Acute Oxycodone Induces the Pro-emetic Pica Response in Rats

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Non-Standard Abbreviations:
Nucleus of the Solitary Tract (NTS)
Paraformaldehyde (PFA)
Norbinaltorphimine (nor-BNI)

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Abstract

Oxycodone, a semi-synthetic opioid analgesic is frequently prescribed for the management of pain. Side effects of nausea and emesis impact patient compliance and limit its therapeutic use. The present study established that an antinociceptive dose of oxycodone (15 mg/kg; oral) induces the pica response. We found sex differences in the temporal course of pica, with females having a longer duration. Opioid receptors mediated the pica response, as naloxone 1.0 mg/kg transiently attenuated, and 2.0 mg/kg blocked pica. A kappa selective antagonist failed to block the response, suggesting mediation by mu opioid receptor. For further validation, we used the well-established kaolin intake model to assess pica with the chemotherapeutic drug cisplatin as a positive control. Oxycodone and cisplatin significantly increased kaolin intake four to seven-fold and the wet weight of stomach was elevated two to three-fold. To examine the underlying neural circuitry, we investigated c-fos activation in the area postrema and nucleus of solitary tract (NTS). Oxycodone treatment significantly increased the number of c-fos positive neurons in the area postrema and NTS compared to water controls. As expected, cisplatin also increased the number of c-fos positive cells in these regions. In the area postrema, the oxycodone effect was greater than cisplatin, especially at 2 hr. These results indicate that antinociceptive dose of oxycodone is associated with the expression of pica, a pro-emetic response.
Introduction

The prescription opioid analgesic oxycodone is a potent mu opioid receptor agonist frequently prescribed for the management of acute and chronic pain (Coluzzi and Mattia, 2005). The therapeutic efficacy of opioids is often limited by severe adverse effects of nausea and emesis (Wheeler et al., 2002). These debilitating side effects compromise patient compliance leading to discontinuation of opioids and subsequently inadequate analgesia (Redmond and Glass, 2005; Miaskowski, 2009); thus, the need to further explore mechanisms that underlie opioid-induced nausea/emasis. Dogs, cats, house musk shrew and ferrets have been the traditional model for emesis research (Florczyk et al., 1982). However these animals are difficult to work with in a laboratory setting and in recent years the rat model of nausea/emasis has emerged as an important tool to investigate the mechanisms underlying drug-induced emesis (Takeda et al, 1993).

Rats, mice and guinea pigs lack a motoric reflex of emesis but display atypical feeding behavior termed the pica response (Takeda et al., 1993; Yamamoto et al., 2004). Pica is an aversive/illness-response, which results in consumption of non-nutritive substances, such as kaolin, following an emetic stimulus (Mitchell et al., 1976). Pro-emetic agents such as cisplatin and apomorphine have been shown to induce the pica response in rats (Takeda et al., 1993). A recent study by Yamamoto et. al (2007) indicated that the magnitude of pica in rats was directly related to their "emetogenic potential" in humans. Cisplatin and cyclophosphamide lead to high and sustained levels of pica, actinomycin D and 5-fluorouracil lead to moderate levels, whereas vincristine induced low levels of the pica response. In addition to these pro-emetic agents, opioids
such as morphine and buprenorphine also induce the pica response in rats (Aung et al., 2004; Bender, 1998). For instance, Aung et al. (2004) showed that morphine induced-pica was attenuated by opioid antagonist methylnaltrexone suggesting an opioid receptor-mediated effect.

In addition to this consummatory behavior, other measures of pica such as impaired gastric function (Liu et al., 2005) and activation of the neural emetic circuitry have also been investigated. Neuroanatomical regions of the area postrema and nucleus of the tractus solitarius (NTS) in the brainstem underlie emetic responding. Vagal afferents from the gastrointestinal tract innervate these areas, which lie outside the blood brain barrier, to mediate the emetic response (Saito et al., 2003; Hornby, 2001). Using pharmacological magnetic resonance imaging in live rats, Chin et al. (2006) found that apomorphine activated the area postrema and NTS at plasma concentrations of drug known to induce emesis (Chin et al., 2006). Additional studies demonstrated that cisplatin selectively increased the c-fos mRNA in the NTS and area postrema, as well as increased the labeling of c-fos immunoreactive positive neurons in these nuclei (Horn et al., 2007; Endo et al., 2004).

The objective of the present study was to investigate whether oxycodone induces the behavioral response of pica and activates the emetic circuitry underlying the pica expression. A novel consummatory behavioral response of bedding intake and the well-established kaolin intake paradigm were assessed to examine pro-emetic effects. In addition, wet weight analysis of the stomach was used as another measure of pica. A separate group of rats were used to determine whether oxycodone induces the expression of c-fos in the brainstem nuclei of area postrema and NTS.
In addition to the above objective, we also assessed sex differences and opioid receptor mediation of pica. The former was important because opioid-induced sex differences have been well documented for measures of analgesia, locomotion, rewarding and aversive effects. Striking sex differences have been observed for responses to the prototypical opioid morphine. Clinical and preclinical evidence indicate that males are more sensitive to the analgesic and sedative effects of morphine. In contrast, females are more sensitive to nausea, reinforcing and locomotor stimulant effects of morphine in comparison to males (Craft, 2008). We recognize that oxycodone has a different pharmacology than morphine, which may lead to other sexual dimorphisms. For instance, clinical data indicate that under conditions of adjusted body weight, women demonstrate 25% slower clearance of oxycodone in comparison to men (Kalso, 2005). Female rats are more sensitive than males to the antinociceptive response to oxycodone following subcutaneous administration (Holtman and Wala, 2006), an effect opposite to that seen with morphine. Since route of administration affects pharmacokinetics (Chan et al., 2008) and can have profound effects on the functional effects, it was also important for us to examine sex differences in the antinociceptive and pro-emetic effects after oral administration.
Methods

Subjects.

Nulliparous adult female or male Sprague-Dawley rats (2-4 months of age; Harlan, Indianapolis, IN) were adapted to an oral gavage or intraperitoneal injection (i.p.) procedure using vehicle. The experimental subjects were group-housed in standard flat bottom plastic cages containing hardwood bedding (SaniChip, PJ Murphy Forest Products; Montville, NJ). Temperature (22°C ± 1°C), humidity (40-50%) and 12 hr light:dark cycle with lights on at 0600 remained constant throughout the experimentation. Food (Teklad, Harlan) and water were available ad libitum throughout the experiment except as noted. The experimental protocol and animal husbandry procedures were approved by the Institutional Animal Care and Use Committee and comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, (publication number 85-23, revised 1985). All experimental assessments were conducted between 1300 and 1700 hr.

Drug Treatments.

Oxycodone HCl (dihydrohydroxycodeinone; Mallinckrodt, St. Louis, MO) (15 mg/kg) or vehicle (water or saline) were administered orally or i.p. in a volume of 1 ml/kg. In preliminary studies, this dose of oxycodone was found to be antinociceptive (See Supplementary Figure 1) with no overt toxicity. Oral gavage was done using an 18-gauge, 7.6 cm long gavage tube (Popper and Sons, New Hyde Park, New York). Naloxone (0.5, 1.0 or 2.0 mg/kg; Sigma-Aldrich, St. Louis, MO) was administered s.c. 30 min prior to oxycodone and the kappa receptor antagonist, norbinaltorphimine (Nor-
BNI; 7.5 or 15 mg/kg s.c.; Tocris, Ellisville, MO) was administered 2 hr before oxycodone. The timing of the naloxone and Nor-BNI pretreatments was based on preliminary studies and Takemori et al. (1988) respectively. Cisplatin (Sigma-Aldrich, St. Louis, MO) at 5 mg/kg was injected in a volume of 3-ml/kg i.p. Saline served as the vehicle control for naloxone, nor-BNI, and cisplatin.

Behavioral expression of pica.

Increased consumption of non-nutritive substances is termed pica behavior. Two measures of pica were used in the present study, ingestion of cage bedding (Experiment 1) and kaolin intake (Experiment 2). The advantage of the bedding ingestion is that temporal analysis of the data is feasible, although the magnitude of the pica response is more difficult to quantify (see supplemental data file for a video clip of the bedding ingestion response). The kaolin consumption measure provides quantitative analysis of the data (i.e., kaolin consumption in g). However, this response is a summation of a 3 hr session, which limits temporal analyses.

Bedding intake. Assessment was conducted by observation of rats in clean, transparent rat cages that were the same dimensions as the home cage. The cages contained equal amounts of standard bedding. Ingestion of bedding into the mouth was scored as a quantal (all or none) response. Paw gnawing and rigidity were also scored at 15 min intervals for 4 hr.

Kaolin intake. Kaolin was made using a standard protocol from literature (Mitchell et al., 1976, Aung et al., 2004). Briefly, 1 g of acacia (gum arabic, Fisher Scientific, Pittsburgh, PA) was mixed with 99 g of kaolin powder (hydrated aluminum silicate,
Fisher Scientific) in a 99:1 ratio, with distilled water to form a thick paste. The paste was rolled on a stainless steel tray, passed through a 5 ml syringe and cut into pieces in a shape and size similar to that of regular rat chow pellets. The pellets were placed on trays and completely dried at room temperature for 72 hr.

The assessment method for kaolin intake was as previously described (Aung et al., 2004) with slight modifications. The procedure consisted of three phases. **Adaptation:** Rats were acclimatized to the presence of pre-weighed kaolin and chow in the cages for 3 hr each during the 3 days of adaptation phase. **Baseline:** Rats were administered the vehicle and baseline measures of kaolin and chow intake were recorded. Kaolin and food pellets were placed in separate areas in the rat cage. **Test Days:** Cisplatin has an extended (>48 hr) pro-emetic action and thus was administered only on test day 1, but the rats were assessed on test days 1 and 2. Oxycodone's duration of action is much shorter and thus was administered on both test days 1 and 2. Kaolin pellets and food pellets were weighed to the nearest 0.1 g prior to and at the conclusion of the 3 hr test session on each day. The difference was recorded as the intake and the two days data were summed and analyzed for treatment effects.

**Stomach Wet Weight.**

Increase in the wet weight of the stomach has also been observed in rats expressing the pica response. The wet weight of the stomach was measured by a procedure previously described (Malik et al., 2007). Briefly, rats were sacrificed at the end of a 3-hr session on test day 2. An incision was made proximal to the gastro-
esophageal junction and distal to the pyloric sphincter to isolate the stomach. Following isolation, stomach was blotted dry and the wet weight was recorded to the nearest g.

**Immunohistochemistry for c-fos.**

**Tissue collection.** The procedure for tissue collection was performed as previously described (Bennett and Semba, 1998) with slight modifications. Briefly, rats were deprived of food or water 3 hr prior to sacrifice to reduce the potential effect of feeding on neural c-fos expression (Horn et al., 2007). Different groups of rats received either oxycodone 15 mg/kg or water via oral gavage and were sacrificed 1 or 2 hr post-treatment. Separate groups of rats received i.p. injection of cisplatin or saline and were sacrificed 6 hr after treatment. The timing for cisplatin treatment was based on optimal results obtained for c-fos activation in the hindbrain region (Horn et al., 2007).

During sacrifice rats were deeply anaesthetized by an injection of sodium pentobarbital (50 mg/kg). The subjects were transcardially perfused with phosphate buffered saline (PBS) to exsanguinate followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). Following fixation, brains were dissected from the skull and stored in 4% PFA in 0.1M phosphate buffer (pH 7.4) for 24 hr. The brains were then passed through 15% sucrose for 24 hr for cryoprotection followed by preservation in 30% sucrose. Three days after cryopreservation, brains were blocked to isolate the forebrain and brainstem. The isolated brainstem was further sectioned at a thickness of 40 µm using a cryostat and serial sections were collected and stored in 24 well plates filled with PBS. Sections were processed for c-fos analysis within a week.
Immunohistochemistry procedures. Based on the bregma coordinates in the atlas, brain sections were sampled between the coordinates of -13.56 mm to -14.40 mm respectively (Paxinos and Watson 2007). Three sections per rat were selected for histochemical analysis which represented the following regions: nucleus of solitary tract rostral to area postrema (NTSr), nucleus of solitary tract medial to area postrema (NTSm) and nucleus of solitary tract caudal to area postrema (NTSc). These sections were also used to survey the area postrema and determine if there were any regional differences in the area postrema across the rostral-caudal axis. Another set of sections adjacent to the above were stained with cresyl violet using standard procedures. This was done to specifically match coordinates from c-fos processed tissue and the plate from the rat atlas.

Brain sections were washed in PBS. The sections were then incubated in 3% hydrogen peroxide (30 min) and then 0.5% BSA (20 min) followed by 1% normal goat serum containing 0.1% Triton-X-100 in PBS for 1 hr. Sections were rinsed in PBS followed by an overnight incubation at 4°C with primary c-fos rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500. Sections were rinsed in PBS and incubated in 0.5% BSA for 20 min. They were then incubated in biotinylated goat anti-rabbit secondary antibody 1:2000 (Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 2.5 hr. Sections were subjected to PBS rinses followed by incubation in 0.5% BSA (20 min) and peroxidase-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) at a dilution of 1:7500 for 30 min. Following PBS rinses, sections were developed and visualized using a...
ImmPACT DAB kit (Vector Laboratories, Burlingame, USA). Finally, sections were mounted, dehydrated, counterstained with hematoxylin and coverslipped.

**Cell Counting.** Pictures of the area of interest on each slide were taken on a microscope equipped with a digital camera. The number of c-fos positive cells was quantified by the presence of positively stained brown cells in the region of interest. Cells with punctate dark brown staining above background were scored positive. C-fos positive cells were counted manually using NIH Image J software (NIH, Bethesda MD). For data analysis, the sum of c-fos positive cells counted across three sections is presented since there were no regional differences.

**Statistical Analyses.**

For the experiments assessing pica as bedding intake, paw gnawing, and rigidity, we had quantal responses (yes or no). None of these behaviors were displayed by the water-treated controls. To avoid inflating our rates of significance by including groups that did not display the behavior at any time point, we used a single 0-hr baseline control (pre-drug) for the measures in Experiment 1. The number of positive responses within each hr across all subjects for a treatment group was summed and graphs depict the % of positive responses across each hour. Each sex was analyzed separately. An initial chi-square contingency analysis was done comparing the responses across the five time points (0, 1, 2, 3, and 4 hr). Fisher’s exact tests were then conducted to compare each group to the 0-hr baseline. For the portion of Experiment 1 that examined receptor mediation, the percent positive responses were analyzed via repeated-measures ANOVA. We used percent positive responses as compared to a
summation of the positive responses because there were unequal n’s across the treatment groups. An ANOVA was used as opposed to a chi-square, since all of the groups had received oxycodone treatment and floor effects were not present. The saline - oxycodone group served as a control in this experiment and the naloxone-oxycodone groups were compared to the control by Tukey’s post-hoc contrasts.

For Experiments 2 and 3, the data were analyzed by a one-way ANOVA followed by post-hoc Tukey test. For Experiment 2, drug treatment was the between subjects measure and in Experiment 3 the between subjects measure was condition (control, cisplatin, 1 hr post-oxycodone or 2 hr post-oxycodone).
Results

Experiment 1: Does oxycodone induce pica response of bedding intake?

Expression of the pica response. Water-treated control rats did not exhibit pica behavior, while the oxycodone-treated rats did (Figure 1a). A chi-square analysis was done within each sex to examine differences between the baseline pre-drug response and the response post-drug. The overall analysis for both males and females was significant ($\chi^2 = 42.64$ and $47.78$ respectively; df = 4, $p < 0.0001$ for both). Subsequent analyses were conducted to determine the time points when the oxycodone-treated subjects differed from baseline using a Fisher's exact test. For males, significant effects were found at all times (1 hr: $\chi^2 = 16.00$, $p < 0.0001$; 2 hr: $\chi^2 = 37.33$, $p < 0.0001$; 3 hr: $\chi^2 = 24.00$, $p < 0.0001$; 4 hr: $\chi^2 = 9.60$, $p < 0.004$ for 4 hr; df = 1 for all). Likewise for females significant effects were found across the 4 hr (1 hr: $\chi^2 = 26.32$, $p < 0.0001$; 2 hr: $\chi^2 = 34.29$, $p < 0.0001$; 3 hr: $\chi^2 = 31.45$, $p < 0.0001$; 4 hr: $\chi^2 = 26.32$, $p < 0.004$ for 4 hr; df = 1 for all). A second set of analyses were conducted to compare males to females for the pica response. There was an overall effect of Sex ($\chi^2 = 6.10$, $p = 0.02$) with females displaying a greater degree of pica than males.

To ensure that the expression of pica was not solely a consequence of the oral route of administration, the experiment was repeated with i.p. injection of oxycodone at two doses, 10 and 15 mg/kg. As shown in Supplemental Data Figure 2, dose-related induction in the expression of pica behavior was also robust. Since similar effects were found with oral and i.p. administration and our objective was to mimic the route of administration used in humans, subsequent studies were conducted with oral administration of oxycodone only. In addition, the temporal pattern of pica expression
did not differ significantly during the third and the fourth hour of treatment when the drug was administered orally. Therefore, in all our future studies we assessed pica behavior for 3 hr only.

Receptor mediation in the temporal expression of pica. We first determined that the three doses of naloxone blocked the antinociceptive effect of oxycodone in female rats (see Supplemental Figure 3). These doses of naloxone were then tested to determine if they block the expression of oxycodone-induced pica behavior. As shown in Figure 1b, naloxone attenuated oxycodone-induced pica behavior in a dose-dependent manner in females, revealing the involvement of opioid receptors in pica behavior. Repeated measures ANOVA revealed a significant Treatment effect ($F_{3,14} = 4.37, p < 0.03$), but no Treatment x Time interaction. A post-hoc Tukey’s test was done comparing the various treatments. There was a significant blockade of the oxycodone-induced pica response in the group receiving 2-mg/kg naloxone ($p<0.05$ for all time points; Tukey post-hoc test).

There was also marginal effect of Time in the repeated measures ANOVA ($F_{2,28} = 3.30, p = 0.052$). Because naloxone is known to have a short half-life, we did a subsequent Bonferroni post-hoc test on the various naloxone doses at each time point. This analysis revealed that naloxone at 1.0 mg/kg transiently attenuated oxycodone-induced pica at 2 hr ($p < 0.05$). In preliminary studies we administered naloxone alone and we failed to see evidence of a pica response (data not shown).

A second set of female rats was analyzed for kappa receptor involvement in the pica response. The rats were treated with 7.5 or 15 mg/kg Nor-BNI prior to oxycodone
administration. There was a significant Time effect ($F_{3,39} = 36.48, p <0.0001$). However, as can be seen in Figure 1c, there was no effect of Treatment or a Treatment x Time interaction. Nor-BNI pretreatment did not affect the expression of the pica response, suggesting that the effect is not mediated by kappa opioid receptors.

**Experiment 2: Does oxycodone induce pica response of kaolin intake?**

**Kaolin intake and oxycodone.** We assessed whether oxycodone induces the pica response of kaolin intake, which is a standard model of nausea/emesis. Initial analyses compared baseline chow and kaolin intake. We did not find any statistical significant differences in the baseline consumption of chow across all the treatment groups. In addition, the rats across all the groups consumed kaolin in minimal quantities on the baseline days (0.6-0.9 grams).

Oxycodone treatment induced a significant increase in the consumption of kaolin ($F_{2,16} = 3.71, p<0.05$; one-way ANOVA). Post-hoc Tukey tests on the data revealed a significant difference for oxycodone in comparison to control ($p <0.05$) as shown in Figure 2a. Cisplatin, which served as a positive control in our study also increased kaolin intake ($p <0.05$).

**Wet weight of stomach as a measure of pica.** Following the above assessment, rats were sacrificed and the wet weight of stomach was recorded. There was an overall effect of Treatment ($F_{2, 11} = 23.58, p<0.0001$; Figure 2b). As expected the pro-emetic agent cisplatin increased the wet weight of the stomach by almost three-fold in comparison to the control group ($p <0.05$; Tukey test). Oxycodone also significantly
increased the wet weight of the stomach by two-fold in comparison to the water-treated controls (p <0.05; Tukey test).

**Experiment 3: Does oxycodone activate pica-related brain regions?**

C-fos immunostaining and oxycodone. We assessed whether oxycodone treatment activated the pro-emetic neuroanatomical regions during the time course of pica behavior. Two controls were used in this study, oral water gavage for the oxycodone treatment and ip saline for the cisplatin-treated subjects. There was no difference between these controls, so they were combined to form a common control group. There also were no regional differences (anterior, medial and posterior) across the rostral-caudal axis for area postrema or NTS and thus the sum of all the regions is reported. Figure 3a shows the pattern of c-fos staining in the area postrema and NTS.

One-way ANOVA revealed a significant Treatment effect (F3, 21 = 509.5, p<0.0001) in the area postrema (Figure 4a). As expected the positive control cisplatin increased the number of c-fos positive cells in the area postrema compared to controls (p < 0.05 post-hoc Tukey test). Importantly, oxycodone at 1 hr and 2 hr led to strong induction of c-fos, increasing the c-fos immunostaining 2.5 – 4 fold over controls (p < 0.05 post-hoc Tukey test for both). Oxycodone increased c-fos protein expression to a greater extent than did cisplatin (p < 0.05 post-hoc Tukey test) and this effect was particularly pronounced at 2 hr, where there were elevated c-fos levels compared to 1 hr post-treatment.

Oxycodone treatment also increased the number of c-fos positive cells in the NTS at 1 hr and 2 hr post-oxycodone treatment in comparison to control (Figure 4b).
One-way ANOVA revealed a significant Treatment effect ($F_{4, 21} = 54.55$, $p<0.0001$). Cisplatin and oxycodone at both time points significantly increased c-fos immunoreactivity compared to the combined controls ($p < 0.05$ post-hoc Tukey test for all comparisons). Although the effect was less pronounced in than in the area postrema, there were more c-fos positive cells after 2 hr of oxycodone treatment than after 1 hr.
Discussion

Prescription opioid-induced emesis during postoperative pain management is a major clinical concern. This is the first report describing that an analgesic dose of oxycodone is able to produce the pro-emetic response of pica in the rat, as assessed by non-nutritive consummatory behavior. The pica was accompanied by gastrointestinal dysfunction and activation of emetic regions in the brain. We used two models to assess the pica response, bedding intake and kaolin consumption. The bedding intake is robust, easy to score, and permits a temporal analysis. But the data are categorical in nature. The second measure utilized kaolin consumption, a well-established model of pica. We found that oxycodone induced a significant increase in kaolin consumption similar to that observed following administration of pro-emetic agents such as cisplatin, apomorphine and copper sulfate (Takeda et al., 1993). We used cisplatin as a positive pro-emetic control in this study. Typically cisplatin-induced pica is monitored over a 24 hr period after administration because of the long duration of cisplatin’s effects. We recorded the kaolin consumption in 3 hr sessions across two days. This change was made to match the shorter duration of action of oxycodone. We acknowledge that cisplatin is an imperfect control for opioids because of this difference in duration, however it does permit us to gauge the magnitude of our effects with a benchmark from the literature. Another challenge in validating pica as a pro-emetic response is whether emesis is viewed as an all-or-none response or if there are gradations. In dogs researchers establish an emetic threshold – the dose of a drug that induces emesis (Burkman, 1982). In rodents that lack the emetic motoric reflex, research has viewed
the pica response as more of a continuous measure and possibly more analogous to nausea.

In Experiment 1 we found that an oral route of oxycodone administration induced the expression of pica. This route was chosen since oxycodone has high oral bioavailability and it is the route most typically used in humans (Kalso, 2005). Importantly, as seen in the supplementary data, i.p. administration also induced a robust pica response, indicating that it is not a route of administration effect. We found strong sex differences in the expression of pica following oral oxycodone. Female rats demonstrated a higher magnitude and prolonged duration of pica in comparison to male rats. Males demonstrated a sharp onset and offset in the expression pattern, whereas females displayed a steadily elevated and prolonged expression. This sex difference may be a result of pharmacokinetic differences. Chan et al. (2008) found sex differences in the plasma concentration of oxycodone following oral administration. The levels of the parent drug oxycodone were higher in females, whereas the males had higher levels of an inactive metabolite of oxycodone (noroxycodone) under similar experimental conditions. Aside from pharmacokinetic effects, there are sex differences in the response to emetic stimuli. Women are more likely to develop nausea and vomiting after acute opiate administration than are males (Cepeda et al., 2003), including oxycodone treatment (Campora, 1991).

Opioids, including oxycodone, generally exert their pharmacological action via interaction with the opioid receptor which can be demonstrated by blockade of opioid actions by the antagonist naloxone (Inturrisi, 2002; Lemberg et al., 2006). We first found that naloxone effectively blocked or attenuated oxycodone-induced antinociception in a
dose-related manner (supplementary data) and then found that the higher doses (1-2 mg/kg) of naloxone attenuated oxycodone-induced pica behavior. The lower dose of naloxone failed to block the pica response. A prominent rebound emergence of pica behavior was observed after 2 hr for the 1-mg/kg naloxone treated group, which clearly delineated the short acting nature of this opioid antagonist. It is important to note that the rats were not undergoing opioid withdrawal during the pica assessment since the naloxone was administered prior to the oxycodone. Nausea and emesis can accompany opioid withdrawal in humans, although we have failed to see pica when we have precipitated withdrawal or permitted rats to undergo spontaneous withdrawal after chronic oxycodone exposure (data not shown).

The present data indicate that oxycodone-induced pica behavior is mediated via the opioid receptor. Interestingly, pica responses from non-opioid drugs may also be mediated by opioid receptors as naloxone was shown to block a ritonavir-induced pica response (Yuan et al., 2009). Many oxycodone effects, including analgesia and gastrointestinal distress, are thought to be mediated by the mu opioid receptor. Naloxone binds to the mu, kappa, delta receptors, but it does have higher affinity for the mu receptor. However, dysphoric effects have been related to the kappa opioid receptor (Knoll and Carlezon, 2010). Additionally, Tsukamoto et al. found that cisplatin increased the immunoreactivity of endogenous kappa opioid receptor ligand dynorphin A in area postrema neurons (Tsukamoto et al., 2007). Thus, we also investigated the role of kappa receptors by administering the kappa selective antagonist, nor-BNI. Nor-BNI was not able to block or attenuate the pica response, consistent with an interpretation of mu opioid receptor involvement.
In addition to nausea and vomiting, opioids produce other types of gastrointestinal distress. We observed an increase in the wet weight of stomach following administration of cisplatin or oxycodone. The magnitude of the pica effect and the stomach wet weight differed between the treatment groups. Oxycodone-treated rats had a greater pica response with respect to kaolin intake than did cisplatin-treated rats. However, for the stomach wet weight, the effect was reversed and the cisplatin-treated rats showed a greater effect. This pattern of data indicates that the wet weight of the stomach was reflecting more than just the pro-emetic pica response. Opioids and cisplatin have different pharmacological actions, but can trigger common mechanisms to modulate the gastrointestinal tract. Common effects include delayed gastric emptying, increased intestinal transit time, impaired gastrointestinal motility and constipation (Bates et al., 2004; Cabezos et al., 2008; Liu et al., 2005; Rudd et al., 2002). It is possible that opioids or cisplatin can trigger these processes individually or act in concert to increase stomach wet weight. Association of emetic events with abnormal patterns of gastrointestinal motility has been previously reported (Takahashi et al., 2007). The goal of the present study was to demonstrate gross alterations in the gastrointestinal events that occur along with central effects of pro-emesis.

In the final experiment, we observed that oxycodone increased the number of neurons that were positive for the protein product from the immediate early gene c-fos in the area postrema and the nucleus of the solitary tract (NTS). These brainstem regions are well studied in regards to the emetic potential of various drugs (Horn et al., 2007). As expected our positive control, cisplatin also increased the number of c-fos positive cells. However, oxycodone induced more robust c-fos expression than did
cisplatin in the area postrema, matching the data for the kaolin intake. There are a few caveats that are important to mention with regard to these data. First, all of the rats in the study were anesthetized with sodium pentobarbital prior to removal of the brain for immunohistochemical processing. We do not think that interfered with assessment of c-fos activation since Morgan et al. (1987) reported that doses up to 80 mg/kg of pentobarbital did not alter c-fos and our dose was below this threshold. Second, it is possible that opiate-induced gastric dysfunction as described above may be activating the brainstem. There are vagal connections that carry information from the gut to the brainstem and stimulate emetic brain regions (Coda, 2006; Danzer et al., 2004). Thus, we cannot discount that some of the c-fos staining was a consequence of gastric feedback signaling. This maybe especially true for the area postrema, where there was a greater effect at 2 hr than at 1 hr. The pica behavior itself may be a feedback stimulus that activates the area postrema and the NTS. It will be important for us to further investigate this possibility by pharmacological blockade of the pica response using opioid (e.g., naloxone) and non-opioid manipulations (e.g., serotonin antagonists). Nonetheless, the data in the present study indicate that brainstem nuclei that underlie processing of pro-emetic stimuli and gastrointestinal distress were activated by oxycodone.

In conclusion, we provide data that indicate an analgesic dose of oxycodone induces the pro-emetic pica response in rats. Future studies will explore mechanisms in the emetic centers of the brain that underlie the expression of oxycodone-induced pica behavior. In particular, a more fine-tuned analysis of the timing and circuitry that underlie nausea and emesis as compared to gastric distress is warranted. These
symptoms are a major reason for discontinuation of analgesic therapy and non-compliance with drug-taking regimens.
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Authorship Contribution

Participated in research design: Batra and Schrott

Conducted experiments: Batra

Performed data analysis: Batra and Schrott

Wrote or contributed to the writing of the manuscript: Batra and Schrott

Other: Schrott acquired funding for the research.
References


Footnotes

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Legends for Figures

Figure 1. (a) Male and female rats (n=6 per group) have increased intake of cage bedding after oral administration of oxycodone (15 mg/kg). The graph depicts the percent of responses that were positive for bedding intake collapsed across each hour. This intake is sustained for 3 - 4 hr. No bedding intake was found prior to drug treatment or after vehicle administration. Overall females displayed a greater pica response than males. (*p <0.05 vs. baseline within each sex; chi-square analysis). (b) Naloxone blocked or attenuated the pica response to oxycodone in female rats. The 2-mg/kg dose decreased the bedding intake across the 3 hr session, while the 1 mg/kg dose transiently attenuated the response at 2 hr. (* p < 0.05 vs. oxycodone; Tukey post-hoc test). N=6 per group. (c) The selective kappa antagonist Nor-BNI did not alter the oxycodone-induced pica response in female rats. N=4 for saline + oxycodone and n=6 for the Nor-BNI + oxycodone groups.

Figure 2 (a) Oxycodone induced pica manifest as increased kaolin intake in female rats (n=4-6). Kaolin intake was monitored over two 3 hr session across 2 days. Data are represented as kaolin intake in grams. Cisplatin also increased kaolin intake compared to controls. (b) Cisplatin and oxycodone significantly increased the wet weight (g) of the stomach compared to the control group in these female rats. * p< 0.05 in comparison to control post-hoc Tukey’s test. N=5-7 per group.
Figure 3. Immunohistochemical staining for oxycodone-induced c-fos activation in emetic circuitry following oxycodone or cisplatin administration. The area postrema is shown in the top panel and the nucleus of the solitary track (NTS) in the bottom panel. In comparison to control, increased c-fos positive cells were observed in cisplatin- and oxycodone-treated rats 1 hr and 2 hr post-treatment. Magnification 10x.

Figure 4. Quantification of c-fos in emesis-related circuitry following cisplatin or oxycodone (OXY) treatment. Controls were collapsed across saline and water-treated groups. Depicted are the mean ± SEM for the number of c-fos positive cells summed across the anterior, medial, and posterior regions. (a) In the area postrema, cisplatin and both oxycodone time points increased c-fos expression, with the greatest effect 2 hr post-treatment. (b) Similar effects were found in the nucleus of the solitary tract (NTS), where oxycodone and cisplatin increased the number of c-fos positive cells. * p<0.05 vs. control; ^ p <0.05 vs. Cisplatin; # p <0.05 vs. Oxycodone 1 hr. n=10 for Controls; n=4 for cisplatin; n= 5-6 for oxycodone-treated.
Supplemental Data and Supplemental Figures 1-3

Establishment of an antinociceptive dose of oxycodone

Initial studies were done to establish effective antinociceptive doses of oxycodone using oral gavage in male and female rats using the hot plate procedure. We initially used a dose range of 1 – 25 mg/kg with a single time point assessment of 30 min. We then further characterized a time course response at 5 and 15 mg/kg oral oxycodone in males and females (n=8 per sex). Latency to lick the hind paws when the rat was placed on a hotplate (52°C; Ugo Basile, Varese, Italy) was recorded. The maximum latency was set at 30 sec in order to avoid any tissue damage. Rats were subjected to three days of baseline testing wherein one trial per day was performed. On the fourth day, rats received water vehicle only followed by testing conducted at 15 min intervals for 3 hr. On the next day they received 5-mg/kg oxycodone. After a three-day clearance break, they received 15 mg/kg.

As can be seen in Supplemental Figure 1, 5 mg/kg led to a mild and transient degree of antinociception, while 15 mg/kg caused a more robust and sustained response, especially in the females. For females, there was a Treatment effect ($F_{2, 21} = 14.68, p < 0.0001$) and a marginal Treatment x Time interaction ($F_{24, 252} = 2.33, p < 0.0007$). For males, there was a Treatment effect ($F_{2, 21} = 3.34, p = 0.05$) and a marginal Treatment x Time interaction ($F_{24, 252} = 1.51, p = 0.06$). A priori comparisons were done between the two doses and the water vehicle treated groups. For females,
both doses induced antinociception ($F_{1, 14} = 7.92$ and $23.34$, p's <0.02 and .0004 for 5 mg/kg and 15 mg/kg respectively). While for males, only the 15-mg/kg dose was significantly different than controls ($F_{1, 14} = 4.82$, p = 0.05). Thus for the subsequent studies examining the pro-emetic pica response, the 15 mg/kg dose was used.

**Supplemental Figure 1:**

**Females**

**Males**
Oxycodone administration i.p. induces the pica response

We had concerns that pica response was a consequence of the oral route of administration, so an additional experiment was conducted using an i.p. route of administration in female rats. We used two doses of oxycodone, 10 mg/kg and 15 mg/kg, and compared the pica response to female rats injected with saline (n=3-4 subjects per group). Pica was assessed as described in the Methods of the main paper. As shown in supplemental data Figure 2, the pica response was found after i.p. injection of oxycodone (Treatment effect: $F_{2, 7} = 174.0, p <0.0001$; Treatment x Time interaction: $F_{4, 14} = 4.97, p <0.02$). Saline-treated subjects did not exhibit the expression of pica behavior. Both doses of oxycodone i.p. induced the pica response across the 3 hr time frame (* $p <0.05$ vs. respective saline control; Tukey’s post-hoc test).

Supplemental Figure 2:

Naloxone attenuates the antinociceptive response to oxycodone

Before determining that naloxone could block the oxycodone-pica response, we determined the doses of naloxone that were effective in attenuating the antinociceptive response to oxycodone. Antinociception was assessed by the hotplate test as described
Female rats (n=6 per group) were subjected to three days of baseline testing wherein one trial per day was performed. On the fourth day (water test day), rats received vehicle only followed by testing conducted at 15 min intervals for 2 hr. On the test day (24 hr later), rats were pretreated with either vehicle or different doses of naloxone (0.5, 1.0 and 2.0 mg/kg s.c.) followed 10 min later by oxycodone (15 mg/kg oral). Following drug treatment, rats were tested on the hotplate every 15 min during a 2 hr session. Data were analyzed by a repeated measures ANOVA.

There was an overall effect of Treatment ($F_{3, 20} = 4.02 \ p <0.03$) and a (Treatment x Time interaction: $F_{15,100} = 3.65, \ p <0.0001$). As can be seen in supplemental Figure 3, each of the naloxone doses was effective in reducing the efficacy of oxycodone in the hotplate antinociceptive test ($p <0.05$ or lower for each post-hoc comparison). Thus, the dose range of 0.5 – 2.0 mg/kg was used in to determine the involvement of opioid receptors in the oxycodone-induced pica response.

**Supplemental Figure 3:**

![Graph showing latency (sec) vs. Time (hr)](image-url)