol-withdrawal-induced anxiety after pretreatment into this site, a view consistent with the present findings. Because of this reported anxiogenic effect of direct 5-HT_{1A} receptor agonist administration into the amygdala, higher doses of buspirone were not tested.

The beneficial effect of prior treatment with buspirone during repeated withdrawals may be due to its interaction with 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, an action which may counteract the exaggerated release of 5-HT that accompanies ethanol withdrawal (File et al. 1993). This finding is consistent with other reports indicating that 5-HT_{1A} receptor agonists have anxiolytic-like effects after administration directly into the both the dorsal and medial raphe nuclei (Andrews et al. 1994; File et al. 1996; Gonzalez et al. 1996). In contrast to the positive anxiolytic effects of buspirone given into the raphe, prophylactic microinjection of the 5-HT_{2C} receptor inverse agonist SB-243213 into the dorsal raphe nucleus during repeated withdrawals did not alter social interaction behavior in ethanol-withdrawn rats.

This outcome suggests that 5-HT_{2C} receptors in the dorsal raphe nucleus are not involved in the anxiety-like behavior observed in ethanol-withdrawn rats. A recent anatomical study has provided evidence for 5-HT_{2C} receptors on GABA interneurons in the dorsal raphe nucleus (Serrats et al. 2005). This finding provides the basis for the inhibitory regulation of 5-HT release by 5-HT_{2C} receptors; however, a 5-HT_{2C} receptor inverse agonist microinjected into the dorsal raphe nucleus would tend to increase, not reduce, 5-HT release and, therefore, would not be expected to counteract ethanol-withdrawal-induced anxiety-like behavior, as was found here. Nevertheless, it must be emphasized that only a single concentration of the 5-HT_{2C} receptor inverse agonist was used in this brain site.

The fact that buspirone administered into the raphe nucleus and SB-243213 administered into the amygdala in rats exposed to control diet did not alter social interaction behavior is consistent with the argument that these drugs are modifying an adaptive change that occurs during the multiple ethanol withdrawals. However, to reinforce this hypothesis, an additional experiment is required. The lighting conditions should be altered to engender anxietylike behavior in the control animals (File and Seth 2003), and then it should be determined whether prior brain site injections of the compounds alter the subsequent social interaction behavior. As rats exposed to CD would not experience an adaptive change, buspirone and SB-243213 should not prevent the light-induced anxiety-like behavior.

The failure of the 5-HT_{2C} receptor inverse agonist and the 5-HT_{1A} receptor partial agonist to counteract the anxiety-like behavior after prior microinjections into the nucleus accumbens is consistent with the limited literature available. An earlier report indicated that the direct injection of the 5-HT_{1A} receptor agonist 8-OH-DPAT into the nucleus accumbens did not modify anxiety-like behavior (Stefanski et al. 1993). Although 5-HT_{2C} receptors are clearly present in the nucleus accumbens (Eberle-Wang et al. 1997), and they regulate dopamine release (e.g., De Deurwaerdere et al. 2004), no studies on the anxiogenic or anxiolytic effects of intra-accumbal 5-HT_{2C} receptor ligands could be identified. The results of our study suggest that 5-HT_{2C} receptors in the nucleus accumbens do not modulate anxiety-like behavior. Even though only single concentrations of buspirone and SB-243213 were used, these doses microinjected into the dorsal raphe nucleus and amygdala, respectively, blocked the anxiety-like response associated with alcohol withdrawal.

It is well-known that multiple (5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C}) receptors in the hypothalamic paraventricular nucleus modulate the HPA axis (Bagdy 1996; Carrasco and Van de Kar 2003; Jorgensen et al. 2002, 2003), but there is little information on the effects of local application of 5-HT_{1A} or 5-HT_{2C} receptor agonists to this site on behavior. Thus, we cannot judge whether the failure of both drugs to counteract the anxiety-like behavior after prior microinjection into the paraventricular nucleus is supported by the literature. These negative effects could have arisen because the concentrations used were below the threshold for this brain region. Nevertheless, the data indicate that the doses of these drugs that modulate 5-HT_{2C} receptors in the amygdala and 5-HT_{1A} receptors in the dorsal raphe nucleus blocked ethanol-withdrawal-induced anxiety-like behavior.

Despite the anxiolytic-like effects of intra-raphe buspirone or intra-amygdala SB-243213, neither of these agents altered line crosses. This outcome confirms contentions put forth by others that line crosses and social interaction are independent behavioral measures in this task (File and Seth 2003; Overstreet et al. 2002). In contrast, evidence from this report and others indicate that 5-HT_{1A} and 5-HT_{2C} receptors as well as GABAergic mechanisms are involved not only in the acute anxiety-like behavior but also in the sensitized anxiety responses seen after multiple withdrawals (File et al. 1989; Moy et al. 1997, 2000; Knapp et al. 2004, 2005; Overstreet et al. 2002, 2003).

In summary, our findings demonstrate that a 5-HT_{1A} partial agonist and a 5-HT_{2C} receptor inverse agonist can ameliorate deficits in social interaction observed after the third withdrawal from ethanol when given during the first and second withdrawals of a multiple withdrawal regimen by interacting with receptors in the dorsal raphe nucleus and amygdala, respectively.

Acknowledgments

We wish to thank Qi Yu, Mili Senapati and Lara Marr for technical assistance and Martyn Wood of GlaxoSmithKline for supporting this project. This work was financially supported by NIAAA grants AA 14284, AA 11605, and AA 14949.

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Fig. 1.

Effects of pretreatment with Buspirone or SB-243213 into the amygdala on subsequent anxiety-like behavior in ethanol-withdrawn rats; see “Materials and methods.” *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. Abbreviations: CD Control diet, ED-V ethanol diet given vehicle, ED-BU ethanol diet given buspirone, ED-SB ethanol diet given SB-243213

Fig. 2. Cannulae location for implants aimed at the amygdala. *Top panel:* schematic showing representative positive locations. *Bottom panel:* digital photomicrograph illustrating target area and needle tracks

Fig. 3.

Dose-related effects of intra-amygdalar SB-243213 in rats multiply withdrawn from ethanol; see "Materials and methods." Data represent the mean $\pm$ S.E.M. of time spent in social interaction. Abbreviations: *CD* Control diet, *ED-V* ethanol diet given vehicle, *ED-SB* ethanol-diet-given SB-243213 at 0.3, 1.0, and 3.0 μ g

*Significantly different from CD
+Significantly different from ED-V

Fig. 4.

Effects of pretreatment with buspirone or SB-243213 into the raphe nucleus on subsequent anxiety-like behavior in ethanol-withdrawn rats; see Materials and methods. *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. See Fig. 1 for abbreviations

Fig. 5. Cannulae locations for implants aimed at the dorsal raphe nucleus. *Top panel:* schematic showing representative positive locations. *Bottom panel:* digital photomicrograph illustrating target area and needle tracks

Fig. 6.

Dose-related effects of intra-raphé buspirone in rats multiply withdrawn from ethanol; see Materials and methods. *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. Abbreviations: *CD* Control diet, *ED-V* ethanol-diet-given vehicle, *ED-BU* ethanol-diet-given buspirone

Fig. 7.

Effects of pretreatment with buspirone or SB-243213 into the nucleus accumbens on subsequent anxiety-like behavior in ethanol-withdrawn rats; see Materials and methods. *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. See Fig. 1 for abbreviations

Fig. 8.

Effects of pretreatment with buspirone or SB-243213 into the paraventricular nucleus on subsequent anxiety-like behavior in ethanol-withdrawn rats; see Materials and methods. *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. See Fig. 1 for abbreviations

Fig. 9.

Effects of pretreatment with buspirone into the raphe nucleus or SB-243213 into the amygdala on subsequent anxiety-like behavior in rats maintained on control diet; see Materials and methods. *Upper panel:* data represent the mean $\pm$ S.E.M. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S. E.M. line crosses. Abbreviations: V-AMY ($N=8$) injected with vehicle into the amygdala; SB-AMY ($N=8$) injected with SB-243213 into the amygdala; V-RN ($N=8$) injected with vehicle into the dorsal raphe nucleus; BU-RN ($N=8$) injected with buspirone into the dorsal raphe nucleus

Fig. 10.

Effects of pretreatment with Ro 60 1075 into the amygdala on subsequent anxiety-like behavior in ethanol-withdrawn rats; see Materials and methods. *Upper panel:* data represent the mean $\pm$ s.e.m. of time spent in social interaction; *lower panel:* data represent the mean $\pm$ S.E.M. line crosses. Abbreviations: *CD* ($N=8$) Control diet, *ED-V* ($N=8$) ethanol-diet-given vehicle, *ED-RO* ($N=8$) ethanol-diet-given Ro 60 1075

Terminal body weights and ethanol intake of rats subjected to repeated withdrawals and treated with drugs interacting with 5-HT receptor subtypes

Table 1

Measure	Treatment groups ¹			
	CD	ED-VEH	ED-BUS	ED-SB
<i>Amygdala</i>				
Final weight	333±2.8 (10)	353±7.8 (9)	344±12.2 (9)	355±10.2 (7)
Ethanol intake	NA	8.8±0.5 (9)	8.4±0.2 (9)	9.1±0.4 (7)
<i>Dorsal raphe nucleus</i>				
Final weight	330±4.8 (16)	359±9.7 (11)	346±9.2 (10)	340±15 (6)
Ethanol intake	NA	9.0±0.6 (11)	8.6±0.2 (10)	7.3±0.6 (6)
<i>Nucleus accumbens</i>				
Final weight	331±4.3 (16)	346±8.0 (13)	331±9.5 (13)	339±8.2 (6)
Ethanol intake	NA	8.9±0.6 (13)	8.7±0.5 (13)	8.6±0.3 (6)
<i>Paraventricular nucleus</i>				
Final weight	334±6.0 (14)	328±6.6 (7)	332±12.1 (7)	338±3.2 (7)
Ethanol intake	NA	8.4±0.4 (7)	8.1±0.5 (7)	8.3±0.3 (7)
<i>SB-243213 dose response</i>				
Final weight	273±2 (7)	328±7 (6)	ED-0.3	ED-1.0 ED-3.0
Ethanol intake	NA 9.9±0.6	10.5±8.7	320±8 (8)	327±5 (7) 323±9 (6)
<i>Buspirone dose-response</i>				
Final weight	368±4 (7)	340±12 (5)	ED-0.1	ED-0.3 ED-1.0
Ethanol intake	NA	9.2±0.3	349±14 (6)	351±14 (5) 335±8 (6)
			8.8±0.6	8.8±0.2 8.7±0.3

¹ Values represent the means for the last 3 days of access to the ethanol diet. Weights are in grams; alcohol intakes are in gram/kilogram. There were no differences in alcohol intake at other time points. The numbers in parentheses are sample sizes CD Control diet, ED ethanol diet, NA not applicable, VEH vehicle, SB SB-243213, ED-0.3 ethanol diet given 0.3 µg of SB-243213 or buspirone, ED-1.0 ethanol diet given 1.0 µg of SB-243213 or buspirone; ED-3.0 ethanol diet given 3.0 µg of SB-243213, ED-0.1 ethanol diet given 0.1 µg of buspirone