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Affective and Neuroendocrine Effects of Withdrawal from Chronic, Long-Acting Opiate Administration

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Abstract

Although the long-acting opiate methadone is commonly used to treat drug addiction, relatively little is known about effects of withdrawal from this drug in preclinical models. The current study examined affective, neuroendocrine, and somatic signs of withdrawal from the longer-acting methadone derivative *l*-alpha-acetylmethadol (LAAM) in rats. Anxiety-like behavior during both spontaneous and antagonist-precipitated withdrawal was measured by potentiation of the startle reflex. Withdrawal elevated corticosterone and somatic signs and blunted circadian variations in baseline startle responding. In addition, fear to an explicit, Pavlovian conditioned stimulus (fear-potentiated startle) was enhanced. These data suggest that anxiety-like behavior as measured using potentiated startle responding does not emerge spontaneously during withdrawal from chronic opiate exposure – in contrast to withdrawal from acute drug exposure – but rather is manifested as exaggerated fear in response to explicit threat cues.

Keywords

acoustic startle; opiate dependence; corticosterone; LAAM; fear-potentiated startle; opiate withdrawal

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1. Introduction

Methadone is a synthetic opiate commonly used to treat drug addiction (Cushman and Dole, 1973; Strain et al., 1999). Methadone has a slower onset and longer duration of action than commonly abused opiates (e.g., heroin). This pharmacokinetic profile helps avoid the repeated cycles of euphoria, withdrawal-induced negative emotionality, and heightened stress reactivity associated with shorter-acting opiates, and contributes to methadone's effectiveness in drug addiction pharmacotherapy (Kreek, 1992; 2000).

Owing to substantial individual differences in pharmacokinetics, however, a proportion of methadone-maintained patients do experience withdrawal when administered the drug on a daily basis (Eap et al., 2002; Hiltunen et al., 1995). Furthermore, methadone users, like other opiate users, show high rates of anxiety and depression (Rounsaville et al., 1985), which can be exacerbated by rapid methadone detoxification (Gold et al., 1979). It is therefore important to characterize more fully the negative emotional states produced during withdrawal from long-acting opiates. The current study investigated the consequences of withdrawal from a long-acting opiate in rats on behavioral measures of anxiety, stress hormone responses, circadian rhythms, and somatic signs.

In this study, withdrawal-induced anxiety was indexed by potentiation of the acoustic startle reflex, an established measure of fear in rodents (Davis, 1992). This measure is well suited as a behavioral tool to investigate the negative affective sequelae of opiate exposure for several reasons. First, the startle reflex can be measured repeatedly in the same subjects (Harris et al., 2004), making it suitable for studying within-subject changes of state across time (Beswick et al., 2003; Rounsaville et al., 1985). Second, the anatomical circuit mediating the startle reflex is relatively simple and well characterized (Lee et al., 1996; Nodal and Lopez, 2003), allowing circuitry involved in modulating startle during withdrawal to be more easily identified. Third, an exaggerated startle response is characteristic of anxiety disorders and drug withdrawal in humans (Dichter and Tomarken, 2008; Krystal et al., 1997; Lang and McTeague, 2009; Orr et al., 2002). Thus, startle has strong utility as a translational measure in anxiety and addiction research.

The startle reflex is potentiated robustly and reliably during withdrawal from acute or intermittent opiate administration, or opiate self-administration (Harris and Gewirtz, 2004; Kalinichev and Holtzman, 2003; Park et al., 2013), and these effects are blocked by anxiolytic drugs (Harris and Gewirtz, 2004; Rothwell et al., 2009). Curiously, however, several studies have reported that withdrawal from chronic, continuous opiate exposure produces either no effect (Fendt & Mucha, 2001) or reductions in the startle reflex (Kalinichev and Holtzman, 2003; Mansbach et al., 1992; but see Higgins and Sellers, 1994). In contrast, other measures of anxiety, as well as of dysphoria and anhedonia, are expressed under similar circumstances (Schulteis et al., 1994; Schulteis et al., 1998). This apparent anomaly may be related to the way in which anxiety is elicited in different behavioral paradigms. Where anxiety has been observed during withdrawal from continuous opiate exposure, the behavioral measure has been evoked in the presence of anxiogenic or fear-eliciting stimuli (Fendt and Mucha, 2001; Harris and Aston-Jones, 2003). Hence, the current study examined potentiation of startle in the presence of threat cues (i.e., fear-potentiated

startle), as well as in their absence (baseline startle). Anxiety generated under these two conditions may be mediated by different neural substrates (Koob and Zorrilla, 2012).

Studies of chronic opiate dependence in rodents typically precipitate a sudden state of withdrawal using an opiate antagonist such as naloxone. Since withdrawal in humans is not typically induced through administration of an opiate antagonist, measures of “spontaneous” withdrawal after cessation of drug treatment may have greater direct relevance to clinical psychopathology (Rothwell et al., 2009). To broaden the applicability of our results, both spontaneous and precipitated withdrawal paradigms were used in these studies. Furthermore, we measured plasma levels of the steroidal stress hormone corticosterone, as well as using startle to assess alterations in circadian rhythms. Both are common outcome measures of opiate withdrawal (e.g., Becker et al., 2008; Beswick et al., 2003; Li et al., 2010) and depression (for a review, see Kronfeld-Schor and Einat, 2012).

Chronic opiate treatment in these studies was achieved using *l*-alpha-acetylmethadol (LAAM) rather than methadone. Methadone has a half-life in rats of approximately 3 hrs (Misra et al., 1973). By contrast, the active metabolites of LAAM have a half-life of more than 24 hrs in rats, more closely resembling the half-life of methadone in humans (Henderson et al., 1977). Therefore, although no longer available for clinical purposes in the United States (U.S. Food and Drug Administration, 2013), LAAM was used to maintain a pattern of opiate exposure that more closely mirrors the time course of drug exposure in methadone-treated patients. We also used two procedures for inducing dependence. The first was closely based on our previous studies with LAAM (Hamilton et al., 2005), whereas the second resembled the chronic, continuous opiate delivery procedures used with other opiates (Fendt and Mucha, 2001; Kalinichev and Holtzman, 2003). Using these approaches, our results suggest that withdrawal from a long-acting opiate induces a negative affective state, but one that is distinct from the state induced during withdrawal from intermittent opiate exposure.

2. Results

2.1 Experiment 1: Precipitated Withdrawal Following 21 Days of LAAM

2.1.1 Weight change during induction of dependence—Rats administered LAAM gained weight more slowly than water-treated rats. In Experiment 1, there was a significant effect of LAAM treatment on weight gain ($F(1, 39) = 15.13, p = .000$), a significant effect of day of treatment ($F(10, 390) = 104.83, p = .000$), and a LAAM treatment x day interaction ($F(10, 390) = 23.15, p = .000$). Follow-up t-tests revealed that LAAM-treated rats gained less weight than water-treated rats during the last half of drug treatment (Figure 1). LAAM produced a similar suppression of weight gain in Experiments 2–5 (data not shown).

2.1.2 Withdrawal measures—Startle was not affected by LAAM and naloxone treatment ($p = .83$; Table 1). There was no potentiation of the startle response above the Water + 0.5 NX group in any of the other groups during withdrawal.

In contrast, there was a naloxone dose-related increase in the incidence of diarrhea (Table 1). Rats administered the higher doses of naloxone displayed diarrhea more frequently than

rats administered lower doses of naloxone or saline following LAAM or rats administered water and a low dose of naloxone ($\chi^2(6, N = 41) = 25.93, p = .000$). Moreover, Table 1 shows that there was a naloxone dose-related increase in weight loss during withdrawal ($F(6, 34) = 4.86, p = .001$). Follow-up Fisher's PLSD tests indicated that rats administered LAAM plus any of the three highest doses of naloxone lost more weight than rats treated with water and a low dose of naloxone.

There was also a significant effect of opiate withdrawal on corticosterone ($F(6, 33) = 4.08, p = .004$). Table 1 shows that administration of LAAM plus all but the lowest dose of naloxone significantly increased corticosterone compared to administration of water plus naloxone. There was no difference in corticosterone concentration between the LAAM-saline group and the water-naloxone group.

2.2 Experiment 2: Precipitated Withdrawal Following 7 Days of LAAM

2.2.1 Withdrawal measures—The startle response was not altered significantly by naloxone-precipitated withdrawal following 7 days of LAAM treatment ($p = .19$). Due to high variability within the treatment groups, treatment with LAAM + naloxone produced a similar % change in startle as treatment with LAAM + saline, water + naloxone, and water + saline (Table 2). Opiate withdrawal produced greater weight loss than LAAM or naloxone treatments or vehicle administration ($F(3, 20) = 5.44, p = .007$). Follow-up Fisher's PLSD tests indicated that rats treated with LAAM + naloxone lost a greater percentage of their body weight than rats treated with either water + saline or water + naloxone. There was no significant difference between rats treated with LAAM + naloxone versus LAAM + saline (Table 2). The presence of diarrhea was also affected by opiate withdrawal ($\chi^2(3, N = 24) = 24.00, p = .000$). Diarrhea was only observed in animals given both LAAM + naloxone (Table 2). Opiate withdrawal increased corticosterone ($F(3, 16) = 6.80, p = .004$). LAAM + naloxone treatment significantly increased the concentration of corticosterone compared to the other groups (Table 2).

2.3 Experiment 3: Spontaneous Withdrawal from 21 Days of LAAM

2.3.1 Withdrawal measures—Animals treated with water for 21 days showed normal diurnal variation in startle (see, e.g., Chabot and Taylor, 1992; Frankland and Ralph, 1995) following cessation of treatment, with startle elevated at 0800 relative to startle at 1800 during 3 circadian cycles (Figure 2, top panel). However, animals withdrawing from either dose of LAAM showed blunted diurnal variation in their startle response, lacking the morning peak observed in control animals approximately 70 hrs after the last treatment (0800 on withdrawal day (W)2; Figure 2, top panel). Although there was no main effect of LAAM treatment ($p = .14$) or significant effect of withdrawal day ($p = .07$), there was a main effect of time of day ($F(1, 29) = 11.75, p = .002$). There was also a significant interaction between time of day and LAAM treatment ($F(2, 29) = 3.91, p = .03$). No other interactions were significant. Follow-up ANOVAs indicated that there was a significant effect of LAAM treatment at 70 hrs ($F(2, 31) = 3.99, p = .03$), but at no other time point. At the 70-hr time point (0800 on W2), rats treated with either dose of LAAM displayed significantly blunted startle compared with water-treated rats (follow-up Fisher's PLSD).

Separate one-way ANOVAs revealed no significant differences between the groups at 0800 on the final two days ($p > .1$), indicating that the blunted response was transient.

Over the 6 days of withdrawal, there was an effect of LAAM treatment on % change in weight ($F(2, 29) = 10.06, p = .001$; Figure 2, bottom panel) and a significant effect of day of withdrawal ($F(5, 145) = 14.72, p = .000$). There was no day x treatment interaction ($p = .15$). Follow-up ANOVAs followed by Dunnett's *t* tests indicated that rats treated with either dose of LAAM had reduced weight gain on the second day of withdrawal.

At the peak of the behavioral effect (i.e., 70 hrs after cessation of LAAM treatment; 0800 on W2), a subset of rats ($n = 8/\text{treatment}$) was sacrificed to determine the corticosterone response. There was an effect of LAAM treatment ($F(2, 21) = 5.63, p = .01$). Those rats previously treated with 0.5 mg/kg or 1.0 mg/kg LAAM showed a higher corticosterone concentration than rats treated with water (Figure 3, top panel). These animals showed a nonsignificant reduction in the startle response (Figure 3, bottom panel; $F(2, 21) = 2.00, p = .16$), but the pattern of responding was similar to that of the larger group evaluated across days.

2.4 Experiment 4: Spontaneous Withdrawal from 7 Days of LAAM

2.4.1 Withdrawal measures—There was a significant main effect of time ($F(1, 10) = 41.01, p = .000$) on the diurnal variation of startle, but no significant effects of LAAM treatment ($p = .51$) or day ($p = .08$). There was also a significant withdrawal day x treatment interaction ($F(5, 50) = 2.68, p = .03$). No other interactions were significant. Follow-up *t*-tests indicated that the effects were driven by a significant effect of LAAM on startle at 0600 on the second day of testing (Figure 4, top panel; 42 hr after LAAM cessation; $t(10) = 3.15, p = .01$). The startle response was blunted by LAAM treatment at this time, but there were no differences between the two groups at any other time point.

Rats were assessed for changes in weight over 6 days following cessation of LAAM treatment. There were significant main effects of LAAM treatment ($F(1, 10) = 26.19, p = .000$) and day ($F(5, 50) = 32.11, p = .000$), and a significant treatment x day interaction ($F(5, 50) = 6.88, p = .000$). Change in weight was significantly less in LAAM-treated than water-treated rats throughout the entire withdrawal period (Figure 4, bottom panel). Although weights began to recover, they did not return to pre-withdrawal levels. Water-treated animals maintained a stable weight gain throughout the withdrawal period.

2.5 Experiment 5: Fear-potentiated Startle During Spontaneous Withdrawal from 7 Days of LAAM

2.5.1 Fear-potentiated startle during withdrawal—Fear-potentiated startle was assessed twice during spontaneous withdrawal, once on the second day and once on the sixth day. Results from Experiments 3 and 4 indicated that differences should exist on day 2 but not day 6 (see Figures 2 and 4). Therefore, separate treatment (water vs LAAM) x time (0600 vs 1800) between-subjects ANOVAs were conducted separately on data from each day. On day 2 of withdrawal, there were significant main effects of treatment ($F(1, 25) = 4.51, p = .044$) and time ($F(1, 25) = 10.28, p = .004$), and a significant treatment x time

interaction ($F(1, 25) = 4.29, p = .05$). Although not significantly different at 0600 ($p = .96$), fear-potentiated startle in LAAM-treated animals was 2.0-fold greater than in water-treated animals at the 1800 time point (Figure 5, top panel; $p = .03$). On day 6 of withdrawal, there were no significant main effects of treatment ($p = .30$) or time ($p = .34$), or an interaction ($p = .91$). Consistent with Experiments 3 and 4, the behavioral effects had dissipated by day 6 of withdrawal (Figure 5, bottom panel).

3. Discussion

The current experiments examined the consequences of both spontaneous and naloxone-precipitated withdrawal from LAAM, a long-acting methadone derivative. Expression of fear-potentiated startle – an established measure of fear and anxiety in a number of mammalian species, including humans – was enhanced substantially during withdrawal from LAAM. Withdrawal also caused a surge in circulating levels of the stress hormone corticosterone, a disruption in startle's circadian rhythm, diarrhea, and weight loss.

Not only are these neuroendocrine and behavioral outcomes consistent with previous reports of a robust LAAM withdrawal syndrome in rats and primates (e.g., Sell et al., 2005; Young et al., 1979), but also with the effects seen during withdrawal from other opiate drugs. Thus, weight loss during both spontaneous and precipitated LAAM withdrawal is congruent with other reports of opiate withdrawal (e.g., Cobuzzi and Riley, 2011; Houshyar et al., 2004; Ikonov et al., 1984; Langerman et al., 2001; Pinter-Kubler et al., 2013). Similarly, the blunting of circadian startle modulation during spontaneous withdrawal is consistent with disruptions of other behavioral measures of circadian periodicity during withdrawal from chronic opiate administration (Brady and Holtzman, 1981; Li et al., 2010; Stinus et al., 1998; Thornhill et al., 1976; van der Laan and de Groot, 1988).

In the current studies, this circadian disruption was accompanied by an elevation in levels of circulating corticosterone. Both disruptions of sleep/wake cycles and persistent elevations in circulating cortisol are common characteristics of depression (Beswick et al., 2003; Tsuno et al., 2005; Young et al., 1994). Thus, it is possible that this constellation of signs indicates an underlying state of dysphoria/anhedonia (De Vries and Shippenberg, 2002; Diana et al., 1995; Harris and Aston-Jones, 2007), although such a relationship would require confirmation using more direct tests (e.g., alterations in thresholds for intracranial self-stimulation).

The substantial increase in potentiated startle to Pavlovian conditioned cues observed here suggests that animals in withdrawal from LAAM are also prone to exaggerated fear. A similar effect has been reported during withdrawal from chronically administered fentanyl (Fendt & Mucha, 2001). In contrast, whereas withdrawal from intermittently administered opiates produces a robust increase in baseline startle (Harris and Gewirtz, 2004; Kalinichev and Holtzman, 2003; Rothwell et al., 2009), such an effect was not seen after chronic exposure to LAAM in the current studies, nor after chronic exposure to other opiates (Fendt and Mucha, 2001; Kalinichev and Holtzman, 2003; Mansbach et al., 1992; but see Higgins and Sellers, 1994).

One difference between fear-potentiated startle (which is increased after chronic opiate exposure) and withdrawal-potentiated startle (which is observed after repeated, acute opiate administration) is the requirement in the former case of an explicit, anxiogenic stimulus for anxiety-like behavior to be manifested. Indeed, other demonstrations of anxiety-like behavior during withdrawal from chronic opiates have also been obtained using explicit, fear-eliciting stimuli (i.e., exposure to a shock-probe, to the lighted portion of a light/dark box, or to the open arms of a plus-maze; Castilho et al., 2008; Harris and Aston-Jones, 2003; Schulteis et al., 1998; Zhang and Schulteis, 2008). Of particular relevance to the behavioral measures used here, withdrawal from chronic, continuous nicotine potentiated startle only when startle was elicited in the presence of a bright (and hence anxiogenic) light (Jonkman et al., 2008). The startle reflex was similarly enhanced in the presence of bright light, but not in the dark, during cocaine withdrawal (Bijlsma et al., 2010).

Taken as a whole, these findings suggest that spontaneous expressions of anxiety versus excessive anxiety-like responses to an acute stressor (e.g., a conditioned or unconditioned fear stimulus) reflect qualitatively distinct states that are induced differentially, depending on drug and dosing regimen (Koob and Zorrilla, 2012). This may be clinically relevant, given that individuals diagnosed with different subtypes of anxiety disorder (e.g., phobia versus posttraumatic stress disorder) are also distinguished by whether startle is exaggerated in the presence versus absence of explicit, anxiogenic cues (Grillon, 2002; Lang and McTeague, 2009).

In summary, these studies demonstrated robust affective, neuroendocrine, and weight-reducing effects of withdrawal from the long-acting opiate LAAM. The efficacy of opiate maintenance therapy is at least partially based on the sustained pharmacokinetics of treatment drugs such as methadone. However, the elimination half-life of methadone shows dramatic individual differences, so that a degree of withdrawal is a daily occurrence in a subgroup of patients (Ferrari et al., 2004). These patients are the most likely to continue to use illicit drugs (Nilsson et al., 1982). Therefore, developing a greater understanding the specific, negative affective sequelae of long-acting opiates, as well as other clinically significant aspects of opiate withdrawal (e.g., hyperalgesia; Harris et al., 2004; Laulin et al., 1998), is an important step towards developing more effective treatments for opiate addiction.

4. Method

4.1 Animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 300–450g at the beginning of the experiment served as subjects. Rats were group-housed 6–8 per cage (40.6 cm x 58.6 cm x 25.4 cm) and were maintained on a 12:12-hr light:dark cycle with lights on at 0800. Food and water were freely available throughout the experiments. Animals were habituated to the vivarium for approximately 2 weeks prior to any experimentation. The rats were then handled daily and habituated to experimental procedures, including oral gavage. All experimental procedures conformed to the *Guidelines for the Care and Use of Laboratory Animals* and were approved by the University of Minnesota Animal Care and Use Committee.

4.2 Drugs

4.2.1 LAAM—The long-acting opiate LAAM HCl (National Institute on Drug Abuse - Research Triangle Institute, Research Triangle Park, NC) was dissolved in water, filtered using a 0.2- μ m filter, and administered via oral gavage (volume = 1.0 ml/kg). Two dosing regimens were used in the present study. In experiments 1 and 3, rats were administered LAAM on days 0, 3, 6, 8, and 10–21 (based on dosing procedures successfully used previously; Hamilton et al., 2005; Lichtblau and Sparber, 1983). In experiments 2, 4, and 5, rats were administered LAAM for 7 consecutive days.

4.2.2 Naloxone—The opiate antagonist naloxone HCl (NX; Sigma, St. Louis, MO) was dissolved in 0.9% saline solution and administered intraperitoneally (volume = 1.0 ml/kg). Naloxone was administered at a range of doses: 0.004, 0.02, 0.1, 0.5, or 2.5 mg/kg in experiments 1 and 2.

4.3 Acoustic Startle

4.3.1 Apparatus—Animals were tested in four identical stabilimeter devices (7.5 cm x 9.0 cm x 17.0 cm) made of Plexiglas cages on four compression springs in sound-attenuating chambers. Cage movement resulted in displacement of a Type 338B35 accelerometer (PCB Piezotronics, Depew, NY) attached to the top of the cage. The accelerometer voltage was amplified by a signal processor (Model 482A20; PCB Piezotronics) and was proportional to the velocity of cage displacement. An Instrunet 100b board (GW Instruments, Somerville, MA) interfaced to a Macintosh PC digitized the analog output of the accelerometer on an arbitrary scale of 0–1000 units. Startle amplitude was defined as the peak accelerometer voltage that occurred during the first 200 ms after the onset of the startle stimulus. High frequency speakers (Radio Shack Supertweeters, range 5–40 kHz) located 5 cm from the cage delivered the 50-ms (rise-decay: 5-ms) bursts of white noise (low pass, 22 kHz) at 90, 95, 100, and/or 105 dB. White noise through the high frequency speakers, together with the ventilation system, elevated the background noise to 58 dB.

The footshock was a 0.5-s, 0.8-mA constant current scrambled shock delivered by a shock generator (SGS-004, BRS-LVE) through the four bars that made up the bottom of the stabilimeter device. Shock intensity was measured with a 1-k Ω resistor connected between two floor bars in each stabilimeter device. Current was defined as the root mean square voltage across the k Ω resistor where $\text{mA} = 0.707 \times 0.5 \text{ peak-to-peak voltage}$. The conditioned stimulus (CS) was a 4-s light, emitted from a bank of 4 LED bulbs, situated approximately 30 cm from the stabilimeter device.

4.3.2 Baseline startle procedure—Animals were grouped into treatments according to their startle responses on the second day of testing to ensure similar mean basal startle responses in each group. A test session consisted of 5-min habituation followed by 20 min of testing. During testing, an acoustic startle stimulus was presented every 30 s, with equal numbers of stimuli of each intensity presented in a semi-random order during the session. In all analyses presented, startle response was calculated as mean startle amplitude collapsed across all decibel levels (Harris and Gewirtz, 2004).

4.3.3 Fear-potentiated startle procedure—Fear conditioning and testing were conducted as described previously (Burman and Gewirtz, 2004). Briefly, rats were habituated to the chambers and startle stimuli as above. Following assignment to drug treatment groups, rats were classically conditioned using a 4-s light CS that coterminated with a 0.5-s, 0.8-mA footshock. The training session consisted of a 5-minute habituation period followed by 12 pairings of the light with the footshock (mean interval between shocks = 2 min, range = 1.5 – 2.5 min).

The test began with a 5-min preperiod followed by a startle habituation protocol comprised of 30 startle stimuli (15 at each of 2 intensities: 95 dB and 105 dB) presented in a semi-random order. Following this, test trials consisted of startle stimuli (95 and 105 dB) presented either alone or 3.5 s after onset of the light CS in a semi-random order, with a 30-s interval between successive startle stimuli.

4.4 Corticosterone Assay

Concentrations of corticosterone in plasma samples were measured using an OCTEIA Corticosterone assay kit (Immunodiagnostic Systems, Fountain Hills, AZ), in accordance with the manufacturer's instructions. Samples were analyzed in duplicate. Briefly, samples were diluted 1:10 in sample diluent. Diluted samples and the enzyme conjugated to horseradish peroxidase were added to each well. Following incubation, the plates were washed, and substrate solution was added to the wells. A stop solution of hydrochloric acid was added, and the absorbance was read at 450 nm using a standard plate reader (Multiskan MCC, Thermo, Waltham, MA). Standards were used to generate a curve from which sample values were interpolated. These values were subsequently converted and expressed as ng/ml plasma. Assay sensitivity was 0.55 ng/ml, and the assay standards ranged from 0 to 186 ng/ml. The intra-assay coefficient of variation was 3.22%, and the inter-assay coefficient of variation was 6.69%.

4.5 Experiment 1: Precipitated Withdrawal Following 21 Days of LAAM

Following 2 days of baseline startle testing, either 1.0-mg/kg LAAM (n = 36 rats) or an equal volume of filtered tap water (n = 5 rats) was administered via oral gavage. LAAM was administered on for 21 days, following the procedure described in Section 4.2.1. On the last day of drug administration (Day 21), rats were re-exposed to the startle apparatus and procedure. On Day 22, a startle test was performed, to serve as a comparison for the withdrawal-induced startle changes (Harris & Gewirtz, 2004). One hr and 20 min later, all rats were weighed and injected with naloxone (0 (n = 6), 0.004 (n = 6), 0.02 (n = 5), 0.1 (n = 7), 0.5 (n = 5), or 2.5 (n = 7) mg/kg for LAAM-treated rats; 0.5 mg/kg for water-treated rats (n = 5)). A similar range of naloxone doses does not affect startle responding, corticosterone, or somatic signs in opiate-naïve animals (Eisenberg and Sparber, 1979; Harris and Gewirtz, 2004; Hijzen et al., 1995). Ten min after the naloxone injection, a final test was given to measure the startle response during withdrawal. To determine the presence of gastrointestinal motility, a prominent somatic sign of withdrawal, rats were weighed and assessed for the presence of diarrhea immediately following completion of the startle test. The rats were then decapitated immediately, and trunk blood was collected (between 1230 and 1330). Blood samples were combined with 15% w/v EDTA solution and stored on ice

until centrifugation. Plasma was aliquoted and frozen at -80°C until analysis for corticosterone concentration.

4.6 Experiment 2: Precipitated Withdrawal Following 7 Days of LAAM

Following startle measures for 2 days, water or 1.0 mg/kg LAAM was administered for 7 consecutive days ($n = 12/\text{treatment}$). On the eighth day, startle was assessed and rats were injected with either saline ($n = 6$ rats from each previous treatment) or 0.5 mg/kg naloxone ($n = 6$ rats from each previous treatment). Ten min later, rats were re-assessed for startle to determine if there was a change in startle during withdrawal. To determine if changes in weight occurred as well, rats were weighed before and after the startle session. Rats were also assessed for the presence of diarrhea as a marker of the withdrawal syndrome. Immediately after withdrawal assessment (i.e., between 1230 and 1330), rats were rapidly decapitated without anesthesia to obtain trunk blood. Blood samples were treated as in Experiment 1.

4.7 Experiment 3: Spontaneous Withdrawal Following 21 Days of LAAM

Two doses of LAAM were used in this experiment: 1.0 mg/kg and 0.5 mg/kg, similar to doses used previously (Hamilton et al., 2005). As in Experiment 1, the first two startle sessions were first conducted to habituate the animals to the startle apparatus. Either LAAM ($n = 19$ rats/dose) or water ($n = 18$ rats) was administered after the second startle session (Day 0 of drug treatment). Treatment continued on days 3, 6, 8, and 10–21, as described in Section 4.2.1. On Day 21 of drug administration and on the morning after the last drug administration, rats were re-exposed to the startle apparatus and given an additional startle session. Beginning at 1800 on the last day of drug treatment (6 hrs after the last LAAM administration), startle was assessed toward the end (1800) and at the beginning (0800) of the light period of the light:dark cycle for three circadian cycles ($n = 8$ rats/treatment). Following the startle testing at 0800 (i.e., approximately 70 hrs after the last LAAM administration) these rats were rapidly decapitated without anesthesia. Blood samples were collected and treated as in Experiment 1. Startle was assessed for two more days in the morning (0800) among the remaining rats ($n = 11$ rats/LAAM dose, $n = 10$ rats/water).

4.8 Experiment 4: Spontaneous Withdrawal From 7 Days of LAAM

Startle was assessed on 2 days prior to drug administration. After assignment to groups, animals were treated with water or 1.0 mg/kg LAAM for 7 consecutive days ($n = 6$ rats/treatment). On the first and final days of LAAM treatment animals were assessed just prior to lights on (0600) and lights off (1800). Following cessation of LAAM or water treatment, rats were again assessed at 0600 and 1800 for 6 days. The shift in timing of startle assessment from 0800 to 0600 was due to the variability of startle responding present at this time point in Experiment 2.

4.9 Experiment 5: Fear-potentiated Startle During Spontaneous Withdrawal from 7 Days of LAAM

Startle was assessed on 2 days prior to drug administration. After assignment to treatment groups, animals underwent Pavlovian conditioning using 12 light-shock pairings. Rats began

either water (n = 14) or 1.0 mg/kg-LAAM treatment (n = 15) the following day. All LAAM administrations took place at 1200 hrs. Fear-potentiated startle was assessed at 0600 (n = 8 rats/ LAAM, n = 7 rats/water) or 1800 (n = 7 rats/treatment) on the second and sixth day following cessation of LAAM treatment.

4.10 Statistics

Primary withdrawal outcomes were withdrawal-potentiated startle (measured as withdrawal test – pretest)/pretest x 100), fear-potentiated startle during withdrawal (measured as startle in presence of CS – startle presented alone)/startle presented alone x 100), % changes in weight, corticosterone levels (ng/ml), and presence of diarrhea. Two subjects showing extreme startle values (>2.0 standard deviations from the mean) in Experiment 5 were defined as outliers and excluded from analysis, per a priori criteria (Burman and Gewirtz, 2004; Cousineau and Chartier, 2010; Geyer and Swerdlow, 1998; Rothwell et al., 2010). In general, data were analyzed using ANOVAs followed by t-tests, Fisher's PLSD tests, or Dunnett's t tests, as appropriate. Preliminary analysis of startle data revealed no main effects on potentiated startle, or interactions, as a function of startle stimulus intensity. This is consistent with other measures of potentiated startle (Walker and Davis, 1997), and hence this factor was excluded from further analyses. Presence of diarrhea was compared between groups using Chi-square analysis.

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Abbreviations

ANOVA	analysis of variance
CRF1	corticotrophin releasing factor-1
CS	conditioned stimulus
HPA	hypothalamic-pituitary-adrenal
LAAM	<i>l</i> -alpha-acetylmethadol
NX	naloxone

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Highlights

- Effects of withdrawal from the long-lasting opiate LAAM were evaluated in rats.
- Circadian variation of the startle reflex was blunted during LAAM withdrawal.
- Corticosterone levels were elevated during LAAM withdrawal.
- Fear-potentiated startle was elevated during LAAM withdrawal.
- LAAM withdrawal produced neuroendocrine, circadian, and anxiety-like effects.

Figure 1.

Over 21 days of LAAM treatment in Experiment 1, LAAM-treated rats ($n = 36$) gained less weight than water-treated rats ($n = 5$). Mean $\pm$ SEM are depicted. * $p < .05$ compared with water-treated rats at the same time point (ANOVA).

Figure 2.

Rats undergoing spontaneous opiate withdrawal after 21 days of LAAM treatment were assessed for changes in startle responding and weight in Experiment 3 ($n = 10$ rats/water, $n = 11$ rats/LAAM dose). Mean $\pm$ SEM are depicted. Top: Rats showed blunted circadian variation in startle responses during spontaneous opiate withdrawal, especially at 0800 on W2. * $p < .05$ 0.5 LAAM compared with water; † $p < .05$ 1.0 LAAM compared with water (ANOVA). Bottom: LAAM-treated rats experienced significant weight loss during

spontaneous withdrawal compared to water-treated rats. * $p < .05$ 0.5 LAAM compared with water; † $p < .05$ 1.0 LAAM compared with water (Dunnett's t).

Figure 3.

A subset of animals was assessed for HPA axis activity at the blunting of the circadian peak ($n = 8$ rats/group) during spontaneous withdrawal after 21 days of LAAM treatment in Experiment 3. These rats were also assessed for their startle response at the same time point. Mean $\pm$ SEM are depicted. Top: Treatment with 0.5 or 1.0 LAAM produced higher levels of corticosterone in plasma; $*p < .05$ compared to water (Fisher's PLSD). Bottom: Startle responding did not differ significantly between groups.

Figure 4.

Changes in circadian variation of startle and weight were assessed during spontaneous opiate withdrawal following 7 days of LAAM administration in Experiment 4. Mean $\pm$ SEM are depicted. Top: Water-treated rats ($n = 6$) showed circadian cyclicity in startle responding, but withdrawing rats ($n = 6$) showed a transient blunting of the morning peak (0600 on W2). * $p < .05$ compared with water at the same time point (ANOVA). Bottom: On withdrawal days (WD) 1 and 2, withdrawing rats lost weight, whereas water-treated rats gained weight ($n=6$ per group). * $p < .05$ compared with water (Fisher's PLSD).

Figure 5.

Fear-potentiated startle during spontaneous withdrawal in Experiment 5. Fear-potentiated startle was assessed on days 2 and 6 of spontaneous withdrawal at 0600 ($n = 8$ rats/LAAM; $n = 7$ rats/water) and 1800 ($n = 7$ rats/treatment). Mean $\pm$ SEM are depicted. * $p < .05$ compared with water (t-test). Top: Percent fear-potentiated startle was elevated significantly in rats withdrawing from LAAM at 1800 on Withdrawal Day 2. Bottom: On day 6 of

withdrawal, there were no differences in fear-potentiated startle between LAAM-treated and water-treated rats.

Table 1

Behavioral, physical, and neuroendocrine measures during naloxone (NX)-precipitated withdrawal following 21 days of LAAM administration. There was no effect of opiate withdrawal on acoustic startle responding. However, there was an effect of opiate withdrawal on circulating corticosterone concentrations and a dose-related increase in the incidence of diarrhea and amount of weight lost during withdrawal in Experiment 1

Treatment Group (n)	% Change in Startle Responding	% with Diarrhea	Weight Change (% change from pre-withdrawal weight)	[Corticosterone] (ng/ml)
Water + 0.5 NX (5)	30.16 ± 25.04	0.00	-0.32 ± 0.24	134.44 ± 45.10
LAAM + Saline (6)	17.11 ± 25.10	0.00	-0.91 ± 0.16	219.38 ± 39.71
LAAM + 0.004 NX (6)	17.32 ± 26.16	16.67	-0.72 ± 0.15	210.20 ± 45.93
LAAM + 0.02 NX (5)	2.10 ± 12.84	40.00	-0.91 ± 0.41	343.92 ± 79.25*
LAAM + 0.1 NX (7)	10.18 ± 18.85	57.14	-1.21 ± 0.25*	350.79 ± 37.42*
LAAM + 0.5 NX (5)	-0.11 ± 17.94	100.00	-1.48 ± 0.43*	349.70 ± 26.73*
LAAM + 2.5 NX (7)	-10.58 ± 5.94	100.00	-2.17 ± 0.27*	361.47 ± 27.15*

* p < .05 compared with Water + 0.5 NX (Fisher's PLSD)

Table 2

Behavioral, physical, and neuroendocrine signs of precipitated withdrawal following 7 days of LAAM administration. Withdrawal (LAAM + NX) increased the incidence of diarrhea and levels of circulating corticosterone, and induced weight loss, compared with the other treatments in Experiment 2.

Treatment Group (n)	% Change in startle responding	% with Diarrhea	Weight Change(% change from pre-withdrawal weight)	[Corticosterone] (ng/ml)
Water + Saline (6)	50.01 ± 20.68	0.00	-0.26 ± 0.47	50.19 ± 7.90
Water + NX (6)	46.02 ± 24.10	0.00	-0.65 ± 0.16	96.72 ± 32.79
LAAM + Saline (6)	22.35 ± 9.28	0.00	-1.33 ± 0.42 *	153.40 ± 58.57
LAAM + NX (6)	0.41 ± 10.63	100.00 *	-2.07 ± 0.21 * [†]	308.10 ± 46.80 *

* p < .05 compared with Water + Saline;

[†] p < .05 compared with Water + NX (Fisher's PLSD).