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Morphine dependence and withdrawal induced changes in cholinergic signaling

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Abstract

Cholinergic signaling is thought to be involved in morphine dependence and withdrawal, but the specific mechanisms involved remain unclear. The current study aimed to identify alterations in the cholinergic system that may contribute to the development of morphine dependence and withdrawal. Acetylcholinesterase (AChE) activity and [³H]-epibatidine binding were evaluated in order to determine if morphine dependence and withdrawal induces alterations in cholinergic signaling or expression of high affinity nicotinic acetylcholine receptors (nAChRs) in the midbrain (MB), medial habenula (MHb) and interpeduncular nucleus (IPN). The effect of cholinergic signaling through nAChRs on morphine-withdrawal induced jumping behavior was then determined. Lastly, the contribution of $\beta 4$ -containing nAChRs receptors in the MHb to morphine-withdrawal induced jumping behavior and neuronal activity as indicated by c-fos expression was assessed. Chronic morphine administration decreased AChE activity in MB and MHb, an effect that was no longer present following precipitated withdrawal. Morphine dependent mice showed increased nicotinic acetylcholine receptor (nAChR) levels in MB. Further, nicotine (0.4 mg/kg) and lobeline (3 mg/kg) decreased jumping behavior while mecamylamine (1 mg/kg) had no effect. Knock-down of $\beta 4$ subunit-containing nAChRs in the MHb attenuated c-fos activation, but did not decrease morphine withdrawal-induced jumping. Thus, morphine withdrawal induces cholinergic signaling in the MHb, but this does not appear to be responsible for the effects of cholinergic drugs on somatic signs of opiate withdrawal, as measured by jumping behavior.

Keywords

Morphine; Dependence; Withdrawal; Acetylcholinesterase; Nicotine; Lobeline

1. Introduction

Behavioral and neurochemical consequences of morphine-induced dependence and withdrawal result from adaptations in multiple neurotransmitter systems across several brain

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regions following repeated drug administration (De Vries and Shippenberg, 2002, Koob *et al.*, 1992). It has been well established that morphine dependence and withdrawal can induce alterations in cholinergic signaling pathways and these changes are thought to underlie at least some of the symptoms associated with morphine withdrawal (Buccafusco *et al.*, 2000, Crossland and Ahmed, 1984, Umberg and Pothos, 2011). Understanding the mechanisms by which altered cholinergic signaling contributes to these adaptations is of particular interest given the modulatory role of acetylcholine (ACh) on other neurotransmitter systems and synaptic plasticity within several neural circuits (for review see Picciotto *et al.*, 2012).

Previous research has indicated that morphine dependence and withdrawal alters cholinergic signaling. While acute morphine administration results in decreased extracellular ACh levels in nucleus accumbens (NAC) of drug-naïve rats, tolerance develops to this effect, as no change in ACh levels are observed in morphine dependent rats in response to a morphine challenge (Rada *et al.*, 1991). Further, naloxone-precipitated opiate withdrawal induces a significant increase in extracellular ACh levels in the NAC, a neurochemical event linked to some behavioral aspects of morphine withdrawal syndrome (Jhamandas and Dickinson, 1973, Rada *et al.*, 1991). Changes in acetylcholinesterase (AChE; an enzyme that breaks down ACh in the extracellular space) activity following morphine dependence and withdrawal have also been reported, an effect that could result in altered extracellular ACh levels (Mohanakumar and Sood, 1983, Sood and Mohanakumar, 1985).

While information regarding changes in ACh neurotransmission within specific brain regions following morphine dependence or withdrawal is limited, several brain regions with cholinergic cell bodies or significant ACh innervation show changes in activity following morphine withdrawal, including the midbrain (MB), medial habenula (MHb) and interpeduncular nucleus (IPN) (Frenois *et al.*, 2002, Wooten *et al.*, 1982). Recent reports have implicated $\beta 4$ subunit-containing nicotinic receptors ($\beta 4^*$ nAChRs) of the MHb in the regulation of nicotine aversion (Frahm *et al.*, 2011). Interestingly, $\beta 4^*$ nAChRs are highly expressed in the MHb-IPN pathway, mediate ACh release in the IPN and may play a role in morphine withdrawal (Grady *et al.*, 2009, Panchal *et al.*, 2005).

Several studies have shown that pharmacological manipulation of the cholinergic system can alter opiate withdrawal-induced behaviors, providing further evidence of a role for cholinergic signaling in morphine dependence and withdrawal. For example, nicotinic agonists can decrease naloxone-induced jumping in morphine dependent mice, while nAChR antagonists have been reported to both increase and decrease this jumping behavior (Brase *et al.*, 1974, Jhamandas and Dickinson, 1973, Zarrindast and Farzin, 1996), suggesting a complex role for nicotinic signaling in opiate withdrawal. Moreover, nicotine administration prior to each morphine injection administered during induction of dependence attenuates withdrawal-induced jumping behavior (Haghpour *et al.*, 2008). Taken together these data support a role of signaling through nAChRs in morphine dependence and withdrawal. However, the effects of morphine dependence and withdrawal on AChE activity within the MHb-IPN pathway warrant further investigation given the importance of these ACh rich regions in the behavioral response to morphine (Taraschenko *et al.*, 2007; Glick *et al.*, 2006). Also, associated specific changes in expression of nAChRs remain unknown.

Here, we investigated alterations in AChE activity and nAChR expression in brain regions that may contribute to the development of morphine tolerance and withdrawal. AChE activity, an indicator of fast adaptation to changing ACh levels (Kaufer *et al.*, 1999), and [3 H]-epibatidine binding was assessed to determine whether morphine tolerance and/or withdrawal induces alterations in extracellular ACh levels or expression of high affinity nAChRs in the MB, MHb and IPN. We then sought to characterize the effects of cholinergic

signaling through nicotinic receptors on escape behavior associated with naloxone-precipitated morphine withdrawal. While nicotine acts as a non-selective agonist at many nAChR subtypes, it can also induce desensitization of the receptors. The effects of lobeline, a nicotinic partial agonist, and mecamylamine, a broad nicotinic receptor antagonist, were therefore assessed to determine whether activation or inactivation of nAChRs mediates effects on opiate withdrawal. Lastly, we determined whether $\beta 4$ -containing nAChRs receptors in the MHb-IPN pathway contribute to morphine-withdrawal induced behavior and neuronal activity as measured by c-fos expression in the MHb.

2. Materials and Methods

2.1. Subjects

Male C57Bl/6J mice (60–180 days of age) were used in these experiments. Mice were housed 4–5/standard cage, received *ad libitum* access to food (Harlan Teklad #2018) and water, and were maintained on a 12-hr/12-hr light/dark cycle. Mice were habituated to the colony for at least 1 week prior to experimental manipulations. All studies were conducted in accordance with the guidelines provided by the National Institutes of Health and were approved by the Yale Animal Care and Use Committee.

2.2. Drugs

Morphine HCL was obtained through the NIDA Drug Supply Program. Nicotine bitartrate salt, mecamylamine hydrochloride, lobeline sulfate and naloxone hydrochloride dihydrate were purchased from Sigma-Aldrich (Saint Louis, MO, USA). All compounds were dissolved in 0.9% NaCl (saline). Nicotine (free base) solutions were adjusted to pH 7.4 with NaOH. All injections were administered IP in a volume of 0.01 ml/g body weight.

2.3. Induction of morphine dependence and naloxone-precipitated withdrawal and evaluation of AChE activity

To induce morphine dependence, mice were administered escalating doses of morphine (20, 40, 60, 80, 100, 100, 100 mg/kg, IP) 3 times a day (0830, 1300, 1830 hr) in the home cage. To assess AChE activity in morphine dependent mice (n=8) compared to saline control (n=9), tissue was harvested 2 hrs after the final morphine injection. In a second group of morphine dependent mice, naloxone (2 mg/kg, IP) was administered 2 hr after the final saline (n=5) or morphine (n=5) injection and tissue was harvested 15 min later. Mice were sacrificed by rapid decapitation and the brain rapidly dissected on an ice-cold surface. The MHb, IPN and MB regions were isolated and immediately placed in 0.5 ml of lysis buffer I on ice. The MB dissection included tissue from the ventral tegmental area (VTA), substantia nigra (SN), periaqueductal grey (PAG), deep mesencephalic area (DMA) and lateral geniculate nucleus (LGN). Brain regions were homogenized by hand in a glass tissue grinder in 0.5 ml of lysis buffer I. The homogenate was centrifuged at 18,000xg for 25 minutes to separate cytosolic and cell membrane-associated proteins. The supernatant was removed and saved as the cytosolic fraction. The pellet (containing nuclei and cell membranes) was re-suspended by hand in 0.5ml lysis buffer I supplemented with 0.5% Triton X-100. Proteins were extracted by incubation at room temperature in 0.5% Triton X-100 for 30 minutes, then the sample was centrifuged for 25 minutes at 18,000xg to remove detergent-insoluble complexes. The supernatant was recovered and used as the membrane-associated protein fraction. All data presented are derived from the membrane fractions. Samples were prepared for rapid colorimetric determination of AChE activity using a QuantiChrom (BioAssay Systems; Hayward, CA) assay kit. Data were normalized to saline control. Briefly, 10 μ l aliquots of tissue extract were added to clear, flat-bottom 96-well plates, and reaction initiated by the addition of 100 μ l of reaction buffer with 2 mg/ml AChE substrate; samples were run in duplicate. The plates were immediately placed in the

spectrophotometer at room temperature and an initial absorbance at 405 nm taken to serve as baseline. Readings were recorded every 5 minutes for 25 minutes thereafter. Linear reaction rates were calculated from within the linear range of the absorbance data in SigmaPlot 2001, with the equation $y = mx + b$, where “y” is absorbance, “x” is time, “b” is any basal absorbance measured at t_0 , and “m” is the slope of the resulting line, which is used as the quantitative measure of AChE activity, expressed in Abs405/min/ μ g protein. Protein content in each sample was determined by the method of Lowry (Lowry *et al.*, 1954). For presentation, data were normalized further as a percentage of saline controls to eliminate any inter-experimental confounds arising from use of different lots of the QuantiChrom assay kit.

2.4. Assessment of nAChR expression in morphine dependent mice

Mice were administered either saline or escalating doses of morphine as described above. Mice were sacrificed by rapid decapitation and the brain rapidly removed and dissected on an ice-cold surface. The MHB, IPN and MB regions were isolated and immediately placed in 0.5 ml of lysis buffer I on ice. The MB dissection included tissue from the VTA, SN, PAG, DMA and LGN. Tissue was homogenized by hand in a glass tissue grinder in 0.5 ml cold lysis buffer I. Tissue homogenates were centrifuged at 18,000xg for 25 min and the pellet re-suspended in 0.5 ml fresh lysis buffer I, and the suspension centrifuged again for 10 min at 18,000xg. This process was repeated an additional two times before the pellet was re-suspended in 0.5 ml 0.1X binding buffer. High-affinity [3 H]-epibatidine binding proceeded essentially as described in Marks et al (2006) with minor modifications. Tissue homogenates (50 μ l aliquots) were incubated in 1.2 ml microtiter tubes arranged in 96-well format with 10 μ l of 4nM [3 H]-epibatidine (specific activity: 55.8Ci/mmol), and the volume adjusted to 500 μ l with the addition of 440 μ l 1.14X binding buffer (final concentrations: 1X binding buffer, 200 pM [3 H]-epibatidine). Nonspecific binding was determined in the presence of 200 μ M cytosine. Binding took place overnight at 4°C with gentle agitation and was terminated by filtration onto glass fiber filters presoaked in 0.5% polyethylinimine using a Packard Cell Harvester. Filters containing trapped [3 H]-epibatidine binding sites were rinsed 3X with ~200 μ l/well of pre-chilled binding buffer and bound radioactivity determined by counting in a Beckman LS6000LL scintillation counter at 55% efficiency.

2.5. Evaluation of the effects of nicotinic receptor ligands on naloxone-precipitated morphine withdrawal-induced jumping behavior

Separate groups of mice were administered either saline or escalating doses of morphine as described above. Mice were transported to the behavioral testing room 30 min prior to administration of the last morphine injection. In the first experiment, mice were given either saline (n = 17) or nicotine (0.2 or 0.4 mg/kg; n = 12 per group) 1 hr 45 min after the final morphine injection. A separate group of morphine dependent mice were given saline (n = 17), mecamylamine (1 mg/kg; n = 11) or lobeline (3 or 10 mg/kg; n = 12 per group) 1 hr 45 min after the final morphine injection. All mice were then given naloxone (2 mg/kg) 15 min later (2 hrs after the final morphine injection) to precipitate withdrawal and immediately placed in a 5L glass cylinder where behavior was digitally recorded for 15 min. This dose of naloxone does not induce escape jumping behavior in drug naïve mice (Way *et al.*, 1969). Video was later assessed for number of escape jumps, a well characterized behavioral outcome of naloxone-precipitated morphine withdrawal (Way *et al.*, 1969; Sharf *et al.*, 2008), by 2 observers blind to the experimental conditions. Although rare, in the case of discrepancies between observers, the video was re-assessed in the presence of both observers. Therefore, the number of jumps counted for each mouse was consistent between the two observers.

2.6. Evaluation of the role of $\beta 4$ -containing nAChRs in the MHb-IPN pathway in naloxone-precipitated withdrawal-induced jumping behavior and c-fos activation in MHb

Mice were placed in a stereotaxic frame (Kopf Instruments, Tijuana, CA) under anesthesia with 4% isoflurane and were infused bi-laterally using a 5- μ l Hamilton micro-syringe (Hamilton, Reno, NE) with a 26G beveled needle with 0.5 μ l of Adeno Associated Virus (AAV)-carrying shRNA targeting *chrb4* (encoding the $\beta 4$ nAChR subunit) or AAV-scramble (control) that have been developed and characterized previously (Mineur *et al.*, 2011). The coordinates for stereotaxic injection (relative to Bregma: Paxinos and Watson) were determined based on atlas coordinates and adjusted by the experimenter following trial infusions with dye (A/P -1.94 mm; M/L ± 0.3 mm; D/V ± 2.6 mm). Viral Infusions were made over 5 min at a rate of 0.1 μ l/min, and the syringe was held in place for an additional 5 min before being removed. Following surgery, mice were allowed to recover for 2 weeks before undergoing behavioral assays.

Two hours after behavioral testing mice were irreversibly anesthetized with chloral hydrate, perfused intra-cardially with cold PBS (0.1 M, pH 7.3) followed by cold 4% paraformaldehyde (PFA) for 5 min each (~ 50 ml of each solution per animal). Brains were taken from the skull and post-fixed for 24 hr in PFA at 4°C, then placed in 30% sucrose/PBS (0.1 M, pH 7.3) for cryoprotection, and finally stored at 4°C until slicing. Brain sections (40 μ m) were cut with a sliding microtome. Verification of infection in the MHb was determined by cryostat sectioning through the infusion site and visual detection of GFP; only mice with infection in the MHb were included in the analysis.

The MHb sends the majority of its projections to the IPN, where the $\beta 4$ nAChR subunit is localized to presynaptic terminals (Grady *et al.*, 2009). Therefore, it is likely that knock down of the $\beta 4$ nAChR subunit in the MHb also results in decreased levels of $\beta 4^*$ nAChRs on nerve terminals in the IPN. Free-floating sections were washed in 0.1M PBS between each of the following steps: pre-blocking in 3% normal goat serum/0.3% Triton X-100 in PBS for 1 hr followed by incubation in the antibody (Texas red pre-conjugated rabbit anti c-fos SC-52, Santa Cruz, CA) in PBS for 48h at 4°C. Sections were washed several times, mounted on slides and coverslipped. Positive cells were visualized and counted with a fluorescent confocal microscope with appropriate filter.

2.7. Data Analysis

All analyses were conducted using IBM SPSS Version 19.0 (Armonk, NY). AChE data were expressed as a percent of saline for each brain region and differences between saline and morphine dependent or morphine withdrawn mice were analyzed with independent t-tests. [3 H]-epibatidine binding was expressed as fmol/mg protein and comparisons were made between saline and morphine withdrawn mice with independent t-tests for each brain region. Each brain region was analyzed as an independent experiment using a 2-tailed t-test. Jumping behavior was analyzed using 2-way between-subjects ANOVAs followed by 1-way ANOVAs with one-tailed independent t-tests employing a Bonferroni correction to assess specific group differences as previous research suggested nicotinic agonists would decrease jumping behavior associated with naloxone-precipitated morphine withdrawal. Jumping behavior and c-fos positive cells between GFP and $\beta 4$ -knockdown mice were analyzed with independent t-tests.

3. Results

3.1. AChE activity following morphine dependence and naloxone-precipitated withdrawal

AChE activity was measured in several brain regions following the induction of morphine dependence and naloxone-precipitated withdrawal. In morphine dependent mice, AChE

activity was significantly decreased in the MB (Fig 1a; $t_{(15)}=4.15$, $p<0.01$) and MHb ($t_{(15)}=2.51$, $p<0.05$), whereas no changes were observed in the IPN. The attenuation of AChE activity in the MB and MHb was no longer evident following naloxone administration to morphine dependent mice, but instead, a increase in AChE activity was observed in the IPN after naloxone injection (Fig 1b; $t_{(8)}=2.20$, $p=0.054$). These data show that changes in cholinergic system signaling occur following precipitated withdrawal in morphine dependent mice.

3.2. Assessment of nAChR levels in morphine tolerant mice

In order to determine if the level of high-affinity nAChRs is changed following induction of morphine tolerance, [^3H]-epibatidine binding was assessed in MB, MHb and IPN. Morphine tolerant mice showed increased nAChR binding in the MB (Fig 2; $t_{(7)}=3.22$; $p<0.05$) and a trend for increased levels in the MHb ($t_{(7)}=1.96$; $p=0.091$). These data provide evidence that morphine dependence alters the number of nicotinic binding sites in MB.

3.3. The effects of nicotinic receptor ligands on naloxone-precipitated morphine withdrawal-induced jumping behavior

The ability of nAChR ligands to decrease behavioral signs of opiate withdrawal was measured by evaluating jumping behavior following naloxone administration to morphine-dependent animals. In groups tested for the ability of nicotine to decrease jumping behavior, the omnibus ANOVA indicated a significant effect of morphine exposure (Fig 3a; $F_{(1,56)}=49.23$, $p<0.001$). Morphine withdrawal alone elicited a significant increase in jumping behavior compared to saline exposure controls ($t_{(23)}=4.59$, $p<0.001$). No significant differences were observed across the saline exposure pretreatment groups ($F_{(2,20)}=1.28$, n.s.). In contrast, there was a significant effect of pretreatment in the morphine exposed groups ($F_{(2,40)}=3.67$, $p<0.05$). The higher dose of nicotine (0.4 mg/kg) significantly decreased naloxone-precipitated jumping behavior compared to saline ($t_{(27)}=2.17$, $p<0.05$). In groups tested for the ability of mecamylamine or lobeline to decrease jumping behavior, the omnibus ANOVA indicated a significant effect of morphine exposure (Fig 3b; $F_{(1,73)}=57.44$, $p<0.001$). Similar to the aforementioned study, morphine withdrawal alone elicited a significant increase in jumping behavior compared to saline exposure controls ($t_{(24)}=6.50$, $p<0.001$). No significant differences were observed across the saline exposure pretreatment groups ($F_{(3,28)}=0.70$, n.s.). However, there was a significant effect of pretreatment in the morphine exposed groups ($F_{(3,51)}=2.80$, $p=0.05$). The non-selective nicotinic receptor antagonist mecamylamine (1 mg/kg) did not affect naloxone-precipitated jumping. In contrast, the lower doses of the nicotinic partial agonist lobeline (3 mg/kg) significantly attenuated jumping behavior compared to saline control ($t_{(27)}=2.77$, $p<0.05$) and a trend was observed for the higher dose to have a similar effect ($t_{(27)}=2.51$, $p=0.07$).

3.4. Role of $\beta 4^*$ nAChRs in the MHb on naloxone-precipitated withdrawal-induced jumping behavior and c-fos activation

Mice infected with AAV-shRNA targeting the nAChR $\beta 4$ subunit in the MHb did not differ from control animals in jumping behavior induced by naloxone-precipitated withdrawal (Fig 4d); however, after knockdown of $\beta 4^*$ nAChRs in the MHb, there was ~ 50% reduction of c-fos immunostaining following naloxone-precipitated morphine withdrawal compared to control animals (Fig 4d; $t_{(22)}=8.81$, $p<0.001$), suggesting that hyperactivity in the MHb during opiate withdrawal is mediated through cholinergic activity at $\beta 4^*$ nAChRs.

4. Discussion

The results from these experiments provide some insight into changes in cholinergic signaling that contribute to morphine dependence and withdrawal. Chronic morphine

administration results in dependence and this coincides with a decrease in AChE activity in the MB and MHb, but not IPN. While not directly reflective of ACh levels, the decrease in AChE activity observed in the MB and MHb could represent a homeostatic response to initial morphine-induced decreases in ACh in the MB and MHb, as has been reported in the NAC (Rada *et al.*, 1991). Interestingly, this morphine-induced decrease in extracellular ACh dissipates across repeated injections (i.e. tolerance develops; Rada *et al.*, 1991), potentially indicating that a decrease in AChE activity occurs in response to lower extracellular ACh levels to re-establish homeostatic levels of extracellular ACh following repeated morphine administration.

The current finding showing that there are no differences in AChE activity in the IPN of morphine dependent animals is consistent with a previous report that relatively high doses of morphine do not change extracellular ACh levels in this region (Taraschenko *et al.*, 2007) and may provide further evidence that AChE activity reflects extracellular ACh levels. In contrast, it has been reported previously that morphine dependent mice show an increase in AChE activity in several brain regions, including the habenula; however, this is likely attributable to the difference in the amount of morphine that was administered to induce dependence, as this previous report administered doses up to 450 mg/kg over 15 days (Mohanakumar and Sood, 1983). Measurements of extracellular ACh in MB and MHb following repeated morphine treatment have not yet been reported.

It is well accepted that AChE can decrease synaptic ACh levels efficiently, but the effects of altered extracellular ACh levels on AChE activity are less clear. Naloxone-precipitated withdrawal alters the pattern of AChE activity observed after induction of morphine dependence. After precipitating opiate withdrawal, there is no longer a difference in AChE activity between the treatment groups in the MB and MHb, whereas an increase in AChE activity that was not present in dependent animals is measured in the IPN, indicating that AChE activity is increased across all these brain areas following withdrawal, compared to the dependent state. The increase in AChE activity following withdrawal could be a compensatory response to increases in extracellular ACh release evoked by precipitated withdrawal in these brain regions, similar to that which has been reported in the NAC (Rada *et al.*, 1991). In addition to regulating ACh levels, AChE has been proposed to have additional effects on cell signaling, including promoting dendritic release of dopamine from neurons in midbrain (for review Soreq and Seidman, 2001), suggesting another potentially significant role for AChE activity in morphine dependence and withdrawal.

The current results are the first to provide evidence that morphine dependence is associated with increased levels of high-affinity nAChRs in the MB and a non-significant trend towards increased high-affinity nAChRs in the MHb. While a wide variety of $\beta 2$ -containing nAChR subtypes are expressed in the MHb and MB, the MHb also has a variety of $\beta 4$ -containing nAChR subtypes (Gotti *et al.*, 2005a, Gotti *et al.*, 2005b, Grady *et al.*, 2009). It has been demonstrated previously that chronic inhibition of AChE can increase nAChR expression, although the specificity of the compounds used and the functional implications of these findings have been questioned (Bhat *et al.*, 1990, Clarke *et al.*, 1994, Takada-Takatori *et al.*, 2009, Schwartz & Kellar, 1985). Thus, the increase in high-affinity nAChR levels in the MB and MHb may have resulted from chronic inhibition of AChE activity or ACh release during the period of morphine treatment. Consistent with this possibility, activity in the VTA, an area of the midbrain, is regulated by nAChR activity (Mansvelder *et al.*, 2002, Pidoplichko *et al.*, 1997), and while the firing rate of DA neurons in the VTA is higher in morphine dependent rats, it returns to that of naïve rats upon naloxone-precipitated withdrawal (Georges *et al.*, 2006). Thus, opiate-dependent modulation of cholinergic signaling through nAChRs could contribute to changes in neuronal activity in key brain areas involved in reinforcement and affect. Future studies characterizing the interactions between AChE

activity and nAChR function using non-pharmacologic manipulations such as viral-mediated gene transfer, may provide therapeutic targets for treating morphine dependence.

Upregulation of nAChRs has been observed following chronic treatment with nAChR agonists and partial agonists, such as nicotine and varenicline (Marks *et al.*, 1983, Turner *et al.*, 2011). Many of the compounds used to inhibit AChE activity in studies examining the effects of decreased AChE activity on nAChR expression interact directly with nAChRs. For example, the AChE inhibitor physostigmine interacts with the allosteric binding site of $\alpha 4\beta 2$ nAChRs and it has been suggested that this pharmacological action may underlie the upregulation of nAChRs following chronic administration (Smulders *et al.*, 2005, Reid *et al.*, 2008). Interestingly, a recent report suggests that morphine acts as a partial agonist at $\alpha 4\beta 2$ nAChRs (Talka *et al.*, 2013). While this effect has not yet been demonstrated *in vivo*, it raises the possibility that chronic morphine exposure may induce nAChR upregulation by direct interaction with nAChR agonist binding sites. Therefore, chronic morphine administration may alter nAChR expression in a manner independent of its effects on ACh neurotransmission and AChE.

Nicotine pre-treatment attenuated jumping behavior, a somatic sign of opiate withdrawal in mice, induced by naloxone-precipitated withdrawal at the higher dose tested here (0.4 mg/kg). These results are consistent with previous reports (Zarrindast and Farzin, 1996). The nAChR antagonist mecamylamine (1 mg/kg) did not have an effect on this behavior on its own in the current study, in contrast to a previous report indicating that a higher dose (5 mg/kg) decreased naloxone-induced withdrawal jumping (Jhamandas and Dickinson, 1973). We should note that 5 mg/kg is a very high dose, and is likely to have off-target effects on other ion channels, including NMDA receptors (O'Dell and Christensen, 1988), so we do not believe that this early study is necessarily indicative of an effect of blocking nAChRs.

Lobeline attenuated jumping at the lower dose (3 mg/kg) tested and there was a non-significant trend for the higher dose (10 mg/kg) to also attenuate jumping behavior. Lobeline has a complex pharmacological profile (for review see Dwoskin and Crooks, 2002). It binds to nAChRs with relatively high affinity (Parker *et al.*, 1998; Boorman *et al.*, 2003) and has historically been thought to function as an agonist at nAChRs. Specifically, it has been suggested that lobeline acts as a full agonist $\alpha 4\beta 4^*$ nAChRs and as a partial agonist at $\alpha 4\beta 2$ nAChRs (Wu *et al.*, 2006). Interestingly, it has also been suggested that lobeline functions as an antagonist at $\alpha 3\beta 2^*$ and $\alpha 4\beta 2^*$ (Miller *et al.*, 2000). In addition to its actions at nAChRs, lobeline inhibits the vesicular monoamine transporter (Teng *et al.*, 1997) and binds to μ -opioid receptors (Miller *et al.*, 2007). It is currently not known whether systemic nicotine or lobeline administration alter AChE activity in the MB, MHb or IPN of morphine dependent mice directly.

While no significant differences were found following pretreatment with nicotine (0.4 mg/kg) or lobeline (3 or 10 mg/kg) in chronic saline-exposed mice, non-specific sedative effects of these drugs on jumping behavior are difficult to assess given the low level of baseline jumping in these groups. However, nicotine (0.3 mg/kg)- and lobeline (3 mg/kg)-induced decreases in locomotor activity have been reported previously (Miller *et al.*, 2003). Since a disruptive affect of locomotor hypoactivity on withdrawal-induced jumping behavior has not been reported, this cannot be ruled out as a potential confound contributing to the present results.

A role for $\beta 4^*$ and $\alpha 5^*$ nAChR signaling in the MHb has been established in regulation of somatic signs and aversive states associated with nicotine withdrawal (Frahm *et al.*, 2011, Salas *et al.*, 2009). In addition, there is evidence that $\beta 4^*$ nAChRs mediate ACh release within the MHb-IPN pathway (Grady *et al.*, 2009) and these receptors may be important for

behavioral and neurochemical aspects of morphine, such as sensitized dopamine release in the NAC (Taraschenko *et al.*, 2007), self-administration (Glick *et al.*, 2006) and precipitated withdrawal-induced behaviors (Panchal *et al.*, 2005). While results from the current study do not support a role for $\beta 4^*$ nAChRs in the MHB in jumping behavior induced by naloxone-precipitated withdrawal, they do suggest that this receptor subunit plays a role in neuronal activity in this brain region, as measured by c-fos activation. However, $\beta 4^*$ nAChR activity may underlie other aspects of the morphine withdrawal syndrome.

In summary, the current data show that alterations in cholinergic signaling contribute to morphine dependence and withdrawal. Further, an increase in nAChR expression was evident following morphine dependence. Future studies characterizing the interactions between AChE activity levels and nAChR activity are warranted. Given the availability of AChE inhibitors that are currently used to treat disease states such as Alzheimer's (Mehta *et al.*, 2012), assessing the ability of these pharmacological agents to treat morphine dependence and withdrawal may provide improved treatment outcomes.

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Highlights

- Chronic morphine administration decreased AChE activity in MB and MHb.
- Morphine dependent mice showed increased nAChR levels in MB.
- Nicotine and lobeline decreased opiate-withdrawal jumping behavior.
- Knock-down of $\beta 4$ subunit-containing nAChRs in the MHb attenuated c-fos activation.
- Cholinergic signaling is altered following morphine dependence and withdrawal.

Figure 1.

a. Decreased acetylcholinesterase (AChE) activity in the midbrain (MB) and medial habenula (MHb) of morphine dependent mice. **b.** Increased AChE activity in the IPN following naloxone-precipitated morphine withdrawal. * $p < 0.05$ compared to saline control. # $p = 0.05$ compared to saline control

Figure 2. Increased nicotinic receptor binding in the midbrain (MB) and a trend towards increased nicotinic receptor binding in the medial habenula (MHb), but not the IPN following induction of morphine dependence. * $p < 0.05$, # $p = 0.09$ compared to saline control.

Figure 3.

a. Nicotine (0.4 mg/kg) attenuates morphine withdrawal-induced jumping behavior. **b.** Lobeline (3 mg/kg) attenuates jumping behavior compared to morphine withdrawn saline controls and there is a trend towards decreased jumping behavior following the higher dose (10 mg/kg). * $p < 0.05$, # $p = 0.07$

Figure 4.

a. Schematic of viral infusion sites in MHb. **b.** GFP expression in MHb following viral infection with AAVs. **c.** Representative photomicrograph of c-fos immunostaining. **d.** Mice with knockdown of the $\beta 4$ nAChR subunit did not differ from control animals in opiate withdrawal-induced jumping behavior. **e.** Mice with knockdown of the $\beta 4$ nAChR subunit had a ~ 50% reduction of c-fos immunostaining following naloxone-precipitated morphine withdrawal compared to control animals. *** $p < 0.001$