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Effects of 5–HT_3 receptor antagonists on behavioural measures of naloxone-precipitated opioid withdrawal*

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Abstract. The effect of the selective 5–HT_3 receptor antagonists, ondansetron and MDL 72222, against various behaviours elicited by naloxone-precipitated morphine withdrawal were examined. Rats made dependent upon morphine by the subcutaneous implantation of a 75 mg pellet, when challenged with naloxone (0.5 mg/kg SC), 3 or 4 days later exhibited a wide range of behaviours including wet dog shakes, paw shakes, salivation and a marked weight loss. Pre-treatment with ondansetron (0.01–1 mg/kg SC) or MDL 72222 (1–3 mg/kg SC) failed to affect the incidence of these responses except weight loss, which was attenuated by both treatments. At doses similar to and below those required to elicit the withdrawal syndrome, naloxone produced a single-trial place aversion in morphine dependent rats. The place aversion produced by naloxone (0.05 mg/kg SC) was antagonized by pre-treatment of ondansetron (0.1–1 mg/kg SC) and MDL 72222 (1 mg/kg SC) prior to conditioning. Chlordiazepoxide (10 mg/kg IP) but not gepirone (3–10 mg/kg SC) was similarly effective. It is concluded that 5–HT_3 antagonists may attenuate some but not all behavioural signs associated with morphine withdrawal. Reasons for this apparent selectivity are discussed.

Key words: Opioid withdrawal – 5–HT_3 receptor – Ondansetron – MDL 72222 – Rat – Place aversion – Withdrawal syndrome

5–HT_3 receptors have attracted considerable interest over the last few years, due primarily to the fact that selective antagonists for this site, e.g. ondansetron, ICS 205–930 and MDL 72222, may have anxiolytic, antipsychotic and antiemetic potential in man (see Miner and Sanger 1986; Costall et al. 1987; Liebundgut and Lancranjan 1987; Jones et al. 1988; Lecrubier 1991). Consistent with these observations, both radioligand binding and autoradiographic techniques have revealed the distribution of 5–HT_3 receptors within mammalian brain, with highest levels found in the area postrema/nucleus tractus solitarii region (Kilpatrick et al. 1989; Barnes et al. 1990b; Pratt et al. 1990), and in the rat at least, a significant proportion of binding sites associated with the dopaminergic mesolimbic pathway; notably the basolateral/cortical nucleus of the amygdala and the nucleus accumbens (Barnes et al. 1990a; Waeber et al. 1990). Indeed a functional role for some of these sites has been implied by microinjection studies showing that amygdaloid and area postrema/nucleus tractus solitarii located 5–HT_3 receptors, may be involved in the putative anxiolytic and antiemetic effects, respectively, of 5–HT_3 receptor antagonists (Costall et al. 1989b; Higgins et al. 1989a, b, 1991).

Recently, a role for 5–HT_3 receptor antagonists in alleviating the symptoms of withdrawal from various substances of abuse has become apparent. For instance, Costall et al. (1989a, 1990a) have reported that ondansetron may ameliorate the behavioural inhibition seen in rodents following abrupt withdrawal from chronic benzodiazepine, cocaine, nicotine and ethanol treatment. Similarly, Oakley et al. (1988) reported a reduction of anxiety related withdrawal behaviours in alcohol dependent marmosets following ondansetron treatment. Furthermore, this agent may also attenuate the weight loss associated with benzodiazepine withdrawal (Costall et al. 1989a; Goudie and Leathley 1990).

Opioid physical dependence has been widely studied by naloxone-precipitated withdrawal in morphine-pellet implanted rats. Both the classical abstinence syndrome, e.g. wet-dog shakes, teeth chattering, jumping, diarrhoea (Blasig et al. 1973; Cicero and Meyer 1973; Wei et al. 1973) and the negative motivational consequences of withdrawal, as measured by place or taste conditioning (Manning and Jackson 1977; Mucha et al. 1986; Mucha 1987; Hand et al. 1988; Stinus et al. 1990) have been studied using this method. As yet, the effect of 5–HT_3

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antagonists on these aspects of morphine dependence has not been investigated, thus in the present study we have examined ondansetron and MDL 72222 against both the somatic signs and the place conditioning induced by naloxone-precipitated morphine withdrawal.

Materials and methods

Animals. Male, Wistar rats (Charles River, Quebec, Canada) weighing 250–300 g at the time of study were used throughout. Rats were housed in groups of four, in a room maintained at 22 ± 1° C temperature and 50% humidity, and allowed 6 days acclimatization to this environment before behavioural testing. Food (Lab Diet, Richmond, Indiana, USA) and water were constantly available except during behavioural conditioning and testing. The light cycle was 0700–1900 hours and all experiments were conducted between 0900 and 1700 hours.

Drugs. The morphine pellets consisted of 75 mg morphine base formulated with an equivalent quantity of microcrystalline cellulose. The placebo pellets were prepared the same way except morphine base was replaced by cellulose.

Naloxone hydrochloride (Sigma), ondansetron hydrochloride (Glaxo), chlordiazepoxide hydrochloride (Roche) and gepirone hydrochloride (Bristol-Myers) were all dissolved in 0.9% sodium chloride solution after gentle warming. A few drops of 0.1 N HCl were first added to MDL 72222 (Research biochemical Inc.) before bringing up to final volume with 0.9% sodium chloride solution. The final pH was adjusted to 5–6 with 1 N NaOH. All controls received the appropriate vehicle alone. The aforementioned drugs were injected subcutaneously in a dose volume of 2 ml/kg, except chlordiazepoxide which was injected intraperitoneally. Final drug concentration referred to that of the free base. All test compounds were administered 30 min prior to naloxone injection, except gepirone (15 min pre-treatment).

Induction of morphine dependence and withdrawal. Under chloral hydrate anaesthesia (400 mg/kg IP; 10 ml/kg dose volume), a single morphine or placebo pellet was implanted subcutaneously in the nape of the rat's neck. The incision was then sealed with surgical clips after the application of antiseptic to the wound area. Implantation of a 75 mg morphine pellet has been reported to produce a sustained plasma concentration in the range of 70–126 ng/ml morphine for up to 9 days (Mucha et al. 1986). Seventy-two or 96 h following pellet implantation (conditioning experiment) or at both times (overt behavioural experiment) the rats were injected with naloxone. For assessment of the abstinence syndrome, a dose of 0.5 mg/kg naloxone was chosen, since previous workers have shown this treatment to produce a mild but nonetheless robust behavioural syndrome suitable for antagonist studies (Sharpe and Jaffe 1989). A range of naloxone concentrations were studied for the place conditioning experiment. An interval of 72–96 h between pellet implantation and naloxone injection was selected based on previous studies (Cicero and Meyer 1973; Wei et al. 1973; Sharpe and Jaffe 1989).

Table 1. Summary of the behaviours studied following naloxone injection in morphine-pellet implanted rats

Behaviour	Unit of measure	Definition
Wet dog shakes	*N*	Total number of distinct whole body shakes recorded during the 30 min test session
Paw shakes	*N*	Total number of bouts of rapid forepaw shakes recorded during the 30 min test session. A bout being defined as distinct episodes of behaviour separated by at least 5 s from another
Startle	Rating scale 0–4	Animals response to a puff of air. 0 = no response, 1 = mild with slight vocalization, 2 = moderate response with vocalization, 3 = marked response/jump with vocalization, 4 = intense with marked vocalization
Mouth movements	*N*	Total number of purposeless chewing movements (i. e. chewing not directed at any particular object) recorded during the 30 min test session
Salivation	g	Weight difference of a cotton bud before and after wiping across the outside of the mouth and inserted into both cheeks
Weight loss	g	Body weight change in the animal recorded 0 and 30 min after naloxone injection
Body temperature change	°C	Rectal body temperature change in the animal recorded 0 and 30 min after naloxone injection

Assessment of somatic signs of morphine withdrawal. Immediately after weighing, the rats were injected with naloxone (0.5 mg/kg) and placed individually in plastic observation chambers (20 × 38 × 48 cm) containing woodchip bedding. For the next 30 min, each rat was continually monitored for the behavioural measures described in Table 1; except startle, salivation and body weights, which were measured after the test session.

During preliminary studies, it became apparent that individual rats differed both qualitatively and quantitatively with respect to certain aspects of the withdrawal syndrome. Because we could observe a similar behavioural response following a second withdrawal 24 h later in the same animal (see Table 2), for the antagonist studies, we adopted a cross-over design such that each animal was pre-treated 30 min before naloxone with either test compound or vehicle in a counterbalanced design. Thus drug effects were compared to vehicle pre-treatments in each animal.

Table 2. Effect of two consecutive naloxone-precipitated withdrawals in morphine-dependent rats. WDS = wet dog shakes; PS = paw shakes; MM = mouth movements; Sal = salivation; St = startle; △Wt = body weight change; △T = body temperature change. For each parameter the responses seen following the first and second withdrawal did not significantly differ

	WDS (*n*)	PS (*n*)	MM (*n*)	Sal (g)	St (0–4)	△Wt (g)	△T (°C)
1st withdrawal (72 h)	18 ± 2	7 ± 2	23 ± 5	0.05 ± 0.02	3	−15 ± 1	−0.6 ± 0.3
2nd withdrawal (96 h)	20 ± 1	5 ± 1	16 ± 3	0.07 ± 0.02	3	−17 ± 1	−0.6 ± 0.4

Place conditioning apparatus. Each place conditioning box was 30 × 60 × 40 cm (width × length × height) and consisted two distinct compartments: one painted white with a roughened perspex floor, the other black with a smooth perspex floor. A central wire gridded, aluminium platform (8 × 31 cm) served as a transitional zone. By means of a central partition coloured to match the appropriate compartment, two rats could be simultaneously conditioned to either compartment. The place conditioning boxes were housed in a sound attenuated room under low white light (approximately 30 lux) and additional red light to aid human visual inspection. All behavioural measurements were made from an adjacent area by means of a camera positioned in the conditioning room.

In preliminary studies, we falled to see any intrinsic preference for either compartment in vehicle conditioned rats. Thus, rats given 4 × 45 min saline pairings to each compartment gave test scores of: black/smooth compartment 365 ± 37 s, white/roughened compartment 352 ± 34 s ($n = 8$).

Assessment of place conditioning to morphine withdrawal. At either 72 or 96 h after morphine pellet implantation, the rats were injected with naloxone and immediately confined to one compartment for 45 min. At the other time period, the animals were injected with vehicle and conditioned to the alternate compartment. All treatments were counterbalanced as closely as possible, according to conditioning compartment and time. Twenty-four hours after the second conditioning phase, each rat was returned to the testing box, placed on the central platform and allowed free access to the entire chamber for 15 min. Preference was measured by the cumulative time spent in the vehicle-paired relative to the naloxone-paired compartment.

Statistics. All data are presented as means ± SEM except startle where median scores are shown. To analyse overt behavioural scores in drug pretreated compared to vehicle pretreated rats, the difference scores for each rat were obtained and compared using a paired *t*-test, except startle which was analyzed using a Wilcoxon matched pairs test. Place preference scores were calculated by subtracting the cumulative time spent on the vehicle-paired side from that on the drug-paired side. Whether an individual dose produced conditioning was determined using a paired *t*-test. The preference scores were also analysed by one-way analysis of variance with between group comparisons made using a Neuman-Keuls test. The accepted level of significance was $P < 0.05$.

Results

Effects of vehicle and naloxone injections in placebo and morphine pellet implanted rats

The injection of saline into either placebo or morphine implanted rats, and naloxone (0.5 mg/kg SC) into placebo implanted rats, failed to consistently affect any of the parameters described in Table 1, except weight loss, which over the 30 min observation period was approximately 2–4 g (Table 3). In contrast, naloxone injections in morphine implanted rats produced a significant incidence of wet dog shakes, paw shakes, mouth movements, heightened startle reflex, diarrhoea and a marked weight loss (Table 3). In addition, some animals displayed rhinorrhoea, penile grooming, writhing, hypothermia and teeth chatter. However, since these effects were inconsistent, they were excluded from subsequent behavioural analysis. The incidence of these behaviours following naloxone treatment in morphine pellet implanted rats only, suggests that at the time of testing the animals had become physically dependent on morphine.

Effects of ondansetron and MDL 72222 on the behaviours induced by naloxone injection in morphine-dependent rats

Pre-treatment with ondansetron (0.01–1.0 mg/kg SC) or MDL 72222 (1–3 mg/kg SC) failed to modify the incidence of wet dog shakes, paw shakes, mouth movements, and startle response in morphine withdrawal rats (see Fig. 1). However, there was a significant dose-related attenuation of the weight loss in these animals with both drugs. Neither drug appeared to affect the incidence of salivation, rhinorrhoea, penile grooming, hypothermia and teeth chatter seen in some animals (data not shown).

Effects of naloxone on place conditioning in morphine-dependent rats

Naloxone (0.05–1.5 mg/kg SC) produced a significant ($P < 0.05$) place aversion following a single conditioning trial in morphine dependent rats (see Fig. 2). However, between these concentrations the aversion was not dose related ($F_{3,26} = 0.51$, n.s.). Interestingly, the most consistent effect appeared to be at the 0.05 mg/kg dose, where the animals displayed very few overt signs of withdrawal (data not shown). In a subsequent experiment, at a lower dose (0.002 mg/kg SC) naloxone failed to produce any significant place conditioning (Fig. 2). It was interesting to note that during testing, some animals displayed certain withdrawal signs, e.g. diarrhoea, wet dog shakes, even though they were untreated and had received the withdrawal conditioning 24–48 h previously. These behaviours were not observed when the rats were within their home cages. In placebo pellet implanted rats, pretreatment with naloxone (0.05–0.5 mg/kg SC) failed to produce any significant place conditioning (Fig. 2).

Table 3. Evidence that the behaviours observed following naloxone challenge in morphine pellet implanted rats are indicative of withdrawal. See Table 2 for list of abbreviations. Placebo = vehicle pellet; mophine = 75 mg morphine pellet; naloxone = 0.5 mg/kg. $N = 6$ per group, $^*P < 0.05$ by comparison to all other groups (see text for details)

		WDS (*n*)	PS (*n*)	MM (*n*)	Sal (g)	St (0–4)	△Wt (g)	△T (°C)
Placebo	Vehicle	2 ± 1	0	2 ± 1	0	1	−4 ± 1	+0.5 ± 0.2
	Naloxone	1 ± 1	0	1 ± 1	0	1.5	−3 ± 1	−0.2 ± 0.1
Morphine	Vehicle	0	0	0	0	2	−2 ± 1	+1.4 ± 0.4
	Naloxone	18 ± 3*	7 ± 2*	23 ± 5*	0.05 ± 0.02*	3*	−15 ± 1*	−0.6 ± 0.3

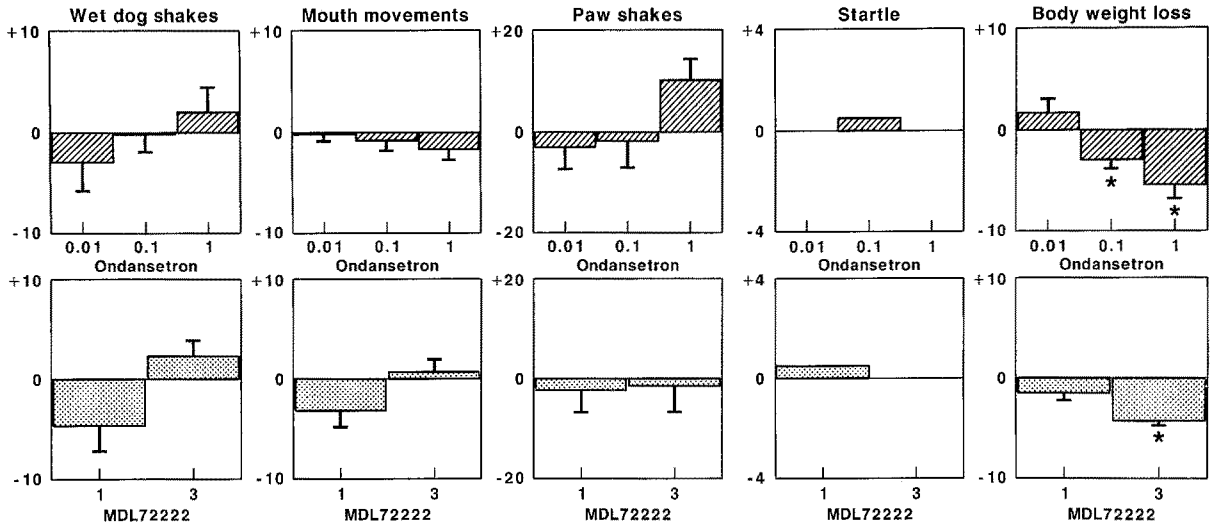

Fig. 1. Effect of ondansetron and MDL 72222 on various behaviours related to the morphine-withdrawal syndrome. The results are expressed as the mean difference scores for each rat between vehicle and drug pretreatment. $N=6$ per group. Units of measure on vertical axes are identified in Table 1. $^*P<0.05$ vs vehicle pretreatment (paired t-test). See text for details

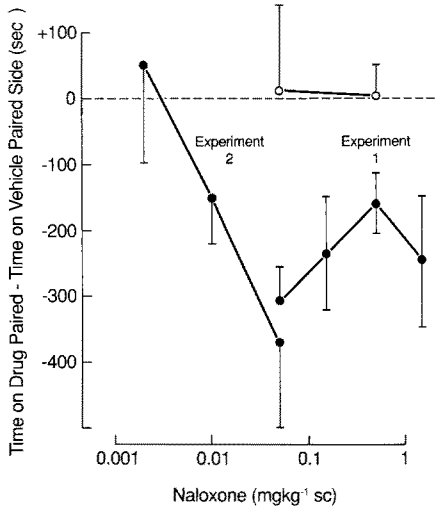

Fig. 2. Effect of naloxone dose on place conditioning in morphine pellet implanted (*filled circles*) and placebo pellet implanted (*open circles*) rats. $N=6$–8 rats per group. See text for details. In morphine dependent rats significant place conditioning was observed with naloxone doses of 0.01 mg/kg and above ($P<0.05$ paired t-test)

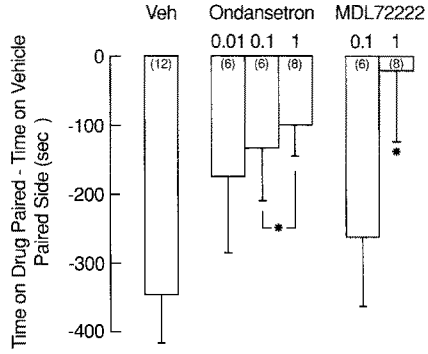

Fig. 3. Effect of ondansetron and MDL 72222 on the place aversion induced by naloxone (0.05 mg/kg) injection in morphine dependent rats. Figures in parentheses denote the number of animals per group. $^*P<0.05$ vs vehicle pre-treatment (Neuman-Keuls test). See text for details

Effects of ondansetron and MDL 72222 against the place aversion produced by naloxone in morphine-dependent rats

The effect of MDL 72222 pre-treatment on naloxone (0.05 mg/kg)-induced place aversion in morphine dependent rats is shown in Fig. 3. There was a significant group effect ($F_{2,23}=3.77$, $P<0.05$) with MDL 72222 at the 1 mg/kg dose producing a significant antagonism of the place aversion ($P<0.05$). In contrast, despite showing an obvious trend towards this effect, ondansetron failed to significantly attenuate naloxone place aversion at a single dose ($F_{3,28}=2.9$, n.s.); however, pooling the 1 and 0.1 mg/kg doses which did not differ significantly [$t=0.41$, $df=12$, t (95%)$=2.18$] revealed a significant interaction ($F_{2,29}=3.87$, $P<0.05$). Thus the combined data from the 1 and 0.1 mg/kg dose levels were significantly different from controls ($P<0.05$). In addition, at the 1 mg/kg dose, ondansetron pretreated rats spent significantly more time in the naloxone paired compartment during testing, by comparison to vehicle pretreated controls (i.e. vehicle: 200 ± 34 s, ondansetron 1 mg/kg: 313 ± 25 s, $t=2.43$, $df=18$, $P<0.05$). Neither MDL 72222 nor ondansetron (both 1 mg/kg) produced any single trial place conditioning when administered alone (i.e. difference scores: MDL 72222 -21 ± 61 s, $n=6$; ondansetron $+39\pm83$ s, $n=6$).

Effects of chlordiazepoxide and gepirone against the place aversion produced by naloxone in morphine dependent rats

Pretreatment with chlordiazepoxide (5–10 mg/kg) prior to naloxone-induced place conditioning revealed a sig-

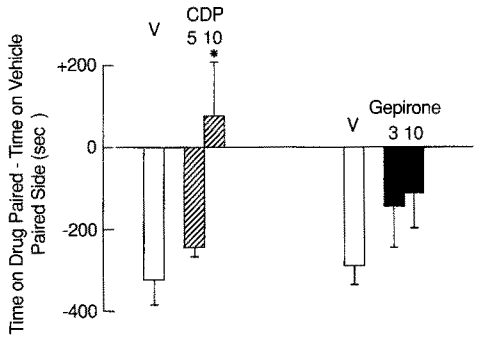

Fig. 4. Effect of chlordiazepoxide (*CDP*) and gepirone on the place aversion induced by naloxone (0.05 mg/kg) injection in morphine dependent rats. $N = 6$–9 rats per group. $^*P < 0.05$ vs vehicle pretreatment (Neuman-Keuls test). See text for details

nificant group effect ($F_{2,13} = 4.97$, $P < 0.05$) with the 10 mg/kg dose producing a significant antagonism of the place aversion (Fig. 4). Gepirone (3–10 mg/kg) on the other hand produced only a modest decrease which failed to reach significance ($F_{2,20} = 1.78$, n.s.).

Discussion

In the first part of the present study, we examined the effect of acutely administered 5–HT_3 receptor antagonists on various components of the morphine-withdrawal syndrome (Blasig et al. 1973; Cicero and Meyer 1973; Wei et al. 1973). At the doses tested, neither ondansetron or MDL 72222 affected the frequency or the incidence of wet dog shakes, paw shakes, mouth movements, elevated startle reflex or salivation. Therefore, one might predict that 5–HT_3 receptors may not be involved in these features of opioid dependence, although it must be noted that the compounds were administered after dependence had been established–it would be of interest to examine their effect following administration during the development of this phenomena. One feature of the withdrawal syndrome that both ondansetron and MDL 72222 did attenuate, however, was that of weight loss. This measure is almost certainly due to the profuse diarrhoea seen in these animals, and so it seems likely that these compounds were in some way reducing this defaecation. It is interesting to compare these observations with the findings of other workers, showing ondansetron to reduce the weight loss following benzodiazepine withdrawal (Costall et al. 1989a; Goudie and Leathley 1990). Whether a common mechanism underlies these responses is unknown and indeed, on the basis of our studies, we are not able to determine a central or peripheral site of action. The effect of 5–HT_3 receptor antagonists with poor CNS penetration, e.g. MDL 72699 (quaternized MDL 72222), against these responses, would be a means to test this experimentally. Perhaps relevant to the present study, Gintzler (1979) demonstrated that a phenylbiguanide-sensitive effect of 5–HT is involved in opioid withdrawal-induced contracture of guinea-pig ileum, and a supersensitivity of ileal 5–HT receptors has been reported during opioid withdrawal (Schultz and Goldstein 1973; Johnson et al. 1978). Thus a direct effect of either ondansetron or MDL 72222 on ileal 5–HT_3 receptors (Richardson and Engel 1986; Pinkus et al. 1989) to reduce this hyperexcitability and resultant diarrhoea, may seem the most plausible explanation for our findings.

In the second part of this study, we examined the motivational consequences of opioid withdrawal by means of place conditioning. Naloxone (0.05–0.5 mg/kg) failed to produce a single trial place aversion in non-dependent rats. These findings are consistent with those reported by Mucha and Iversen (1984) who only observed significant place aversion to a dose of 0.5 mg/kg naloxone following at least three pairings to the conditioning compartment. However, in morphine-dependent rats we observed significant place aversion following a single pretreatment with these same doses of naloxone. Such an effect is in accord with related studies using this model (Mucha et al. 1982, 1986; Mucha 1987; Hand et al. 1988; Stinus et al. 1990). The most marked aversion seen in morphine dependent animals was following a dose of naloxone (0.05 mg/kg) which failed to consistently produce any signs of an observable abstinence syndrome. At higher doses of naloxone, significant place aversions were recorded but the response became more variable. This may imply that the distress shown by the rats at these doses was impairing processes underlying place conditioning. Treatment of the rats with either MDL 72222 or ondansetron before the withdrawal conditioning produced a significant antagonism of the place aversion produced by naloxone (0.05 mg/kg). This could have been due to the 5–HT_3 receptor antagonists inducing place conditioning in their own right, however, following a single conditioning trial we failed to observe any place conditioning following 5–HT_3 antagonist pretreatment alone.

The finding that place aversions can be elicited without the demonstration of an observable abstinence syndrome and because 5–HT_3 receptor antagonists appear to block the former but not latter response, suggests the independence of these behaviours. Indeed other workers have failed to observe any obvious correlation between these two events (Mucha et al. 1982; Mucha 1987; Hand et al. 1988). There is substantial evidence for an involvement of the periaqueductal grey in the development of opioid physical dependence (Laschka et al. 1976; Wise 1988 for review). However, a recent study involving the microinjection of methylnaloxone into various brain regions in morphine dependent rats revealed the nucleus accumbens and amygdala to be particularly sensitive to the place aversion produced by this treatment (Stinus et al. 1990). Furthermore, the same workers reported very few overt signs of withdrawal in these animals, especially following amygdaloid injection. The identification of the amygdala as a region involved in the motivational consequences of withdrawal is of relevance to this study, since a relatively high density of 5–HT_3 receptors are localized in this structure (see Introduction). Evidence suggests the amygdala is involved in some behavioural effects of 5–HT_3 receptor antagonists (Costall et al. 1989b; Higgins et al. 1989a, 1991) and indeed Costall et al. (1990b)

have demonstrated that intra-amygdaloid injections of ondansetron alleviate the behavioural inhibition seen in mice following diazepam, cocaine, ethanol and nicotine withdrawal. Since anxiety is a core feature of withdrawal (see Emmett-Oglesby et al. 1990 for review), it could be hypothesized that the antagonism of place aversion reported in this study and the effects reported by Costall et al. (1989a, 1990a, b) are manifestations of a similar response mediated by 5-HT_3 receptor antagonists–namely that of anxiolysis.

To test this hypothesis we studied the effects of two pharmacologically distinct anxiolytic agents, chlordiazepoxide and gepirone (Sellers 1978; Csanalosi et al. 1987) in this paradigm. Complete blockade ot the naloxone-induced place aversion was observed in chlordiazepoxide but not gepirone-treated animals. The failure of gepirone to antagonize this effect could have been due to the use of inappropriate doses, although those chosen in this study have been shown to be pharmacologically active in other animal models (Kehne et al. 1988; Koenig et al. 1988). The ineffectiveness of gepirone may not necessarily be in conflict with an anxiolytic affect as it is feasible that their profile against this place aversion may reflect differences previously reported with these drugs across various anxiety models (cf Chopin and Briley 1987; Jones et al. 1988). Alternatively, the efficacy of chlordiazepoxide but not gepirone in this model may in part be due to the amnestic properties of benzodiazepine-type drugs (Roy-Byrne et al. 1987; Preston et al. 1988). If this were the case then such an effect would be unlikely to account for the responses observed with the 5-HT_3 receptor antagonists, for evidence suggests that these drugs may enhance cognitive performance (Barnes et al. 1990c).

Therefore, despite their ineffectiveness against most of the overt signs of morphine withdrawal, both MDL 72222 and ondansetron did ameliorate a conditioned behaviour which may represent a negative emotional stimulus associated with this response. Our observations could therefore suggest that 5-HT_3 receptor antagonists have clinical utility in opiate dependence where conditioned drug effects are of particular importance (Childress et al. 1988; Siegel 1988).

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