Opiate Withdrawal and Conditioned Withdrawal Enhance Consolidation of Object Memory in Male Sprague-Dawley Rats

by

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ABSTRACT

OPIATE WITHDRAWAL AND CONDITIONED WITHDRAWAL ENHANCE CONSOLIDATION OF OBJECT MEMORY IN MALE SPRAGUE-DAWLEY RATS

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The present study explored the effects of acute precipitated and conditioned withdrawal on object memory. Using the object recognition task, it was demonstrated that 3 mg/kg naltrexone enhanced memory in morphine-maintained (osmotic mini-pumps; 10 mg/kg/day) rats when administered immediately but not 6 h post-sample. Importantly, post-sample naltrexone enhanced memory in the same animals in a drug-free state following removal of mini pumps 7 days later. To establish that conditioned withdrawal could also alter memory, morphine-maintained animals received naltrexone in a distinctive context (CS+) and vehicle in a separate context (CS) alternatively over 10 days. At the end of conditioning, confinement to this CS+ produced a conditioned locomotion suppression. More important, immediate but not delayed post-sample exposure to CS+ enhanced memory. These experiments indicate that both acute precipitated and conditioned withdrawal have significant facilitatory effects on memory consolidation and thus can play a role in the development of addictive behaviours.
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General Introduction

Opioid addiction is a public health issue that contributes substantially to global disease burden (Degenhardt et al., 2014). The extent of this epidemic was clearly echoed in a 2018 report by the United Nations Office on Crimes and Drugs estimating that about 70% of negative health impact related to prescribed and illicit drug use can be attributed to misuse of opioids.

Preclinical and clinical evidence have suggested that the effects of drugs on memory processes is a core feature of the progression from occasional drug use to a compulsive and relapsing pattern that defines opioid addiction (Everitt, 2014; Goodman and Packard, 2016; Hyman, 2005; Kelley, 2004; Milton and Everitt, 2012; O’Brien et al., 1998). In fact, the automatic repertoire of behavioural actions performed efficiently by dependent individuals are facilitated by the action of drugs of abuse on the formation of habitual memories (Hyman, 2005; Torregrossa et al., 2011). Conceived specifically, the memory consolidation hypothesis of drug addiction predicts that addictive behaviours are well remembered partly because drugs of abuse can enhance memory traces (White, 1992). Recent evidence has indicated that contextual stimuli that become associated with these substances can also modulate memory (Holahan and White, 2013; Wolter et al., 2019).

Can withdrawal impact memory consolidation? This is an important question because reduced agonistic activity at opioid receptors in dependent individuals caused by metabolism of the last dose, cessation of use, or administration of an antagonist (i.e., precipitation of withdrawal) produces a negative affective state central to the addiction
cycle (Koob and Volkow, 2010). That is, increased intensity of negative emotional/motivational symptoms, termed “hyperkatifeia” (Shurman et al., 2010), is highly aversive and reinforces behaviors leading to its termination or avoidance (negative reinforcement). In fact, there is convincing experimental evidence suggesting that opioid withdrawal causes the release of stress hormones (Culpepper-Morgan and Kreek, 1997), can induce long term potentiation (Salmanzadeh et al., 2003), and enhances central noradrenergic and cholinergic transmission (Frederickson, 1975; Rasmussen et al., 1990) all key processes involved in stabilization of memory traces (Roozendaal and McGaugh, 2011). Furthermore, opioid withdrawal can also be triggered by environmental stimuli that have been consistently paired with withdrawal state (Frenois, 2005; O’Brien et al., 1992; Schnur, 1992; Wikler and Pescor, 1967) and avoidance of conditioned withdrawal is another critical aspect of the addiction cycle (Goldberg and Schuster, 1967; Hellemans et al., 2006; Kenny et al., 2006; Koob and Le Moal, 2001). Therefore, the experiments in this thesis were designed to explore whether acute precipitated withdrawal and conditioned withdrawal could impact memory consolidation.

**Neurobiology of memory consolidation**

Newly acquired memories initially exist in a trace or in a labile state. Endogenous processes involving stress hormones induced by emotional arousing situations play an important role in making memory traces more permanent (McGaugh, 2000). This notion is supported by studies indicating that pharmacological increase in stress hormones including vasopressin, epinephrine, glucocorticoids, adrenocorticotropin immediately
after a memory task enhanced fear-based task in rodents (Roozendaal and McGaugh, 2011) and humans (Abercrombie et al., 2006; Cahill et al., 2003).

A common mechanism by which these hormones exert facilitatory actions on memory consolidation involves activation of the amygdala (Cahill et al., 1995). In fact, selective lesions to the basolateral amygdala blocks the memory enhancing effects of stress hormones (Roozendaal and McGaugh, 1997). Additionally, there is evidence that these hormones influence memory in part by increasing noradrenergic activity in the basolateral amygdala (Beldjoud et al., 2015). Indeed, infusion of adrenergic antagonists in the basolateral amygdala can block memory enhancing effects of epinephrine (Liang et al., 1986) and glucocorticoids for inhibitory avoidance tasks (Ferry et al., 1999; Hatfield and McGaugh, 1999) and high noradrenaline levels in the basolateral amygdala predict better memory performance in rodents (McIntyre et al., 2002; Quirarte et al., 1998). Furthermore, there is evidence that increased cholinergic transmission in the basolateral amygdala is also essential for neuromodulatory influence by a variety drugs on memory consolidation and that lesions to the amygdala cholinergic input from the nucleus basalis magnocellularis impaired memory for inhibitory avoidance tasks (Power and McGaugh, 2002)

The amygdala is not the locus for the storage of memories and therefore interact with other brain regions through its major projections to process different kinds of memory (McGaugh, 2013; McGaugh et al., 1996). Thus, lesions to basolateral amygdala or it major efferent projections mediates the memory enhancing effects of different drugs infused in the hippocampus, entorhinal cortex, striatum, anterior
cingulate, caudate nucleus and nucleus accumbens (Ikegaya et al., 1997; Malin et al., 2007; Roesler et al., 2002a; Roozendaal et al., 2001).

**Drugs of abuse as memory modulators**

In 1992, White & Milner suggested that drugs of abuse facilitate learning of addictive behaviors by enhancing memory consolidation, a time-dependent process during which memories are stabilized (McGaugh, 2000). In other words, behavioral responses to drug-associated stimuli followed by the administration of addictive substances end up being remembered better and therefore more effective in driving subsequent behavior.

To test the hypothesis of memory enhancing action of drugs of abuse in the laboratory, a drug must be administered shortly after a learning/memory task in a non-contingent manner to selectively modulate memory consolidation and not memory encoding or retrieval. If the drug enhances memory consolidation, then subjects treated with the drug immediately following training display better performance of the task when tested drug-free (White, 1992, 1996). Importantly, when the drug is delivered several hours following training, no memory enhancement is observed and suggests an action of the drug on a time-limited widow that consolidation occurs (Roozendaal and McGaugh, 2011). Using this approach, it has been found that cocaine, amphetamine, alcohol, nicotine, caffeine and glucose can all enhance memory consolidation in a variety of species and learning tasks (Alkana and Parker, 1979; Blaiss and Janak, 2007; Borota et al., 2014; Holahan and White, 2013; Lee and Ma, 1995; Leri et al., 2013;
Messier and White, 1987; Roozendaal et al., 2002b; Soetens et al., 1993; Wolter et al., 2019).

**Opioids and memory consolidation**

Opiates are chemical compounds that are natural derivatives from the opium poppy and include natural alkaloids such as morphine, codeine and thebaine (Goodman et al., 2011). An opioid is a broader term that refers to any agent that has the pharmacological properties of an opiate and include synthetic agonists (e.g. oxycodone, and fentanyl), antagonists (e.g. naltrexone and naloxone) as well as endogenous peptides (enkephalins, endorphins, dynorphins) that exert their effects by binding to opioid receptors (mu, delta and kappa) in the brain (Satoh and Minami, 1995).

Testing the memory consolidation hypothesis of drug abuse with opiates and opioid agonists has revealed variable findings. In some cases, enhancement of memory has been found (Levy et al., 2009; Mondadori and Waser, 1979; Stäubli and Huston, 1980; White et al., 1978) while other studies have reported impairments (Castellano et al., 1994; Introini et al., 1985) in a variety of species and learning/memory tasks. It remains unclear what causes this variability, although it has been proposed that the emotional nature of the memory task (motivated by incentive or aversive stimuli) and the selection of doses may be critical to the direction of the effect (Calabrese, 2001; Cloke et al., 2014; Izquierdo et al., 1980; Mondadori and Waser, 1979). With regard to opioid antagonists, the findings have consistently revealed that they can enhance memory consolidation. Thus, post-training administration of naloxone and naltrexone can improve memory for avoidance tasks (Castellano et al., 1989; Gallagher, 1978; Introini-
Conditioned memory modulation

An interesting question is whether cues that have been repeatedly paired with addictive substances through classical conditioning processes can modulate memory. This is an important question because these conditioned stimuli can acquire the ability to elicit behavioral responses in the absence of the drug and play a role in addiction (Robbins et al., 2006). Using a post-training approach, it has been confirmed that cues previously paired with consumption of sucrose solution enhance consolidation of a conditioned cue preference task in rats (Holahan and White, 2013). Consistent with this finding, recent evidence from our laboratory suggests that exposure to contextual stimuli paired with nicotine and cocaine elicited a conditioned locomotion response and enhanced object recognition memory (Wolter et al., 2019). In agreement, we have recently shown that exposure to contextual cues paired with heroin can also produce a conditioned motor response and enhanced object recognition memory (Huff et al., in preparation). These findings are important to the current study because they demonstrate that conditioned stimuli can significantly impact memory.

Opioid withdrawal

Opioid withdrawal is expressed when there is reduced agonistic activity at opioid receptors after the metabolism of the last dose or termination of opioid agonist (Pinelli and Trivulzio, 1997; Wang et al., 2001). This leads to a spontaneous withdrawal state characterized by somatic and psychological symptoms (Burma et al., 2017). Somatic symptoms include increases in body temperature and blood pressure, hot or cold
flashes, diarrhea, and perspiration in humans (Kanof et al., 1992) and paw tremors, suppressed locomotion, rapid weight loss, diarrhea, tearing, wet dog shakes, lacrimation, and irritability to handling in rodents (Redmond and Krystal, 1984). In both humans and rodents, psychological symptoms include changes such as anxiety, hyperalgesia, and profound negative emotional state (Maldonado et al., 1992; Shurman et al., 2010).

Interestingly, withdrawal can also be precipitated by opioid antagonists. In fact, an acute withdrawal state can be induced by administration of opioid antagonists after brief exposure to opioid and opiate agonists in animals (Becker et al., 2010; Schnur, 1991) and humans (Kanof et al., 1992). Acute precipitated withdrawal is characterized by similar somatic and psychological symptoms observed during spontaneous withdrawal, although acute precipitated withdrawal has a faster onset and a shorter duration (Maldonado et al., 2013).

There is emergence of a protracted withdrawal phase characterized by symptoms common to acute withdrawal but appears weeks and months after acute withdrawal has subsided. There are alterations in temperature, heart rate, and metabolic responses. Psychological symptoms include anxiety, depression, sleep disturbances, drug craving and irritability in humans (SAMHSA, 2010).

Similar protracted effects of withdrawal have been observed in rodents. For example, while opioid antagonists have no effect on locomotor activity and operant response in control animals, they suppress locomotor activity and disrupt operant
responding for food in both opioid dependent and post-dependent animals, a pattern common to acute withdrawal (Baldwin et al., 1993; Brady and Holtzman, 1981). Additionally, rodents exhibit anxiety and dysphoric behaviors after acute withdrawal has subsided (Aston-Jones and Harris, 2004).

**Conditioned withdrawal**

Wikler (1967), proposed that environmental stimuli can trigger a conditioned withdrawal response through classical conditioning because they have been repeatedly present during drug withdrawal. Indeed, experimental evidence in humans supports the notion that environmental stimuli can predict the onset of withdrawal-like symptoms. For instance, exposure to a tone and odor of peppermint repeatedly paired with naloxone-precipitated withdrawal lead to the occurrence of conditioned autonomic changes similar to the acute withdrawal state in opioid dependent humans. (O'brien et al., 1992).

Conditioned withdrawal responses have also been observed in rodents. For example, morphine-maintained monkeys were trained to press a lever for food on a fixed ratio schedule. Subsequently, acute precipitated withdrawal was continually paired with a tone. Presentation of the tone alone produced conditioned responses including suppression of food-lever response rate and physiological changes including bradycardia, emesis and excessive salivation, which were observed during acute withdrawal (Goldberg and Schuster, 1967). More recently, it has been demonstrated that presentation of environmental stimuli previously associated with naloxone-precipitated withdrawal triggered conditioned withdrawal like symptoms, increased
heroin intake and caused changes in brain reward thresholds in opioid maintained rodents (Hellemans et al., 2006; Kenny, 2006).

**Opioid withdrawal and memory**

The literature on the effects of opioid withdrawal on memory processes indicates that it can interfere with initial acquisition and retrieval of older memories. However, the effects of opioid withdrawal on memory consolidation has never been empirically explored although experimental evidence indicate that it actions on memory consolidation may be facilitatory. In fact, as discussed below there are at least five lines of experimental evidence indicating that opioid withdrawal activates neural mechanisms that underlie the memory stabilization.

**Acquisition and retrieval**

Experiments that induce a withdrawal state prior to training subjects on a learning/memory task have revealed that spontaneous or antagonist-precipitated withdrawal impairs memory acquisition in Morris Water Maze (Dougherty et al., 1996) spatial recognition (Ma et al., 2007), object recognition (Rabbani et al., 2009a) and radial maze (Sala et al., 1994). Moreover, these deficits are reversed by drugs that act on glucocorticoid receptors and the endocannabinoid system (Mesripour et al., 2008; Vaseghi et al., 2012).

When studying memory retrieval, withdrawal is induced shortly before testing subjects on a particular learning/memory task. In other words, subjects are tested in a withdrawal state. Generally, these experiments indicate that recall is impaired. Hence,
withdrawal impairs verbal memories (Rapeli et al., 2006) and object recognition memory in rodents (Mesripour et al., 2008; Rabbani et al., 2009a). Interestingly, these deficits are observed during early withdrawal and is recovered in late withdrawal (Rabbani et al., 2009b; Rapeli et al., 2006).

Consolidation

The effect of opioid withdrawal on memory consolidation has never been empirically explored. This may be because such a study is technically difficult to perform. In fact, to selectively investigate the effect of withdrawal on memory consolidation, subjects need to experience the withdrawal state immediately, or shortly after, training on a learning/memory task (i.e., post-training method). Moreover, testing is typically conducted when subjects are in a drug-free state, and in the case of withdrawal this would mean testing them when back to a dependent state, or after dissipation of withdrawal.

Arguably, these methodological difficulties should not preclude the investigation of the phenomenon because exploring whether/how withdrawal influences the consolidation of memories could be key to understanding why addiction is cyclical (Koob and Le Moal, 2001) and relapsing (Stewart, 2008).

Stress hormones are involved in both memory consolidation and opioid withdrawal

It has been shown that pharmacological stimulation of stress hormones including glucocorticoids, vasopressin, corticotropin releasing factor (CRF), and adrenocorticotropin releasing hormone (ACTH) shortly after training on a variety of
emotionally arousing learning tasks leads to better performance when subjects are retested stress free (Bohus et al., 1978; De Kloet et al., 1999; de Wied et al., 1976; Mitchell and Meaney, 1991; Roozendaal et al., 2007; Schwabe et al., 2012). Moreover, selective blockade of glucocorticoid receptors immediately after training impairs memory (Oitzl and de Kloet, 1992), and elevated cortisol levels predict better memory for emotionally-charged material when subjects are presented with stressful situations post-learning (Abercrombie et al., 2003, 2006; Cahill et al., 2003; Smeets et al., 2008).

Opioid withdrawal could promote memory consolidation by causing the release of stress hormones. Thus, in both opioid maintained human and animal subjects, spontaneous and acute precipitated withdrawal are accompanied by elevated levels of CRF, ACTH, glucocorticoids and vasopressin (Culpepper-Morgan and Kreek, 1997; García-Pérez et al., 2016; Zhang et al., 2008; Zhou et al., 2013, 2008), and blocking glucocorticoid and CRF receptors decreases the severity of several symptoms of withdrawal (Contarino and Papaleo, 2005; Navarro-Zaragoza et al., 2014, 2012; Papaleo et al., 2008).

The amygdala is involved in both memory consolidation and opioid withdrawal

Three lines of evidence suggest that the amygdala is involved in memory consolidation. First, concurrent activation of the amygdala is required for the memory enhancing effects of stress hormones (Mcgaugh, 2002; Roozendaal et al., 2002a) and selective lesions to the basolateral amygdala block the memory enhancing effects of corticosterone in avoidance tasks (McGaugh et al., 1996; Roozendaal et al., 2009; Roozendaal and McGaugh, 1997). Second, clinical evidence suggests that patients with
bilateral lesions to the amygdala have significant impairments in consolidating long-term declarative memories (Adolphs et al., 1997; Cahill et al., 1995). Indeed, studies employing imaging techniques have confirmed that increased activity in the amygdala is necessary for the consolidation of long-term declarative memories (Canli et al., 2000; Hamann et al., 1998; Phelps and Anderson, 2004). Third, studies in animals have ascertained that the basolateral complex of the amygdala is necessary for the processing of different forms of memories in brain regions including striatum, entorhinal cortex, nucleus accumbens, anterior cingulate cortex and hippocampus (Malin et al., 2007; McGaugh, 2004; Roesler et al., 2002b).

Opioid withdrawal could have a facilitatory effect on memory consolidation because it also activates the amygdala. Hence, morphine-dependent rats injected with different doses of naloxone express high levels of Fos protein in the central and basolateral complex of the amygdala (Le Guen et al., 2001; Stornetta et al., 1993). Interestingly, activation of the amygdala during opioid withdrawal is necessary for the expression of somatic symptoms, as well as learning about withdrawal-predicting cues. Thus, microinjection of naloxone in the amygdala can precipitate symptoms of withdrawal in dependent animals (Calvino et al., 1979; Tremblay and Charton, 1981), and an intact basolateral amygdala is necessary for learning the associations between a neutral stimulus and the aversive properties of antagonist precipitated withdrawal in rodents (Frenois et al., 2005, 2002; Koob et al., 1992; Schulteis et al., 2000; Stinus et al., 1990).
Increase noradrenergic activity underlies both memory consolidation and opioid withdrawal

In 1972, Seymour Kety suggested that activation of noradrenaline (NA) is necessary for the consolidation of new learning within the cortex. This hypothesis has been supported by animal studies indicating that infusion of beta adrenergic agonists in brain regions such as the hippocampus and amygdala immediately post-training enhance memory for inhibitory avoidance tasks (de Wied et al., 1976; Ferry et al., 1999; Hatfield and McGaugh, 1999; Liang et al., 1990; Quirarte et al., 1998) and that the beta adrenergic antagonist propranolol impairs object recognition memory (Roozendaal et al., 2008). Additionally, post-training exposure to stressful experiences also enhance memory consolidation partly by elevating NA levels in the amygdala (Quirarte et al., 1997). Consistent with this finding, in vivo microdialysis studies have shown that NA levels are elevated in the amygdala for 2 hours after training on an inhibitory avoidance task and this predicts subsequent memory performance (McIntyre et al., 2002). It has been proposed that drugs/stressful experiences modulate memory consolidation by directly activating NA cells in the nucleus of the solitary tract (NTS) and the locus coeruleus (LC) that projects to the hippocampus and the amygdala (Roozendaal et al., 2008; Roozendaal and Hermans, 2017; Walling and Harley, 2004).

Opioid withdrawal could enhance memory consolidation also through its regulation of NA activity. Hence, studies in laboratory animals have shown that during withdrawal there is upregulation of NA activity in the LC and that this mediates the severity of withdrawal symptoms (Done et al., 1992; Ivanov and Aston-Jones, 2017; Maldonado, 1997; Mazei-Robison and Nestler, 2012; Rasmussen, 1995; Rasmussen et
al., 1990; Silverstone et al., 1991; Streel et al., 2006). Furthermore, drugs that decrease NA activity in the LC such as clonidine and propranolol reduce the expression of opioid withdrawal symptoms (Maldonado, 1997; Redmond and Krystal, 1984; Taylor et al., 1998), noradrenergic neurons in the NTS express more Fos protein following antagonist-precipitated withdrawal (Stornetta et al., 1993), and stimulation of the noradrenergic neurons in the NTS can produce severe withdrawal symptoms in rodents maintained on opioid agonists (Navarro-Zaragoza et al., 2012).

**The cholinergic system is involved in both memory consolidation and opioid withdrawal**

Three main lines of evidence support the role of acetylcholine (ACh) in memory consolidation. First, studies in animals using the post-training approach have shown that muscarinic activation can enhance consolidation of inhibitory avoidance tasks (Baratti et al., 1984; Castellano et al., 1996; Introini-Collison and Baratti, 1992; Power et al., 2003; Rudy, 1996). Second, elevation of ACh levels within the amygdala, hippocampus, and striatum enhance memory consolidation on different learning tasks (Barros et al., 2002; Kopf et al., 2001; Power, 2005; Power et al., 2003; Ragozzino et al., 1996; Toumane et al., 1988; Vazdarjanova and McGaugh, 1999). Third, the memory-modulatory influence of stress hormones requires concurrent release of ACh within the amygdala (Paré, 2003).

Opioid withdrawal could facilitate memory consolidation also by stimulating cholinergic systems. Hence, there is evidence that opioid withdrawal increases the release of ACh in cholinergic neurons throughout the cortex (Frederickson, 1975). Both
spontaneous and precipitated withdrawal lead to an increase in brain level ACh in morphine-exposed rats (Large and Milton, 1970), levels of cortical ACh mediates the severity of withdrawal symptoms in rodents (Crossland and Ahmed, 1984; Domino and Wilson, 1973), and deletion of the muscarinic ACh receptors significantly attenuates both the somatic and affective symptoms of naloxone-induced morphine withdrawal (Basile et al., 2002).

**Opioid withdrawal enhances long-term potentiation**

Long term potentiation (LTP), a process by which synapses are strengthened due to constant neural excitability, is believed to underlie the molecular mechanisms of memory consolidation (Abel and Lattal, 2001; Dudai, 2004; Morris, 2003). LTP occurs in a time-limited manner shortly after acquisition of a learning task (Genzel et al., 2017) and can be blocked by NMDA receptor antagonists and inhibitors of protein synthesis when administered shortly after training (Alberini and Kandel, 2014; Dudai, 2004; Stern and Alberini, 2013). Neural plasticity changes that occur at synapses during long term potentiation are mediated in part, by expression of activity-regulated cytoskeletal-associated protein (Arc) in central memory systems (Bramham et al., 2010; McIntyre et al., 2005; McReynolds et al., 2014, 2010; Ploski et al., 2008).

Opioid withdrawal could promote memory consolidation by enhancing LTP. It has been demonstrated that applying naloxone to hippocampal slices of morphine-dependent animals increases LTP as measured by theta-frequency primed-burst tetanic stimulations (Mansouri et al., 1997; Salamanzadeh et al., 2003). This has been confirmed in another study by Ito et al., (2001), who reported that withdrawal from chronic
morphine administration enhanced LTP in synapses of the dentate gyrus (DG) of the hippocampus. Moreover, LTP enhancement induced by opioid withdrawal is associated with intracellular changes that lead to increase synaptic excitability (Robinson et al., 1982), and administration of an NMDA receptor antagonist or a voltage-dependent calcium channel blocker inhibited cellular excitability and blocked morphine withdrawal induced LTP in the hippocampus (Pourmotabbed et al., 1998). Finally, opioid withdrawal has been observed to increase Arc activity in the amygdala (García-Pérez et al., 2017, 2016; Valero et al., 2018).

**Object recognition memory task**

The current study employed the object recognition task to assess withdrawal effects on memory consolidation. This task assumes that rats with intact memory will explore a novel object more than a familiar one due to their innate preference for novelty. Typically, it involves three main phases including habituation, sample or training, choice or test. The habituation phase involves allowing animals to explore the apparatus without any objects for a specified time, usually between 5-10 minutes. To prevent the possibility of spatial information as a confounding variable, recent studies habituate animals to a Y-apparatus rather than an open field (Winters et al., 2008). During the sample or training phase, animals explore two identical novel objects (A1 and A2) placed at the end of the exploratory arms of the Y-apparatus for a predetermined time. The choice or testing phase involves replacing an identical object with a novel object (A3 and B) and allowing animals to explore for a specified time. The sample and choice phases are separated by a delay that depends on the goal of the
study. The objects and location of the objects are counterbalanced across all subjects and phases.

The object recognition task was considered suitable for this research study for several reasons. First, the object recognition task requires no external motivation such as food or foot shocks that can influence memory, and can be completed in a short time (Antunes and Biala, 2012). Second, it does not involve any obvious experimental stress such as foot shocks that can impact memory (Ennaceur, 2010). Third, previous studies have shown that the object recognition task is sensitive to post-training exposure to drugs of abuse and drug-paired stimuli (Rkieh et al., 2014; Wolter et al., 2019). Finally, it can be carried out in a within-subjects experimental design.

**Hypothesis and predictions**

There is convincing evidence that withdrawal can activate neural processes required for the stabilization of newly created memories. Hence, the current experiments were designed to test the hypothesis that opioid withdrawal can enhance memory consolidation. This hypothesis generates three predictions. First, immediate post-training naltrexone should enhance object recognition memory in animals maintained on morphine. Second, considering that protracted withdrawal symptoms can be observed long after acute withdrawal, we predicted that naltrexone would alter memory in drug-free animals that had been previously maintained on morphine. Third, immediate post-training exposure to a context paired with withdrawal would elicit a conditioned withdrawal response and enhance object memory. Therefore, three main experiments were designed to test these predictions. In Experiment 1, we determined
the dose of naltrexone that would be effective in eliciting a conditioned locomotion response in morphine-naive and morphine-maintained animals. Experiment 2 explored the effects of immediate or delayed post-sample naltrexone on object memory in morphine-naive and morphine-maintained animals. Importantly, the post-training effects of naltrexone were re-tested in the same animals following a period of withdrawal. Finally, Experiment 3 assessed the effects of immediate or delayed post-sample exposure to contextual stimuli associated with withdrawal on object memory.
Materials and methods

Subjects

Subjects were Male Sprague-Dawley rats (Charles River, Quebec, Canada), weighing between 225-250 g at the beginning of each experiment. Upon arrival, rats were individually housed in standard rat cages (polycarbonate; 50.5×48.5×20 cm) with standard bedding and environmental enrichment and acclimatized to the facility on a reverse light-dark schedule (lights off at 7:00; on at 19:00). Each rat was handled twice for 10 minutes prior to the start of every experiment. Rats had access to 20 g per day of standard rat chow, and water was available ad libitum in home cages. All behavioral testing was conducted during the dark period. All experiments were approved by the Animal Care Committee of the University of Guelph and were carried out in accordance with recommendations provided by the Canadian Council on Animal Care.

Surgery

Morphine maintenance was established by subcutaneous implantation of osmotic mini pumps (Alzet model 2ML2, 0.5 l/h for 14 days, Durect Corporation, Cupertino, CA), which were surgically implanted and removed as described previously (Leri et al., 2006). Briefly, rats were anesthetized with isoflurane (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON, Canada) and a small incision was made between the scapulae in the skin, spreading apart subcutaneous tissues to create a small pocket for pumps. Osmotic mini pumps were inserted with the flow moderators directed away from the incision. Mini pumps were removed using the same procedure as in implantation.
Testing Apparatus

Locomotion chambers

Locomotor activity was monitored in 6 semi-transparent Plexiglas chambers (30 × 40 × 26 cm) lit by individual LED lights (42 diodes) and was covered by black wire mesh to allow video tracking with EthoVision (v11.5, Noldus, The Netherlands). Each rat was tested in the same two chambers consisting of either vertical black and white stripes on the wall or a checkered box pattern with a large square on the wall designated as (CS-) or (CS+) during locomotor tests. Chambers designated as “CS+” had ceramic tiles to create a distinct context compared to “CS-.” During the conditioning phase of Experiments 1 and 3, animals received vehicle in the CS- chamber and naltrexone in the CS+ chamber.

Object recognition

Object recognition was tested in a Y- apparatus previously described (Winters et al., 2004). Briefly, the Y-apparatus was made of three identical arms (40 ×27 ×10 cm) constructed from solid white Plexiglas to prevent encoding of spatial or contextual information in the room. Specifically, one arm was designated the start arm and consisted of a guillotine door placed 18 cm from the rear of arm to confine rats at the start of each trial. The remaining two arms were designated as exploratory/choice arms. Objects used consisted of copies made from ceramics, plastic and glass with varying height (ranging from 10 to 20 cm), tactile and visual qualities. Objects were fixed to the floor using a reusable adhesive putty and wiped with 50% ethanol after each trial to
minimize olfactory cues that could influence exploration. A JVC Everio digital camera was mounted on a tripod placed above the Y-apparatus to record each trial.

**Drugs and doses**

Morphine sulphate (Pharmascience, Montreal, QC) and Naltrexone-Hydrochloride (Sigma Aldrich) were dissolved in physiological saline. Morphine (10 mg/kg/day s.c.) was administered via osmotic mini-pumps because it readily produces morphine dependence in rats (Adams and Holtzman, 1990a). Naltrexone (1 and 3 mg/kg s.c.) was injected subcutaneously and chosen based on previous findings in our laboratory indicating these dose reliable precipitate withdrawal in opioid exposed animals (Daniels et al., 2016).

**Assessment of morphine withdrawal**

*Loss of body weight*

Rats were weighed prior to and after naltrexone injections. Change in body weight was measured as percentage body weight loss in 2 hours.

*Wet dog shakes*

During locomotion testing, rats were videotaped for 2 hours and instances of wet dog shakes was scored. Only the first 30 minutes was scored. Wet dog shakes were defined as a sudden uncontrollable twitch of the body.
Procedures

Experiment 1: Conditioned withdrawal in morphine-maintained rats

This experiment investigated whether naltrexone could induce a conditioned locomotion response in morphine-naive and morphine-maintained rats. This experiment included a total of 48 animals. Hence, morphine-naive animals (n=12 each) received 1 or 3 mg/kg in CS+, and morphine-maintained (n=12 each) received 1 or 3 mg/kg in CS+. Prior to conditioning, all rats were habituated to locomotor chambers for 30 minutes by allowing them to explore the apparatus without any injections. The following day, morphine-maintained animals were implanted with morphine-filled osmotic mini-pumps. Contextual conditioning began 48 h following implantation of mini pumps. On day 1 of conditioning, half of the animals from each group received 1 or 3 mg/kg naltrexone and confined to CS+ for 2 hours. The other half were injected with vehicle and confined immediately to CS- for 2 hours. This was repeated on 9 additional days alternatively (5 naltrexone parings and 5 vehicle parings in total). To measure naltrexone-induced conditioned locomotor suppression, two tests each lasting 30 minutes were conducted over two days that involved placing animals in the CS- & CS+ chambers, counterbalanced across subjects, without naltrexone injections. The difference in locomotor activity in the CS- and CS+ chambers during test session was used as index of conditioned locomotor suppression.

Experiment 2a: The effects of immediate post-sample naltrexone-precipitated withdrawal on object memory

This experiment investigated the effect of post-training 0 or 3 mg/kg naltrexone on object memory in morphine-naive (n=6) and morphine-maintained (n=12) rats.
Morphine-naive animals received a sham surgery. This dose was chosen because it was found to produce a robust conditioning effect in Experiment 1. On days 1 and 2 of the experiment, rats were habituated to the Y-apparatus for 5 mins without any objects 48 hr following implantation of mini-pumps. On day 3, rats were exposed to the sample phase of object recognition where they were allowed to explore two identical objects placed at the ends of the Y-apparatus. The sample phase ended when animals explored both objects for a total of 25 seconds or a maximum of 3 minutes in the Y-apparatus whichever occurred first. Immediately following the sample phase, rats were injected with 0 or 3 mg/kg naltrexone and locomotor activity was assessed as an index of withdrawal. After a 72-h retention interval, each animal was exposed to a choice phase for 2 minutes, during which they were presented with a copy of the sample object in one arm and in the other a novel object. A 72-h retention interval was used because it is a suboptimal condition in which drug-naive rats do not typically show memory (Melichercik et al., 2012; Wolter et al., 2019). All animals were tested with each dose of naltrexone and the order of the dose was counterbalanced.

*Experiment 2b: The effects of delayed post-sample naltrexone-precipitated withdrawal on object memory*

This experiment examined the effect of delayed post-training naltrexone on object recognition memory. The same procedure as in Experiment 2a was employed, the only difference being that a separate group of morphine-maintained animals (n=6) received either 0 or 3 mg/kg naltrexone 6 hours following the sample phase.
**Experiment 2c: The effects of immediate post-sample naltrexone on object memory after a period of withdrawal.**

This experiment employed the same animals as in Experiment 2a, and it was designed to assess whether naltrexone could alter memory in drug-free animals previously maintained on morphine after a period of withdrawal. Hence, 7 days following removal of mini pumps after the choice phase in experiment 2a, each animal was exposed to a sample phase and immediately thereafter injected with 0 or 3 mg/kg naltrexone and locomotor activity was assessed. The choice phase was conducted after a 72-h delay interval. A 7-day period was selected because body weight recovered by this time.

**Experiment 3a: The effects of immediate post-sample exposure to withdrawal-paired environment on object memory**

The objective of this experiment was to assess the effect of post-sample exposure to withdrawal-paired chamber CS+ on object memory. Hence, this experiment involved two main phases, contextual conditioning and object recognition testing, in the same animals. In Phase 1, the same conditioning procedure as in Experiment 1 was employed. Briefly, rats (n=7) were habituated to chambers by allowing them to explore it without naltrexone injections. The following day, all rats were implanted with morphine-filled osmotic mini-pumps. Contextual conditioning began 48 h after mini-pumps implantation. On day 1 of conditioning, half of the rats received 3 mg/kg naltrexone and were confined to CS+ for 2 h. The other half were injected with vehicle and confined immediately to CS- for 2 h. This was repeated alternatively on 10 consecutive days (5 naltrexone parings and 5 vehicle parings in total) and counterbalanced.
Phase 2 involved object recognition testing following the removal of morphine-filled osmotic mini-pumps 7 days after the last day of conditioning. Rats were habituated to the Y-apparatus used for object recognition on two consecutive days. Next, all animals were exposed to the sample phase and half of them were immediately confined to their designated naltrexone-paired compartment (CS+) for 30 min. The other half were confined immediately to their designated vehicle paired compartment (CS-) after exposure to sample objects. The choice phase of object recognition was conducted after a 72-h delay interval. Twenty-four hours after the choice phase, the same animals experienced another sample phase with different objects and were immediately exposed to alternate chambers (CS+ and CS-). The final object recognition choice phase was conducted after a 72-h delay interval. Therefore, all animals were subjected to post-sample exposure to CS- and CS+, counterbalanced across subjects on two object recognition trials with different objects during sample and choice phases.

*Experiment 3b: The effects of delayed post-sample exposure to withdrawal-paired environment on object memory*

The aim of this experiment was to assess the effect of delayed post-training exposure to the withdrawal paired compartment CS+ on object recognition memory. The procedure used for this experiment was identical to that of Experiment 3a, the only difference being that a separate group of rats (n=6) were exposed to conditioning chambers 6 hours following the sample phase.
Data analysis

Mixed design and repeated analyses of variance (ANOVA) were used as appropriate. Significant main effects, and/or interactions, were further analyzed by Student-Newman-Keuls post-hoc tests. A t-test was used to compare locomotor activity in CS+ and CS- in experiment 3, locomotor activity post-sample following naltrexone injections and total exploration during sample and choice phases. Two factor ANOVAs were performed using Sigma Stat (v.3.5 for Windows) while three factor ANOVAs were performed using SPSS (v.25, Windows, SPSS Inc). The exact values of nonsignificant analyses are not reported.

Exploration was defined as active sniffing within < 2 cm of the object and/or touching the object with the nose. To determine novelty preference, a discrimination ratio DR (novel object exploration – familiar object exploration) / total exploration was calculated for both sample and choice phases. In the sample phase, as all objects should be equally novel, a discrimination ratio around zero is expected. In the choice phase, a discrimination ratio significantly greater than zero is indicative of intact memory. The DR score during sample and choice phases were compared to indicate recognition memory. For all object recognition testing, total exploration was analysed to control for treatment effects on exploration.
Results

Experiment 1

Table 1 represents mean (SEM) percent body weight loss and average wet dog shakes during conditioning in the CS+ only. For body weight, the ANOVA revealed a significant interaction between Pairing and Group \[F(4,176) = 14.12, P< 0.001\], Group and Dose \[F(1,176) = 19.09, P= 0.01\] as well as main effects of Pairing \[F(4,176) = 17.161, P< 0.001\], Group \[F(1,176) = 530.15, P< 0.001\] and Dose \[F(1,176) = 12.13, P= 0.001\]. Multiple comparisons further indicated that, morphine-maintained animals injected with 1 or 3 mg/kg naltrexone lost significantly more weight than morphine-naive animals.

For wet dog shakes, the ANOVA revealed only a significant main effect of Group \[F(1,16) = 262.55, P< 0.001\]. Pairwise comparison indicated that compared to morphine-naive animals, morphine-maintained rats displayed more wet dog shakes when they were injected with 1 or 3 mg/kg in CS+.

Figures 2A and 2B represent mean (SEM) distance moved during conditioning with vehicle in CS- and 1 mg/kg naltrexone for morphine-naive and morphine-maintained rats, respectively. The ANOVA revealed a significant interaction between Pairing, Dose and Group \[F(4,176) = 10.92 ,P< 0.001\], Group and Dose \[F(1,176) = 94.86, P< 0.001\] as well as main effects of Group \[F(1,176) = 6.21 ,P= 0.01\] and Dose \[F(1,176) = 107.36 ,P< 0.001\]. Multiple comparisons further indicated that morphine-maintained animals injected with 1 mg/kg naltrexone moved less in CS+ compared to vehicle injections in the CS- as well as morphine-naive animals.
Figures 2C and 2D represent mean (SEM) distance moved during conditioning with vehicle in CS- and 3 mg/kg naltrexone in CS+ for morphine-naive and morphine-maintained rats, respectively. The ANOVA revealed a significant interaction between Pairing, Dose and Group \([F(4,176) = 2.90 , P = 0.02\], Group and Dose \([F(1,176) = 68.05, P< 0.001]\], Pairing and Chamber \([F(4,176) = 2.43, P = 0.04]\) Pairing and Group \([F(4,176) = 68.50, P = 0.001]\] as well as main effects of Pairing \([F(4,176) = 25.88 , P< 0.001]\] and Dose \([F(1,176) = 59.45, P< 0.001]\). Multiple comparisons further indicated that morphine-maintained animals injected with 3 mg/kg naltrexone moved less in CS+ compared to vehicle injections in the CS- as well as morphine-naive animals.

Figures 2E and 2F represent mean (SEM) distance moved during test of conditioned locomotion for morphine-naive and morphine-maintained rats, respectively. The ANOVA revealed a significant interaction between Chamber and Group \([F(1,44) = 12.64 , P= 0.001]\] and main effects of Group \([F(1,44) = 39.82, P< 0.001]\] and Chamber \([F(1,44) = 4.11 , P= 0.04]\]. Multiple comparisons further indicated that, when confined in the CS+ without naltrexone injections, morphine-maintained rats displayed suppressed locomotion.

**Experiment 2a**

Figure 3A represents mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone for morphine-naive animals. The ANOVA revealed no significant effects. Figure 3B represents mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections of 0 or 3 naltrexone for morphine-maintained rats. The ANOVA revealed a
significant interaction between Phase and Dose \[F(1,11) = 10.750, P= 0.007\], as well as main effects of Phase \[F(1,11) = 5.46, P= 0.03\] and Dose \[F(1,11) = 5.27, P= 0.04\]. Multiple comparisons further indicated that, when animals were injected with 3 mg/kg naltrexone, their choice discrimination ratio was significantly higher compared to their sample discrimination ratio, as well as 0 mg/kg naltrexone choice discrimination ratio.

Experiment 2b

Figure 5A represents mean (±SEM) discrimination ratio produced during sample and choice phases when naltrexone injections were delayed 6 hours following the sample phase. The ANOVA revealed no significant effects.

Experiment 2c

Figure 3C represents mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone for morphine-naive rats re-tested 7 days following removal of mini-pumps. The ANOVA revealed no significant effects. Figure 3D represents mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone for morphine-maintained rats re-tested 7 days following removal for pumps. The ANOVA revealed a significant interaction between Phase and Dose \[F(1,11) = 8.23, P= 0.01\], and main effect Dose \[F(1,11) = 16.28, P= 0.002\]. Multiple comparisons further indicated that, when animals were injected with 3 mg/kg naltrexone, their choice discrimination ratio was significantly higher compared to their sample discrimination ratio, as well as 0 mg/kg naltrexone choice discrimination ratio.
Experiment 3a

Table 2 represents mean (SEM) percent body weight loss and average wet dog shakes during conditioning with vehicle in CS- and 3 mg/kg naltrexone in CS+. For weight, the ANOVA revealed a significant interaction between Dose and Pairing \([F(4,24) = 3.78, P= 0.01]\), as well as main effect of Dose \([F(1, 24) = 119.59, P< 0.001]\) and Pairing \([F(4, 24) = 6.69, P< 0.001]\). Multiple comparisons further indicated that, when morphine-maintained animals were injected with 3 mg/kg naltrexone in CS+ they lost significantly more weight compared to when they are injected with vehicle in CS- for all the pairings. Additionally, more wet dog shakes were observed when animals were injected with 3 mg/kg naltrexone in CS+ compared vehicle in CS- \([t(5)= -12.03, P <0.001]\).

Figure 4A represents the mean (SEM) distance moved in chambers paired (pairings 1–5) with injections of 0 mg/kg naltrexone in CS− and 3 mg/kg naltrexone in CS+. The ANOVA revealed a significant interaction between Dose and Pairing \([F(4,24) = 5.12, P= 0.004]\), and a main effect of Dose \([F(1,24) = 213.83, P<0.001]\). Multiple comparisons further indicated that, when animals were injected with 3 mg/kg naltrexone in CS+, they moved less compared with vehicle injections in the CS- for all the pairings (1-5). Figure 4B represents mean (SEM) distance moved following post-sample test of conditioned locomotion in chambers CS- and CS+. A t-test indicated that animals moved less in the CS+ compared to the CS- \([t(6)= -9.96, P <0.001]\). Figure 4C represents mean (±SEM) discrimination ratio produced during sample and choice phase of OR following exposure CS- and CS+. The ANOVA revealed a significant interaction...
between Phase and Chamber [F(1,6) = 9.01, P= 0.02] and main effect of Chamber [F(1,6) = 6.56, P= 0.04]. Multiple comparisons further indicated that, when rats were exposed to the CS+ chamber post-sample, their choice discrimination ratio was significantly higher when compared to their sample discrimination ratio and CS− choice discrimination ratio.

**Experiment 3b**

Figure 5B represents mean (±SEM) discrimination ratio produced during sample and choice phases when exposure to CS+ and CS- was delayed 6 hours following the sample phase. The ANOVA revealed no significant effects.
Discussion

The current study tested the hypothesis that opioid withdrawal can enhance memory consolidation. Using the OR task, it was demonstrated that immediate but not 6-h delay post-sample naltrexone enhanced object memory in morphine-maintained animals only. Importantly, post-sample naltrexone enhanced object memory for drug-free animals that had been previously maintained on morphine following a period of withdrawal. To establish that conditioned withdrawal could also alter memory, we employed a contextual conditioning procedure whereby morphine-maintained animals received naltrexone in a distinctive context (CS+) and vehicle in a separate context (CS-) alternatively over 10 days. During conditioning in CS+, naltrexone suppressed locomotor activity, caused a rapid body weight loss and increased frequency of wet dog shakes. Importantly, confinement to this CS+ immediately but not 6-h after the acquisition phase of the OR task enhanced memory. Therefore, this study reports for the first time that both acute precipitated and conditioned withdrawal can have significant facilitatory effect on consolidation of object memory.

Naltrexone enhanced object memory in morphine-maintained animals.

Naltrexone-precipitated withdrawal elevates stress hormones including corticosterone, corticotropin releasing factor and vasopressin (Culpepper-Morgan and Kreek, 1997; García-Pérez et al., 2016; Zhang et al., 2008; Zhou et al., 2013, 2008), enhances central noradrenergic and cholinergic transmission (Basile et al., 2002; Frederickson, 1975; Krystal et al., 1990; Maldonado, 1997; Navarro-Zaragoza et al., 2014), can induce long-term potentiation (Mansouri et al., 1997; Salmanzadeh et al., 2003) and therefore
should enhance memory consolidation (McGaugh, 2015, 2000; Roozendaal, 2002; Schwabe et al., 2010) as hypothesized. The enhancement of object memory is likely to reflect a precipitation of withdrawal by naltrexone other than non-specific effects for several reasons. First, we confirmed that locomotor activity was decreased only in morphine-maintained animals following post-sample naltrexone injections. Second, object memory was no longer facilitated when naltrexone was delayed by 6 hours following the sample phase suggesting that enhancement of memory is exclusively to a time-dependent consolidation window rather than effects on encoding or retrieval (Roozendaal and McGaugh, 2011) and rules out non-specific effects of withdrawal on sensory, perceptual, cognitive or motor functions. Third, exploration of the novel objects is less likely to be a learned aversion to the sample objects or location of the objects because they are counterbalanced across all subjects on both sample and choice phases. Moreover, naltrexone can be aversive in opioid-naive subjects (Daniels et al., 2016; Hollister et al., 1981). Thus, it would be expected that morphine-naive animals would also explore the novel object more as indicated by a high choice discrimination ratio.

Interestingly, naltrexone enhanced memory in drug-free animals that had been previously maintained on morphine following a period of withdrawal. A withdrawal-like state can be elicited when opioid antagonists are administered to subjects formerly maintained on opioids following a period of withdrawal (Adams and Holtzman, 1990b; Baldwin et al., 1993; Brady and Holtzman, 1981). In fact, compared to morphine-naive controls, naltrexone suppressed locomotor activity in the animals previously maintained
on morphine, a pattern consistent with acute withdrawal state. Additionally, spontaneous morphine withdrawal is evident for 2-4 days after removal of morphine filled osmotic mini-pumps, and by a week there is a recovery in body weight and no disruption of operant responding (Adams and Holtzman, 1990a); we also observed a recovery in body weight by 7 days following removal of mini-pumps. Therefore, it is likely that in our study there was total elimination of morphine in plasma and that physical withdrawal symptoms might have subsided. Prolonged changes in the brain occur many weeks beyond acute withdrawal (Koob and Le Moal, 2001). For instance, functional neuroadaptations in the amygdala and noradrenergic neurons persist long after acute opioid withdrawal has subsided (Aston-Jones and Harris, 2004), and these brain systems are critical for the consolidation of emotional memories (Cahill et al., 1995; McGaugh, 2015; McIntyre et al., 2002).

To determine that conditioned withdrawal would also impact memory consolidation, morphine-maintained rats received naltrexone in CS+ and vehicle in CS- (Experiment 3a) over 5 pairings. During these pairings, naltrexone suppressed locomotor activity, decreased body weight and increased frequency of wet dog shakes in CS+. Increased wet dog shakes, loss of body weight and suppression of locomotor activity have been shown to be indicative of withdrawal and confirms that naltrexone effectively precipitated withdrawal in the CS+ (Gellert and Holtzman, 1978). Additionally, when locomotion was tested without naltrexone injections, animals moved significantly less in CS+ in comparison to CS-, clearly indicating that the CS+ effectively produced a conditioned response on motor behaviour. Consistent with previous studies, this study
suggests that environmental stimuli can be associated with opioid withdrawal through classical conditioning processes (Amitai et al., 2006; Baldwin et al., 1993; Becker et al., 2010; Hellemans et al., 2006; Kenny, 2005; O'brien et al., 1992; O'Brien et al., 1976; Schnur, 1992; Schulteis et al., 2000). More importantly, we extend these findings by demonstrating that confinement to withdrawal associated contextual stimuli post-training can enhance memory. This finding most likely reflects enhancement of object memory by the CS+ rather than non-specific effects associated with confinement to conditioning chambers because when exposure to CS+ was delayed no memory effects were observed.

Conditioned effects on memory have been demonstrated with cocaine and nicotine conditioned contextual stimuli (Wolter et al., 2019), conditioned sucrose stimuli (Holahan and White, 2013) and conditioned aversive stimuli (Holahan and White, 2002). Exposure to environmental cues associated with withdrawal activates the basolateral amygdala (Frenois, 2005; Schulteis et al., 2000), which has been suggested to be responsible for modulation of memory by unconditioned aversive stimuli (Mcgaugh, 2002) and conditioned aversive stimuli (Goode et al., 2016; Holahan and White, 2002). In fact, the amygdala sends afferents to the perirhinal cortex (Pikkarainen, 2001), which is required for object recognition (Winters et al., 2008), supporting its possible role in the enhancement of object memory induced by conditioned withdrawal.

Finally, post-training naltrexone failed to enhance object memory in morphine-naive animals. This is in disagreement with previous studies showing that post-training opioid antagonists enhance memory for avoidance and appetitive memory tasks
(Castellano et al., 1989; Fulginiti and Cancela, 1983; Gallagher, 1982; Introini-Collison et al., 1989; Introini-Collison and Baratti, 1986). Three main possible factors may contribute to this discrepancy. First, drug effects on memory consolidation vary from task to task (Roozendaal and McGaugh, 2011), and these results have been revealed in aversive or appetitive motivated memory learning tasks. Object recognition task does not involve any obvious aversive or appetitive manipulations (Ennaceur, 2010). Second, if naltrexone is administered as a single injection shortly after training, it does not enhance memory, although it enhances memory when administered as two injections 30 minutes apart following training (Gallagher, 1978). This suggests that the use of a single injection in the current study could be contributing factor. Third, memory enhancement has been found with doses of naltrexone ranging from 0.5 to 2 mg/kg in rodents (Castellano et al., 1989; Gallagher, 1982)

There are several limitations to the current work that should be addressed by future studies. First, different memory systems are required for different tasks. It is unclear whether our manipulations would reveal the same findings with other learning tasks. Second, in Experiment 2c, the post-training effects of naltrexone on object memory were tested 7 days after removal of mini pumps. It is unclear whether the post-training effects of naltrexone could still be observed after an abstinence period longer than a week. Third, in Experiment 2c, it was not determined whether naltrexone would alter memory when administered 6 hours post-sample that might lead to the conclusion that memory enhancement may be due to non-specific sensory, perceptual or cognitive effects. However, such an experiment would be identical to Experiment 2b, in which
naltrexone was delayed 6 hours following the sample phase in morphine-maintained animals and no memory effects were found. Fourth, an additional control group would be animals with vehicle mini-pumps for the conditioning experiments because it can be argued that the mini-pumps can have effects on motor activity. However, it has been shown that locomotor activity is unaffected in drug-naive animals inserted with vehicle-filled pumps (Lapointe et al., 2019). Finally, there is evidence that sex plays a role in the expression of opioid withdrawal (Becker and Koob, 2016; Diaz et al., 2005; Santoro et al., 2017). This study included only Male Sprague Dawley rats. It is therefore not clear whether similar findings would be replicated in female rats.

Despite these limitations, the current study provides important directions for future research. Previous studies have established that the basolateral amygdala is involved in conditioned drug responses (Luo et al., 2013) and the same brain region is involved in the expression of conditioned withdrawal responses (Frenois, 2005; Schulteis et al., 2002). Our laboratory has recently shown that post-training heroin conditioned stimuli enhance memory. Given that the amygdala plays a major role in conditioned memory modulation, it raises the question as to whether the same mechanism is involved in memory enhancement induced by heroin conditioned stimuli and conditioned withdrawal. Moreover, it has been suggested that extinction learning involves the formation of new memories (Santini et al., 2018). Considering that opioid withdrawal can enhance memory, an interesting question that could be explored by further studies is whether post-training antagonist-precipitated withdrawal can facilitate the extinction of heroin conditioned cues.
Again, further studies could also explore the role of stress, the amygdala, noradrenergic and cholinergic transmission in memory facilitation induced by acute withdrawal and conditioned withdrawal. Moreover, previous studies have indicated that facilitation of memory storage by various pharmacological agents is associated with cellular changes (Stern and Alberini, 2013). One such cellular change is high expression of Arc protein and increase of dendritic spines in the hippocampus (Bramham et al., 2010; Dudai, 2004; Ploski et al., 2008). Hence further studies would explore whether memory enhancement induced by acute withdrawal and conditioned withdrawal is associated with memory related plasticity changes in the hippocampus. Finally, further studies would determine whether extinction of conditioned locomotor suppression in the CS+ would lead to a parallel extinction of memory facilitation induced by exposure to the CS+.

The findings from this study support three main conclusions. First, post-training naltrexone enhanced object memory in morphine-maintained rats which supports our hypothesis that opioid withdrawal can enhance memory consolidation. Second, the post-training effects of naltrexone can be observed in a drug-free state in animals previously maintained on morphine. Third, environmental stimuli that have been repeatedly present during a state of withdrawal can trigger a conditioned withdrawal state. More important, such conditioned withdrawal stimuli can modulate memory. This suggests that actions that are performed and stimuli that are encountered during drug seeking in a withdrawal or conditioned withdrawal state could be enhanced and hence play a significant role in development and maintenance of addictive behaviours.
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Figure and Table Legends

**Table 1.** mean (SEM) percent body weight loss and average wet dog shakes during conditioning in the CS+ only (Experiment 1). The * denotes significant difference compared to morphine-naive animals.

**Table 2.** mean (SEM) percent body weight loss and average wet dog shakes during conditioning in CS+ and CS- (Experiment 3a). The * denotes significant difference compared to animals injected with 0 mg/kg naltrexone in the CS-.

**Figure 1.** (A) Experimental timeline for Experiment 1 (B) Experimental timeline for Experiments 2a, 2b and 2c. (C) Experimental timeline for Experiments 3a, 3b.

**Figure 2.** (A) and (B) mean (SEM) distance moved during conditioning with Vehicle in CS- and 1 mg/kg naltrexone in CS+ for morphine-naive and morphine-maintained rats respectively. The * denotes significant difference compared to animals injected with 0 mg/kg in the CS- and morphine-naive animals. The # denotes significant difference in comparison to all pairings for morphine-naive animals. (C) and (D) mean (SEM) distance moved during conditioning with Vehicle in CS- and 3 mg/kg naltrexone in CS+ for morphine-naive and morphine-maintained rats respectively. (E) and (F) mean (SEM) distance moved during test of conditioned locomotion for morphine-naive and morphine-maintained rats respectively. The * denotes significant difference compared to CS-.

**Figure 3.** (A) mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone in morphine-naive animals (sham surgery). (B) mean (±SEM) discrimination ratio produced during sample and choice
phase of OR following injections 0 or 3 naltrexone for morphine-maintained rats. (C) mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone for morphine-naive rats tested 7 days later. (D) mean (±SEM) discrimination ratio produced during sample and choice phase of OR following injections 0 or 3 naltrexone for morphine-maintained rats tested 7 days following removal of pumps. The * denotes a significant difference compared to 0 mg/kg naltrexone choice phase discrimination ratio. The # denotes a significant difference compared to sample discrimination ratio.

**Figure 4.** (A) mean (±SEM) distance moved in chambers paired (pairings 1–5) with injections of 0 mg/kg naltrexone in CS− and 3 mg/kg naltrexone in CS+. (B) mean (±SEM) distance moved following post-sample test of conditioned locomotion in CS− and CS+. The * denotes significant difference compared to CS−. (C) mean (±SEM) discrimination ratio produced during sample and choice phase of OR following exposure CS− and CS+. The * denotes significant difference compared to CS− choice phase discrimination ratio. The # denotes a significant difference compared to sample discrimination ratio.

**Figure 5.** (A) mean (±SEM) discrimination ratio produced during sample and choice phases when naltrexone injections were delayed 6 hours following the sample phase (B) mean (±SEM) discrimination ratio produced during sample and choice phases when exposure to CS+ and CS− was delayed 6 hours following the sample phase.
Tables and figures

Table 1. Experiment 1
Mean (SEM) percent body weight loss and average wet dog shakes. The * denotes significant difference compared to morphine-naive animals.

<table>
<thead>
<tr>
<th>Pairing</th>
<th>Body weight</th>
<th>Wet dog shakes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>morphine-naive</td>
<td>morphine-maintained</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>1</td>
<td>-1.0 (0.3)</td>
<td>-0.9 (0.3)</td>
</tr>
<tr>
<td>2</td>
<td>-1.4 (0.2)</td>
<td>-1.4 (0.2)</td>
</tr>
<tr>
<td>3</td>
<td>-0.4 (0.2)</td>
<td>-1.0 (0.2)</td>
</tr>
<tr>
<td>4</td>
<td>-0.7 (0.1)</td>
<td>-1.2 (0.3)</td>
</tr>
<tr>
<td>5</td>
<td>-0.7 (0.1)</td>
<td>-0.8 (0.2)</td>
</tr>
</tbody>
</table>
Table 2. Experiment 3a

Mean (SEM) percent body weight loss and average wet dog shakes. The * denotes significant difference compared to 0 mg/kg

<table>
<thead>
<tr>
<th>Pairing</th>
<th>Body weight</th>
<th>Wet dog shakes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/kg</td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>1</td>
<td>-1.9 (0.3)</td>
<td>-2.6 (0.7) *</td>
</tr>
<tr>
<td>2</td>
<td>-2.4 (0.2)</td>
<td>-5.7 (0.7) *</td>
</tr>
<tr>
<td>3</td>
<td>-2.1 (0.2)</td>
<td>-6.7 (1.1) *</td>
</tr>
<tr>
<td>4</td>
<td>-2.2 (0.4)</td>
<td>-6.6 (0.7) *</td>
</tr>
<tr>
<td>5</td>
<td>-2.7 (0.2)</td>
<td>-7.6 (1.1) *</td>
</tr>
</tbody>
</table>
Figure 2

A. Morphine-naive (vehicle in CS- vs. 1 mg/kg in CS+)

B. Morphine-maintained

C. Morphine-naive (vehicle in CS- vs. 3 mg/kg in CS+)

D. Morphine-maintained

E. Morphine-naive (CS- vs. CS+)

F. Morphine-maintained (1 mg/kg vs. 3 mg/kg)
Figure 3

Pump IN

Morphine-naive

Mean (SEM) discrimination ratio

0 mg/kg  3 mg/kg

-0.4  0.0  0.2  0.4

Sample
Choice

Morphine-maintained

Mean (SEM) discrimination ratio

0 mg/kg  3 mg/kg

-0.4  0.0  0.2  0.4

#

Pump OUT

Morphine-naive

Mean (SEM) discrimination ratio

0 mg/kg  3 mg/kg

-0.4  0.0  0.2  0.4

Sample
Choice

Morphine-maintained

Mean (SEM) discrimination ratio

0 mg/kg  3 mg/kg

-0.4  0.0  0.2  0.4

#
Figure 4

A

Vehicle in CS-  
3 mg/kg in CS+

Mean (SEM) distance moved (cm)

*  *  *  *  *

Pairing

1  2  3  4  5

B

CS-  
CS+

Mean (SEM) distance moved (cm)

*  *

C

Discrimination ratio

Sample  Choice

CS-  CS+
Figure 5

A. Delayed naltrexone

B. Delayed CS

Mean (SEM) discrimination ratio

-0.4 to 0.8

0 mg/kg to 3 mg/kg

CS- to CS+

Sample

Choice
Assessment of locomotor activity post-sample following naltrexone injections (Experiment 2a and 2c)

**Figure 6.** (A) Mean (SEM) total distance moved following post-sample naltrexone injections for morphine-naive. A t-test revealed no significant difference. B) Mean (SEM) total distance moved following post-sample naltrexone injections for morphine-maintained animals. A t-test indicated that morphine-maintained animals moved less when injected with 3 mg/kg naltrexone compared to 0 mg/kg naltrexone [t(11) = 10.82, P < 0.001]. (C) Mean (SEM) total distance moved following post-sample naltrexone
injections for morphine - naive tested 7 days later. A t-test revealed no significant
difference. D) Mean (SEM) total distance moved following post-sample naltrexone
injections for animals that had been previously maintained on morphine. A t-test
indicated that animals previously maintained on morphine moved less when injected
with 3 mg/kg naltrexone compared to 0 mg/kg naltrexone [t(11)= 2.53, P=0.02]].

Assessment of body weight over 7-day abstinence

![Graph showing body weight changes over 7 days](image)

**Figure 7.** Mean (SEM) Body weight following removal of mini pumps. The
ANOVA revealed a significant difference [F(7,88) = 18.53, P <0.001]. Multiple
comparison further indicated that, in comparison to baseline weight (day 0), animals lost
significantly more weight on days 1 to 4 following removal of mini pumps. * denotes
significant difference compared to day 0.
Total exploration during object recognition tasks

Experiment 2a

Figure 8. Mean (SEM) Total exploration during sample and choice phase. No significant difference between morphine – maintained and morphine – naive total exploration on both sample and choice phase.

Experiment 2b

Figure 9. Mean (SEM) Total exploration during sample and choice phase for animals injected with 0 mg/kg and 3 mg/kg naltrexone. No significant difference
between 0 mg/kg and 3 mg/kg naltrexone total exploration on both sample and choice phase.

**Experiment 2c**

![Figure 10](image)

**Figure 10.** Mean (SEM) Total exploration during sample and choice phase. No significant difference between morphine – maintained and morphine – naive total exploration on both sample and choice phase.

**Experiment 3a**

![Figure 10](image)
**Figure 11.** Mean (SEM) Total exploration during sample and choice phase. No significant difference between CS+ and CS- total exploration on both sample and choice phase.

*Experiment 3b*

![Graph showing mean (SEM) total exploration during sample and choice phase.](image)

**Figure 12.** Mean (SEM) Total exploration during sample and choice phase. No significant difference between CS+ and CS- total exploration on both sample and choice phase.