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Relative potency of the opioid antagonists naloxone and 6-alpha-naloxol to precipitate withdrawal from acute morphine dependence varies with time post-antagonist

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ABSTRACT

The current study compared the potency of naloxone versus 6-alpha-naloxol to precipitate opioid withdrawal under varying conditions of morphine pretreatment history using suppression of operant responding for food reward as the index of withdrawal. Male Wistar rats trained to respond on a lever for food reward received pretreatment with either Vehicle (Morphine-Naïve), a single subcutaneous (SC) injection of 5.6 mg/kg morphine (Single Morphine), or two morphine injections at 24 h intervals (Repeat Morphine), with varying doses of naloxone or 6-alpha-naloxol injected SC 4 h post-morphine and 5 min prior to the 30 min test session. When responding over the entire 30 min operant session was examined, naloxone was only 5-fold more potent than 6-alpha-naloxol in suppressing operant responding under Morphine Naïve conditions, but this increased to a 65-fold potency difference after Single or Repeat Morphine pretreatment. Examination of the relative potency of these antagonists in the Early Phase of operant testing (5–15 min post-antagonist) revealed an even greater 100-fold potency difference between naloxone and 6-alpha-naloxol, but in the Late Phase of testing (25–35 min post-antagonist), this had declined to a 9-fold potency difference, comparable to the relative potency of naloxone to 6-alpha-naloxol under Morphine–Naïve conditions. The results confirm a differential potency of naloxone to its reduced conjugate 6-alpha-naloxol *in vivo*, and extend the observation of this phenomenon to an acute (single) pretreatment with a low dose of morphine and an additional sign of opioid withdrawal to those previously used. However, the results also indicate that delay in onset of action of 6-alpha-naloxol at opioid receptors in the central nervous system may contribute significantly to its reduced potency relative to naloxone under certain morphine pretreatment conditions.

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1. Introduction

Leftward shifts in opioid antagonist dose-effect functions resulting from opioid agonist exposure are a well-established quantitative index of neuroadaptive changes associated with opioid dependence (Villereal and Castro, 1979; Way et al., 1969). Using this quantitative approach, numerous human and animal studies have revealed that even a single injection of an opioid agonist can elicit a state of "acute dependence" as measured by increased potency of opioid antagonists to precipitate a variety of withdrawal signs ranging from somatic/physiological to affective/subjective (Adams and Holtzman, 1990; Azar et al., 2003; Azorlosa et al., 1994; Bickel et al., 1988; Cheney and Goldstein, 1971; Easterling and Holtzman, 1997; Easterling et al., 2000; Harris and Gewirtz, 2005; Heishman et al., 1989a,b; Kalinichev and Holtzman, 2003; Liu and Schulteis, 2004; Parker and Joshi, 1998; Schulteis et al., 1997; Schulteis et al., 2004, 2003; Shoblock and Maidment, 2006, 2007; Wang et al., 2001; Wang et al., 2004; Young, 1986; Zhang and Schulteis, 2008). As would be expected if acute dependence reflects the early stages in the development of a state of chronic opioid dependence, repeated treatments with morphine at daily or weekly intervals can progressively increase the severity of withdrawal-like signs elicited upon antagonist administration (Adams and Holtzman, 1990; Azorlosa et al., 1994; Liu and Schulteis, 2004; Schulteis et al., 1999; Schulteis et al., 2004, 2003; Zhang and Schulteis, 2008).

At first glance, the most plausible explanation for antagonist-induced precipitation of withdrawal from either acute or chronic morphine would appear to be displacement of agonist from opioid receptors. However, it has been demonstrated that significant somatic (body weight loss), endocrine (increased plasma corticosterone release) and aversive stimulus (conditioned place aversion) indices of withdrawal from acute or chronic morphine pretreatment can be elicited by antagonists given up to 24–48 h post-morphine (Kishioka et al., 1995; Parker and Joshi, 1998; Schulteis et al., 1997; Shoblock and Maidment, 2006, 2007) at a time when there are negligible amounts

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of morphine present in the system (Kishioka et al., 1995). Recently a possible explanation of the ability of opioid antagonists to elicit withdrawal signs in the absence of residual morphine has been offered, based on the observation that mu and delta opioid receptors, like other G-protein-coupled receptors, demonstrate some basal signaling activity as demonstrated by G-protein-coupled second messenger activity in the absence of agonist binding (Burford et al., 2000; Sadee et al., 2005). Pretreatment with agonist can increase the level of basal signaling activity of so-called "constitutively active" mu and delta opioid receptors (Liu and Prather, 2002, 2001; Wang et al., 2001, 2004, 2000). Some opioid antagonists such as the commonly used naloxone and naltrexone actually take on inverse agonist properties at constitutively active opioid receptors, and are able to suppress basal second messenger activity after agonist pretreatment, even when no agonist remains to occupy the receptor (Sadee et al., 2005; Wang et al., 2001, 2004). In contrast, other antagonists such as the mu-selective peptide CTAP, and naloxone/naltrexone conjugates with a reduced C atom in position 6 of the ring portion of the antagonist molecule (6-alpha- and 6-beta-naloxol and naltrexol), remain neutral antagonists, capable of blocking agonist activity at the receptor, but not altering basal signaling in the absence of agonist (Bilsky et al., 1996; Raehal et al., 2005; Sadee et al., 2005; Wang et al., 2001, 2004).

Interestingly, the time course of increased potency of naltrexone to precipitate withdrawal jumping after 3 days of repeated intermittent morphine injection is correlated closely with the time course of increased basal mu opioid receptor signaling in brain tissues removed from mice treated with an identical morphine regimen (Wang et al., 2004). In contrast, the redox-modified naloxone and naltrexone conjugates, which lack inverse agonist properties *in vitro*, precipitate withdrawal jumping after either single treatment with 100 mg/kg morphine or chronic morphine treatment (repeated injection of 20–30 mg/kg or pellet implantation) only at times when agonist remains in the system (e.g. 2–10 h post-morphine), but elicit little if any withdrawal jumping at extended time points (20–48 h), timepoints where naloxone or naltrexone remain effective (Sadee et al., 2005; Shoblock and Maidment, 2006, 2007; Wang et al., 2001, 2004). Similar findings have been reported with additional somatic signs of withdrawal such as paw tremors, wet dog shakes, increased respiration, and increased defecation (Divin et al., 2008; Raehal et al., 2005), and with measures of the aversive motivational consequences of opioid withdrawal (e.g. conditioned place aversion (Shoblock and Maidment, 2006, 2007). Based on these observations, Sadee and colleagues (2005) postulated that an increase in constitutively active mu opioid receptors in response to morphine pretreatment may be a primary factor in the leftward shift in potency of naloxone or naltrexone to precipitate opioid withdrawal.

However, whilst the inverse agonist properties of naloxone and naltrexone after morphine pretreatment versus neutral antagonist properties of 6-alpha- and 6-beta-naloxol and naltrexol as demonstrated *in vitro* are well-established (Bilsky et al., 1996; Sadee et al., 2005; Wang et al., 2001, 2004), it is not entirely clear that relative potency differences of these compounds to precipitate withdrawal *in vivo* are accounted for entirely by an agonist-induced increase in constitutively active opioid receptors. Some argue that constitutive opioid receptor activity is a "prerequisite mechanism involved in acute opioid withdrawal" (Freye and Levy, 2005), and there is evidence that weak inverse agonists or neutral antagonists exhibit little ability to precipitate somatic withdrawal at lower doses of morphine, but do elicit withdrawal after high dose morphine pretreatment (Walker and Sterious, 2005). However, others have argued that differential rate of access to opioid receptors in the central nervous system (CNS) may account for differences in potency of antagonists such as naltrexone and 6-beta-naltrexol *in vivo* (Divin et al., 2008).

The current study sought to further characterize the conditions under which the antagonists naloxone and 6-alpha-naloxol show differential potency in their ability to precipitate withdrawal following acute morphine pretreatment *in vivo*. Naloxone and its conjugate 6-alpha-naloxol were chosen based upon our laboratory's extensive characterization of naloxone-precipitated opioid withdrawal as measured by numerous somatic (Criner et al., 2007; Schulteis et al., 1999, 1997) and negative emotional/aversive indices (Azar et al., 2003; Criner et al., 2007; Liu and Schulteis, 2004; Schulteis et al., 2004, 2003; Zhang and Schulteis, 2008). Because 6-alpha-naloxol was made available to us in only limited supply from the Research Resources Drug Supply System of the National Institute on Drug Abuse (NIDA, Bethesda, MD, USA), a single index of withdrawal, suppression of operant responding for food reward, was carefully chosen from among those we have characterized, based on the following advantages:

1) Suppression of operant responding has been repeatedly demonstrated to be well suited to quantitative analysis of the relative magnitude of leftward shift in antagonist potency under varying morphine pretreatment conditions (Adams and Holtzman, 1990; Schulteis et al., 1997; Schulteis et al., 1994, 2004, 2003; Young, 1986);
2) Antagonist-induced suppression of operant responding is observed after pretreatment with very low doses of morphine (1.0–5.6 mg/kg; [Schulteis et al., 1997, 2004, 2003] relative to those used in prior acute dependence studies (20–180 mg/kg; [Divin et al., 2008; Raehal et al., 2005; Sadee et al., 2005; Walker and Sterious, 2005; Wang et al., 2001, 2004]), thereby permitting a direct test of the hypothesis that weak inverse agonists or neutral antagonists cannot effectively precipitate withdrawal after low dose agonist pretreatment (Walker and Sterious, 2005); and
3) Collection of data in discrete epochs across the 30 min operant session enables examination of possible time-dependent differences in naloxone versus 6-alpha-naloxol potency (e.g. early [Min 1–10] versus late [Min 21–30] phases of testing), thereby providing further evaluation of the possibility that differential rate of access of these compounds to opioid receptors in the CNS may contribute to observed *in vivo* potency differences for withdrawal precipitation.

2. Materials and methods

2.1. Animal subjects

Male Wistar rats (n=109, Harlan Labs, Livermore, CA, USA) weighing 300–400 g at the time of testing were used. All rats were group housed (2–3/cage) in a temperature- and humidity-controlled room with a 12 h light/12 h dark cycle (lights on at 06:00). Once operant training began, rats were maintained on 15 g/rat of standard rat chow per day in addition to the food pellets earned in the operant boxes (total food intake was approximately 20–22 g/rat/day), but had ad *libitum* access to water at all times except during the 30 min operant sessions. All training and testing took place between 12:00 and 16:30. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System, an AAALAC-accredited facility, and are in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (revised 1996).

2.2. Drugs

Morphine sulfate and 6-alpha-naloxol HCl were generously provided by the Research Resources Drug Supply System of the National Institute on Drug Abuse (NIDA, Bethesda, MD, USA), and naloxone HCl was purchased from Sigma (St. Louis, MO, USA). All drugs were prepared for injection in physiological saline, and all injections were made subcutaneously (SC) in a volume of 0.1 ml/100 g body weight. Morphine was administered at a dose of 5.6 mg/kg, selected from earlier work demonstrating effective induction of acute

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