JPET Fast Forward. Published on January 13, 2021 as DOI: 10.1124/jpet.120.000423
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Title Page

Enhancement of opioid antinociception by nicotinic ligands

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JPET Fast Forward. Published on January 13, 2021 as DOI: 10.1124/jpet.120.000423
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Running Title Page

Running title: Varenicline and epibatidine as opioid adjuvants

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Number of Pages: 17

Number of Tables: 1

Number of Figures: 4

References: 48

Abstract: 233 words

Introduction: 524 words

Discussion: 1210 words

Nonstandard abbreviations: nAChR- nicotinic acetylcholine receptor; MOR- μ-opioid receptor; KOR- κ-opioid receptor; DHβE- dihydro-β-erythroidine

Conflict of Interest: No author has an actual or perceived conflict of interest with the contents of this article.

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Abstract

Nicotine previously has been shown to augment the antinociceptive effects of μ-opioid agonists in squirrel monkeys without producing a concomitant increase in behavioral disruption. The present studies were conducted to extend these findings by determining the ability of the nAChR agonist epibatidine and partial α4β2 nAChR agonist varenicline to also selectively augment the antinociceptive effects of the MOR full agonist fentanyl, the MOR partial agonist nalbuphine, and the KOR agonist U69,593 in male squirrel monkeys. Results indicate that both nAChR ligands selectively increased the antinociceptive effects of nalbuphine and epibatidine increased the antinociceptive effects of U69,593 without altering effects on operant behavior. However, neither epibatidine nor varenicline enhanced the antinociceptive effects of fentanyl, perhaps due to its high efficacy. The enhancement of nalbuphine's antinociceptive effects by epibatidine, but not varenicline, could be antagonized by either mecamylamine or DHβE, consistent with α4β2 mediation of epibatidine's effects but suggesting the involvement of non-nAChR mechanisms in the effects of varenicline. The present results support previous findings showing that a nAChR agonist can serve as an adjuvant for MOR antinociception and, based on results with U69,593, further indicate that the adjuvant effects of nAChR drugs also may apply to antinociception produced by KOR. Our findings support the further evaluation of nAChR agonists as adjuvants of opioid pharmacotherapy for pain management and point out the need for further investigation into the mechanisms by which they produce opioid-adjuvant effects.

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Significance Statement

Nicotine has been shown to augment the antinociceptive effects of MOR analgesics without exacerbating their effects on operant performance. The present study demonstrates that the nAChR agonist epibatidine and partial α4β2 nAChR agonist varenicline can also augment the antinociceptive effects of nalbuphine, as well as those of a KOR agonist, without concomitantly exacerbating their behaviorally disruptive effects. These findings support the view that nAChR agonists and partial agonists may have potential as adjuvant therapies for opioid-based analgesics.

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Introduction

Opioids are among the most commonly prescribed medications for the management of pain; however, their use is limited by adverse effects (e.g., respiratory depression, behavioral inhibition, addiction; Schug et al., 1992; Paulozzie and Ryan, 2006; Clark and Schumacher, 2017). One approach to this problem has been to identify novel drugs (e.g., the development of biased agonists or non-opioid ligands) that may be effective analgesics absent of opioid-like deleterious effects. However, though conceptually appealing, this approach has not yet been fruitful. An alternative strategy involves the adjuvant use of selected non-opioids to increase the analgesic potency of prescription opioids (for review, see: Li, 2019). According to this strategy, combination of the adjuvant and opioid drugs should permit the use of lower doses of opioids for effective pain relief and, thereby, reduce the intensity of adverse effects. A wide range of non-opioid drugs (e.g., NMDA antagonists, cannabinoid agonists, imidazoline I_2 agonists) have been investigated preclinically to evaluate this "opioid-sparing" approach (Maguire and France, 2014, 2018; Siemian et al., 2016; Li, 2017), which has enjoyed some clinical success. For example, α2 adrenergic agonists, such as clonidine, have been shown to reduce the need for post-operative morphine, suggesting that these drugs may effectively function as adjuvants for μ-opioid receptor (MOR) mediated analgesia (Blaudszun et al., 2012; Engelman and Marsala, 2013).

Recently, using an assay combining nociception (warm water tail withdrawal) and operant food-maintained behavior in squirrel monkeys (Withey et al., 2018a), we found that the nicotinic acetylcholine receptor (nAChR) agonist nicotine increased the antinociceptive potency of MOR agonists at doses below those that exacerbated their disruptive effects on operant behavior (de Moura et al., 2019). These effects of nicotine were nAChR subtype-selective (i.e., not evident with muscarinic ligands) and inversely related to efficacy at MOR (i.e., nicotine was more effective in enhancing the antinociceptive effects of the low-efficacy agonist nalbuphine than the high-efficacy agonist fentanyl). These findings were of particular interest because nicotinic ligands previously have been reported to produce antinociception in their own right (Wewers et al., 1999; Zarrindast et al., 1999; Berrendero et al., 2002; Kyte et al., 2018), providing a functional framework for the idea that a nAChR agonist may have utility as opioid adjuvants.

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The present studies were conducted to explore the ability of nAChR agonists to augment the antinociceptive effects of opioids. Using assay conditions previously employed to study nicotine (Withey et al., 2018a; de Moura et al., 2019), the effects of epibatidine and varenicline alone and in combination with the MOR agonists fentanyl or nalbuphine, or the κ-opioid receptor (KOR) agonist U69,593 were investigated in squirrel monkeys. Epibatidine, a high-efficacy agonist that selectively activates the α4β2 nAChR, previously has been evaluated as a candidate analgesic in its own right, whereas varenicline, a relatively non-selective agonist with low efficacy at α4β2 nAChRs, currently is marketed for smoking cessation (Grady et al., 2010). These ligands, as well as the MOR agonists fentanyl and nalbuphine, were selected for study primarily to evaluate the role of α4β2 nAChR and MOR efficacy in their expected adjuvant effects. The inclusion of U69,593 in these studies permitted an initial evaluation of whether nicotinic agonists might also enhance KOR-mediated antinociception.

Methods

Subjects. Adult male squirrel monkeys (n=4; Saimiri sciureus) were housed in stainless steel cages in a climate-controlled vivarium under a 12-h light/dark cycle at the McLean Hospital Animal Care Facility (licensed by the United States Department of Agriculture). Subjects had unrestricted access to water and were fed a daily allotment of high protein primate chow (Purina Monkey Chow, St. Louis, MO) supplemented with multivitamins and fruit, per veterinary recommendation. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at McLean Hospital, and housing was compliant with guidelines provided by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, Commission on Life Sciences, National Research Council (2011). All subjects previously served in similar studies with nAChR and MOR agonists (de Moura et al., 2019).

Apparatus. Experimental sessions were conducted 5 days a week, between 9 am to 1 pm. During experimental sessions, subjects were placed in customized Plexiglas chairs as previously described (Withey et al., 2018a; de Moura et al., 2019). Briefly, the seated subject faced two levers, each 8 cm below a stimulus light that could be illuminated during the session. The subjects also faced and had easy access to a customized Plexiglas receptacle between the levers into which fluid could be delivered for consumption. During sessions, a syringe pump (Med Associates, Inc., Georgia, VT, Model PHM-100-10) outside the chamber could be operated to deliver sweetened condensed milk via Tygon tubing into the receptacle's reservoir (0.15 ml in 0.84 s). The rear portion of the chair was designed so that the tail of the subject could hang freely, permitting its end to be immersed in a water-filled container; only the distal 3-4 cm of the tail was immersed in water during experiments, allowing the subject to remove its tail from the water at any time without assistance. All experimental events were programmed and recorded through a commercially available interface (MED-PC, Med Associates Inc., St. Albans, VT, USA).

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Behavioral Procedures. The procedures in this study have been previously described (Withey et al., 2018a; de Moura et al., 2019). Briefly, the daily session was composed of four 15-minute components, each divided into a 10-minute long timeout followed by a 5-minute test period. The test period was initiated by the illumination of red stimulus light above one lever. The completion of 10 responses (FR10) on that lever within 20 seconds (limited hold 20-s) terminated the red lights and triggered the delivery of 30% milk in water (v/v) into the reservoir. A 30-s short timeout followed the completion of the FR requirement or the expiration of the limited hold, after which the red stimulus lights were re-illuminated and the FR10 schedule was again in effect. During the 30-s timeout, the distal portion of the subject's tail was immersed in 35, 50, 52, or 55ºC water, and the latency to withdraw the tail from the water was recorded. The trial was terminated by withdrawal of the tail from water by the subject or, if 10 s elapsed without tail-withdrawal, removal of the heated water by the experimenter. The 10-s cutoff served to avoid the possibility of tissue damage. These session parameters permitted at least 6 tail-withdrawal measurements within each component. The order of water temperatures within and across components was randomized, with the proviso that determinations in each test component occurred no more than once at 50, 52, or 55ºC and at least 3 times at 35ºC. The water temperature of 35°C was selected to control for conditioned responses to water because previous data have shown that it does not induce a tail-withdrawal response under non-drug conditions (Withey et al., 2018a).

Drug Testing Procedures. Dose-response functions for antinociception and decreases in operant response rate produced by the MOR agonists fentanyl, nalbuphine, and the KOR agonist U69,593 (La Regina et al., 1988) were determined in all subjects using cumulative dosing procedures, i.e., by administering graded increasing intramuscular (i.m.) doses at the beginning of successive components (i.e., at the onset of the 10-min timeout). For example, if the starting dose was 0.32 μg/kg fentanyl, prior to second component, an i.m. injection of 0.68 μg/kg was administered, 2.2 μg/kg was administered prior to the third component, and 6.8 μg/kg was administered before the fourth and final component, so that a dose-response function including the doses of 0.32, 1, 3.2, and 10 ug/kg fentanyl would be measured through the experimental session. In other experiments,

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the effects of each opioid also were determined after i.m. injections of the nAChR agonists epibatidine (0.56 μg/kg) or varenicline (0.032 mg/kg). These doses of the nAChR agonists were selected on the basis of previous data (Withey et al., 2018b) and dose-ranging studies showing that these doses were the highest that did not completely abolish food-maintained responding. In the present experiments, saline or nAChR agonists were administered immediately prior to the session to permit evaluation of their effects in the first component, and the first dose of each opioid was administered in the second component. Subsequent studies to evaluate antagonism of nicotinic modulation of opioid effects were conducted by administering either the non-selective nAChR antagonist mecamylamine or the selective α4β2 nAChR antagonist dihydro-β-erythroidine (DHβE; Grady et al., 2010; de Moura and McMahon, 2017) 5 min prior to the administration of the nAChR agonists or saline vehicle. The doses of mecamylamine and DHβE were selected on the basis of previous studies in squirrel monkeys (Withey et al., 2018b). Drug testing occurred no more than once per week throughout the present studies.

Data Analyses. Latency to withdraw the tail from water was expressed as the elapsed time from immersion of the tail in water to its removal by the subject or experimenter; operant response rate was measured as responses per second and expressed as a percentage of the subject's overall rate of responding during control sessions. The effects of successive injections of saline on both tail-withdrawal latency and response rates across components were determined at least monthly and provided baseline values (control sessions). Overall baseline values for tail-withdrawal latency and response rate in the present study are expressed as the mean of control values across subjects (± S.E.M). Results from drug experiments are similarly calculated for individual subjects and expressed as the mean of data across subjects (± S.E.M).

Effects of nAChR agonists alone or combined with nAChR antagonists. A repeated measures one-way ANOVA was used to compare response rates after administration of saline and varenicline (0.032 mg/kg) or epibatidine (0.56 μg/kg), alone or in the presence of mecamylamine (0.1 mg/kg) or DHβE (0.1 mg/kg). Linear regression was used to fit lines to the linear portion of dose-response functions in individual subjects (GraphPad

Prism version 5.0 for Windows; GraphPad Software, San Diego, CA). For response rate, the linear portion of the curve was defined to include not more than one dose that produced <20% of control response rate, and not more than one dose that produced >80% of control response rate. For tail-withdrawal latency, the linear portion of the curve was defined to include not more than one dose that produced <2-s latency, and not more than one dose that produced >8-s latency.

Effects of opioids alone or after treatment with nAChR agonists or nAChR agonists-antagonists combinations. Dose response data for opioids alone or following treatment with nAChR agonists or nAChR agonist-antagonist combinations were fitted to straight lines and were considered significant if the slope differed significantly from 0. The slopes and intercepts of the dose-response functions for an opioid alone or after each type of treatment were compared using an *F*-ratio test (GraphPad Prism), with statistical significance set at $p<0.05$. ED_{50} values for each type of treatment were determined using linear interpolation. If the dose-response function for a nAChR agonist in combination with an opioid had a slope >0 but the group average latency was not at least 5 sec, the ED_{50} value was estimated using linear regression, provided that tail-withdrawal latency in at least half the subjects was >5. Linear regression was used to determine a common slope to calculate potency ratios with corresponding 95% confidence limits (95% CL) per Tallarida (2000). If the 95% confidence limits of a potency ratio did not include 1, the ED_{50} values were considered significantly different.

Drugs. All doses were calculated in terms of the drug's base weight and were administered intramuscularly. Drugs were fentanyl citrate (Sigma-Aldrich, St. Louis, MO), nalbuphine hydrochloride hydrate (Sigma-Aldrich), (±)-epibatidine dihydrochloride hydrate (Sigma-Aldrich), varenicline tartrate (Sigma-Aldrich), dihydro-β-erythroidine (DHβE; Tocris Biosciences, Minneapolis, MN), and mecamylamine hydrochloride (Sigma-Aldrich).

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Results-

Control Response Rate and Tail-Withdrawal Latencies. Control rates of operant responding (i.e. after saline administration) were 2.81 (±0.82) responses/second. Tail-withdrawal latencies (mean ±SEM) following the administration of saline averaged 1.3 (±0.19), 1.2 (±0.14), and 1.1 (±0.08) sec at 50, 52, and 55°C, respectively (Figure 1).

Effects of opioids alone. Dose response data for the effects of opioids on operant behavior and tail-withdrawal latency are shown in Figures 1-3; ED_{50} values are summarized in Table 1. The high efficacy MOR agonist fentanyl dose-dependently decreased rates of responding, while concomitantly increasing tail-withdrawal latencies at all warmed water temperatures (Figure 1, left panels: Table 1). In contrast, doses up to 3.2 mg/kg of the low efficacy MOR agonist nalbuphine did not appreciably alter rates of responding and increased tail-withdrawal latency at only two of the three water temperatures (50 and 52°C) (Figure 2, Table 1). Nalbuphine was without antinociceptive effect when the water temperature was 55°C. The KOR agonist U69,593, like fentanyl, dose-dependently decreased the rate of operant responding and increased tail-withdrawal latencies at all water temperatures (Fig 3).

Overall, the magnitude of antinociception produced by each of the three opioids was inversely related to the water temperature (Table 1). Raising the temperature of water from 50 to 55°C decreased the maximum latency to tail-withdrawal from 8.1 (±1.1) to 7.0 (±2.0) sec following fentanyl, from 7.6 (±1.4) to 1.8 (±0.30) sec following nalbuphine, and from 10 (±0) to 8.1 (±1.1) sec following U69,593. Decreases in the magnitude of antinociceptive effects in 55°C water were accompanied by decreases in opioid potency from values recorded in 50°C water, as reflected by >2-fold decreases in potency for fentanyl [2.5 (0.6-11)] and U69,593 [2.2 (0.7-6.4)].

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Modification of opioid effects by varenicline and epibatidine. Varenicline (0.032 mg/kg) alone or following injection of mecamylamine (0.1 mg/kg) or DHβE (0.1 mg/kg) did not significantly disrupt operant response rate ($F_{3,15}$=4.1, p=0.11), whereas epibatidine (0.56 μg/kg) significantly decreased rates of responding. The rate-decreasing effects of epibatidine were not significantly antagonized by either mecamylamine or DHβE ($F_{4,37}$=2.5, p=0.058). When administered prior to saline, neither varenicline nor epibatidine significantly increased tail-withdrawal latencies to <2.5 s (Figures 1 and 2).

Varenicline and epibatidine did not significantly alter the potency with which fentanyl or nalbuphine decreased rates of operant responding (p>0.05; Figures 1 and 2). Similarly, neither varenicline (0.032 mg/kg) nor epibatidine (0.56 μg/kg) significantly enhanced the antinociceptive effects of the high efficacy MOR agonist fentanyl regardless of water temperature p>0.05; Figure 1). In contrast, the antinociceptive effects of the low efficacy MOR agonist nalbuphine were significantly augmented by both epibatidine and varenicline p>0.05; Figure 2; Table 1). Epibatidine markedly increased the antinociceptive potency of nalbuphine at 50°C and 52°C warmed water, and increased the tail-withdrawal latency from 55°C water for nalbuphine alone (from 1.8 [±0.30] sec to 6.5 [±2.2]) sec; Figure 2, bottom panels). The effects of the low efficacy α4β2 nAChR agonist varenicline on nalbuphine's antinociceptive effects were of a lesser magnitude than those of the high efficacy α4β2 nAChR agonist epibatidine, and varenicline approximately doubled the latency to tail-withdrawal from 55°C water compared to nalbuphine alone (to 3.5 (±2.2) sec; Figure 2, top panels).

The nonselective and α4β2-selective nAChR antagonists mecamylamine (0.1 mg/kg) and DHβE (0.1 mg/kg), respectively, were used to examine mechanisms by which epibatidine and varenicline might enhance nalbuphine's antinociceptive effects. Pretreatment with either nAChR antagonist attenuated the augmentation of the antinociceptive effects of nalbuphine by epibatidine (50°C: [mecamylamine: $F_{2,28}$=4.7, p=0.018], [DHβE: $F_{2,28}$=5.3, p=0.011]; 52°C: [mecamylamine: $F_{2,28}$=4.1, p=0.027], [DHβE: $F_{2,28}$=3.6, p=0.045]) (Figure 2, bottom panels). However, neither nAChR antagonist blocked varenicline's ability to enhance the antinociceptive effects of nalbuphine (50°C: [mecamylamine: $F_{2,19}$=1.5, p=0.26], [DHβE: $F_{2,19}$=0.69, p=0.51]; 52°C: [mecamylamine: $F_{2,19}$=0.41, p=0.67], [DHβE: $F_{2,19}$=0.58, p=0.57]) (Figure 2, top panels).

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As shown in Figure 3, epibatidine did not significantly alter the rate-decreasing effects of U69,593 ($F_{2,15}$=0.26, p=0.77) but did enhance its antinociceptive effects. Based on ED_{50} values, the potency of U69,593 to engender tail-withdrawal latency from water temperatures was increased up to 5.1-fold following epibatidine pretreatment (Table 1).

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Discussion

The present results show that the high-efficacy MOR agonist fentanyl dose-dependently increased tail-withdrawal latency at all temperatures (50, 52, and 55°C) and consistently decreased rates of operant responding, whereas the low-efficacy MOR agonist nalbuphine increased tail-withdrawal latencies at 50 and 52, but not 55°C, and had no significant rate-decreasing effects. The efficacy-related effects of fentanyl and nalbuphine in the present study are consistent with previously described effects of other MOR agonists on concomitantly-measured nociception and operant behavior (Withey et al. 2018; de Moura et al. 2019). The present results also extend previous findings with nicotine (de Moura et al. 2019), showing enhancement of the MOR partial agonist nalbuphine's antinociceptive effects by both the full α4β2 agonist epibatidine and the partial α4β2 agonist varenicline.

In contrast to previous findings with nicotine (de Moura et al., 2019), neither epibatidine nor varenicline enhanced the antinociceptive effects of the MOR high-efficacy agonist fentanyl at doses studied here. These findings strengthen the idea that the effectiveness of nAChR ligands in enhancing MOR antinociception may be inversely related to the efficacy of the MOR agonist. However, the relationship between the reported efficacy of nicotinic ligands and their adjuvant effects on opioid antinociception are less certain. On the one hand, the greater enhancement of opioid antinociception by epibatidine than varenicline in the present studies suggests that the magnitude of such adjuvant effects also may be related to the efficacy of the nAChR ligand, presumably at the α4β2 subtype of nAChR. On the other hand, nicotine (de Moura et al., 2019), but not epibatidine (present study), enhanced fentanyl's antinociceptive effects, even though both nAChR ligands are considered to be full α4β2 agonists.

The inability of epibatidine to enhance the antinociceptive effects of fentanyl in the present study was unexpected but may have been related to the dose of the epibatidine tested. In an earlier study (de Moura et al., 2019), the dose of nicotine that enhanced fentanyl's antinociceptive potency without disrupting operant

behavior was ¼ log unit higher than the dose that increased the antinociceptive potency of nalbuphine whereas, in the present study, the highest dose of epibatidine (0.0056 mg/kg) that did not substantively decrease operant behavior, was the same with both fentanyl and nalbuphine. Possibly, a higher dose of epibatidine would have enhanced the antinociceptive effects of fentanyl, albeit with a loss of behavioral selectivity- i.e., increases in antinociception coupled with decreases in the rates operant responding. Alternatively, the differing effects of nicotine and epibatidine may reflect functionally meaningful differences in their nAChR subtype selectivity. Nicotine is relatively non-selective in activating different subtypes of nAChRs whereas epibatidine is approximately 10-fold more selective than nicotine in binding to α4β2 sites than to other nAChR subtypes (e.g., α7; Grady et al., 2010). Perhaps, actions mediated via other subtypes of nAChR contributed to the adjuvant effects of nicotine in a behaviorally selective manner, i.e., enhancing opioid antinociception without disrupting operant behavior. However, this explanation, while attractive, does not easily reconcile the ability of the α4β2-selective antagonist DHβE, like the non-selective antagonist mecamylamine, to attenuate the adjuvant effects of epibatidine with nalbuphine. In the absence of data with other subtype-selective nAChR antagonists, the ability of nAChR ligands to activate α4β2 nAChRs seem to be the most likely mechanism mediating nAChR enhancement of opioid antinociception.

Like nicotine and epibatidine, the α4β2 nAChR partial agonist varenicline enhanced nalbuphine's antinociceptive effects without evidence of behavioral disruption. Surprisingly, the adjuvant effects of varenicline were not antagonized by doses of DHβE or mecamylamine that could attenuate the adjuvant effects of nicotine and epibatidine. This lack of antagonism may indicate that the doses of either DHβE or mecamylamine were not sufficient to attenuate the adjuvant effects of varenicline. However, this suggestion is difficult to reconcile with the idea that the nAChR mechanisms that mediate varenicline's opioid-adjuvant effects are similar to those that mediate the effects of nicotine or epibatidine. An alternative explanation is that non-α4β2-mediated mechanisms contribute more prominently to the opioid-adjuvant effects of varenicline than to those of nicotine or epibatidine. For example, in contrast to the α4β2 subtype selectivity of epibatidine the affinity of varenicline at the α7 subtype of nAChR is comparable to its affinity at the α4β2 subtype (Grady et

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al., 2010). Previous reports also have noted varenicline is insensitive to *in vivo* antagonism by DHβE (de Moura and McMahon, 2016, 2017, 2019; but see: Moerke et al., 2017). From this perspective, varenicline also may be less sensitive to mecamylamine's non-surmountable actions at nAChRs, thus requiring higher pretreatment doses for antagonism. Due to its high sequence homology to nAChRs (Werner et al., 1994), the 5HT3 receptor has also been investigated as a potential off-target site of varenicline. Indeed, varenicline has been shown to be a potent full agonist at 5HT3 receptors (Lummis et al., 2011). Thus, it is possible that the effects of varenicline on 5HT3 receptors could be producing the opioid antinociceptive enhancing effects, thereby explaining why mecamylamine and DHβE failed to antagonize varenicline. However, this is unlikely inasmuch as inhibition, but not activation, of 5HT3 receptors has been shown to produce analgesia or antinociception (Riering et al., 2004). At this time, it is unclear whether and how non-α4β2 nAChR actions of varenicline may have contributed to the present results.

KOR agonists have well-documented antinociceptive properties (Negus et al., 2010; Le Naour et al., 2014) but, due to unacceptable side effects, have limited promise for the management of pain (Pfeiffer et al., 1986; Wadeberg, 2003; MacLean et al., 2013; Ranganathan et al., 2012). Nevertheless, KOR agonists continue to be investigated as candidate medications for pain management because, unlike most MOR agonists, they do not display significant abuse potential (Raganathan et al., 2012) and have only limited respiratory depressant effects (Shook et al., 1990). The ability of epibatidine to enhance the antinociceptive potency of the KOR agonist U69,593 without concomitant behavioral disruption suggests that nAChR agonists could play a role in the further development of KOR-based analgesics. However, the present data with U69,593 must be viewed cautiously, as the magnitude of epibatidine's effect on KOR antinociception was comparatively low—less than its enhancement of the effects nalbuphine. It may be that the ability of nAChR ligands to modulate KOR antinociception is less pronounced than their ability to mediate MOR antinociception. Alternatively, considering that U69,593 is a high efficacy KOR agonist (Butelman et al., 2004), it also may be that epibatidine more effectively enhances the antinociceptive effects of low-efficacy than high-efficacy KOR agonists, as appears to be the case for low- verses high-efficacy MOR agonists. Additional studies comparing the ability of nicotinic

ligands to modulate antinociception produced by KOR agonists varying in efficacy are necessary to evaluate the viability of this novel strategy for pain management.

In summary, the present results support the hypothesis that nAChR agonists can, under suitable conditions, serve as adjuvants for opioid-based analgesia. Furthermore, our findings show that the magnitude of such effects may be inversely related to opioid agonist efficacy and, perhaps, directly related to nAChR agonist efficacy, suggesting that full agonists at nAChRs combined with opioid partial agonists, e.g., epibatidine with nalbuphine, may optimize adjuvant enhancement of opioid analgesia. Finally, the adjuvant effects of nAChR agonists appear to be mediated, in part at least, by $\alpha4\beta2$ nAChR activation, although the role of other nAChR mechanisms deserves closer scrutiny.

Acknowledgements:

The authors would like to thank Dr. Roger D. Spealman for comments on an earlier version of this manuscript and Margaret Branham for her technical assistance.

Authorship Contributions:

Participated in research design: Moura, Bergman

Conducted experiments: Moura

Performed data analysis: Moura

Wrote or contributed to the writing of the manuscript: Moura, Bergman

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Footnotes:

This work was supported by the National Institutes on Drug Abuse [Grant DA035857]; and Harvard Medical School Livingston Fellowship.

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Figure Legends

Figure 1: Rate-decreasing effects and tail-withdrawal latencies at 50, 52, and 55ºC for fentanyl alone (open circles) or in combination with varenicline (top panels, gray circles) or epibatidine (bottom panels, gray circles) (n=4). *Left Panel:* Ordinate- response rate normalized to baseline rate of responding. *Right Panels:* Ordinate- tail-withdrawal latency in seconds. Error bars depict ± S.E.M.

Figure 2: Rate-decreasing effects and tail-withdrawal latencies at 50, 52, and 55ºC for nalbuphine alone (open circles) or in combination with varenicline (top panels, gray circles), varenicline+ 0.1 mg/kg mecamylamine (top panels, filled circles), epibatidine (bottom panels, gray circles), or epibatidine + 0.1 mg/kg mecamylamine (bottom panels, filled circles) (n=4). *Left Panel:* Ordinate- response rate normalized to baseline rate of responding. *Right Panels:* Ordinate- tail-withdrawal latency in seconds. Error bars depict ± S.E.M.

Figure 3: Rate-decreasing effects and tail-withdrawal latencies at 50, 52, and 55ºC for nalbuphine alone (open circles) or in combination with varenicline (top panels, gray circles), varenicline + 0.1 mg/kg DHβE (top panels, filled squares), epibatidine (bottom panels, gray circles), or epibatidine + 0.1 mg/kg DHβE (bottom panels, filled squares) (n=4). *Left Panel:* Ordinate- response rate normalized to baseline rate of responding. *Right Panels:* Ordinate- tail-withdrawal latency in seconds. Error bars depict ± S.E.M.

Figure 4: Rate-decreasing effects and tail-withdrawal latencies at 50, 52, and 55ºC for U69,593 alone (open circles) or in combination with epibatidine (gray circles) (n=4). *Left Panel:* Ordinate- response rate normalized to baseline rate of responding. *Right Panels:* Ordinate- tail-withdrawal latency in seconds. Error bars depict ± S.E.M.

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Table 1- ED_{50} values and potency ratios of fentanyl, nalbuphine, and U69,593 alone and in combination with various doses of epibatidine or varenicline.

ED_{50} values in mg/kg with corresponding 95% confidence limits for fentanyl, nalbuphine, and U69,593 alone and in the presence of various doses of epibatidine or varenicline in 50, 52, and 55ºC warm water. Potency ratios are calculated when available with corresponding 95% confidence limits. Increases in antinociceptive potency are considered statistically significant if the 95% confidence limits of the potency ratios of drug alone or in the presence of nicotine does not include 1.

Drug	50ºC		52ºC		55ºC	
(in mg/kg)	ED_{50} (95% CL)	PR (95% CL)	ED_{50} (95% CL)	PR (95% CL)	ED_{50} (95% CL)	PR (95% CL)
Fentanyl	0.001 (0.0002-0.007)		0.002 (0.0008-0.007)		0.003 (0.001-0.006)	
+ 0.032 Var	0.001 (0.0002-0.005)	0.9 (0.2-4.3)	0.002 (0.0005-0.006)	1.4 (0.4-5.3)	0.004 (0.002-0.008)	0.7 (0.2-2.4)
+ 0.00056 Epi	0.0007	1.5	0.001	2.1	0.004	0.7

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	(0.0003-0.001)	(0.4-6.5)	(0.0004-0.003)	(0.7-6.5)	(0.001-0.009)	(0.2-2.6)
Nalbuphine	1.2		1.6		NA	
	(0.28-5.0)		(0.26-10)			
+ 0.032 Var	0.15	7.7	0.25	6.3	NA	NA
	(0.041-0.51)	(1.9-31)	(0.019-3.2)	(0.9-46)		
+ 0.00056 Epi	0.018	74	0.021	62	NA	NA
	(0.0011-0.28)	(49-113)	(0.0076-0.056)	(16-242)		
U69,593	0.033		0.037		0.068	
	(0.012-0.087)		(0.025-0.054)		(0.031-0.15)	
+ 0.00056 Epi	0.006	5.1	0.013	2.7	0.018	3.7
	(0.003-0.013)	(2-13)	(0.007-0.027)	(1.2-6)	(0.007-0.047)	(1.3-11)

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Figure 1

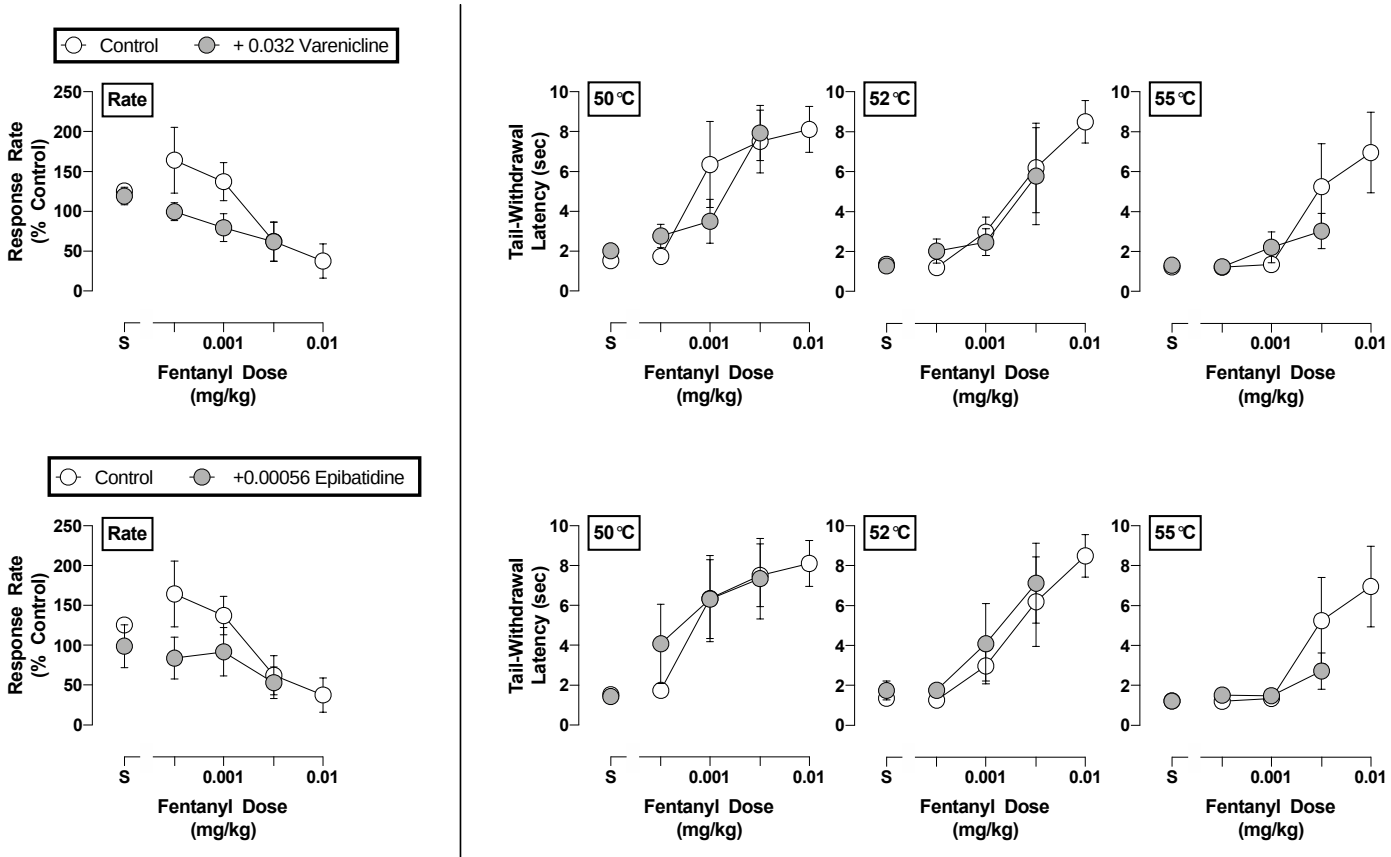

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Figure 2

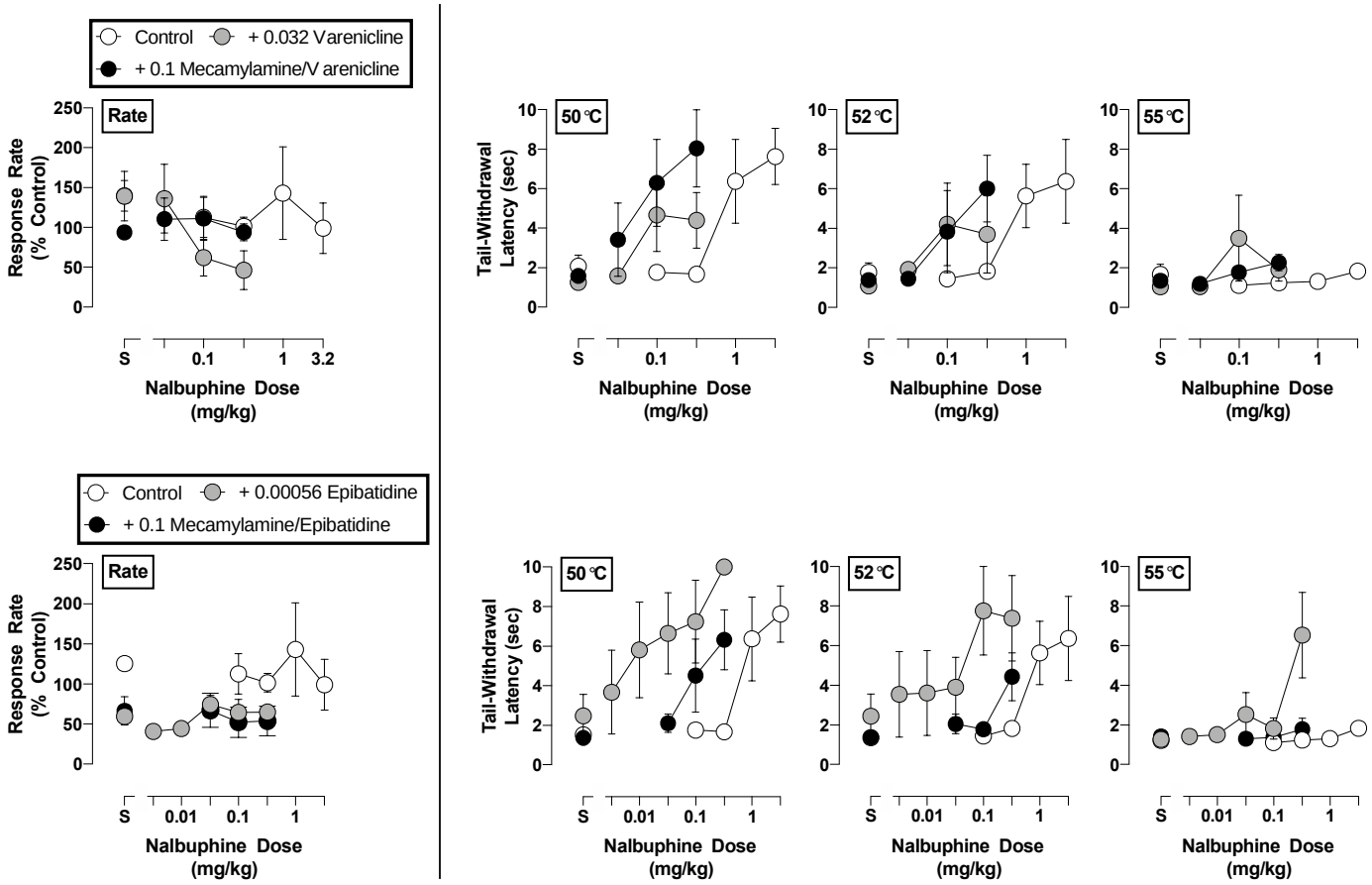

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Figure 3

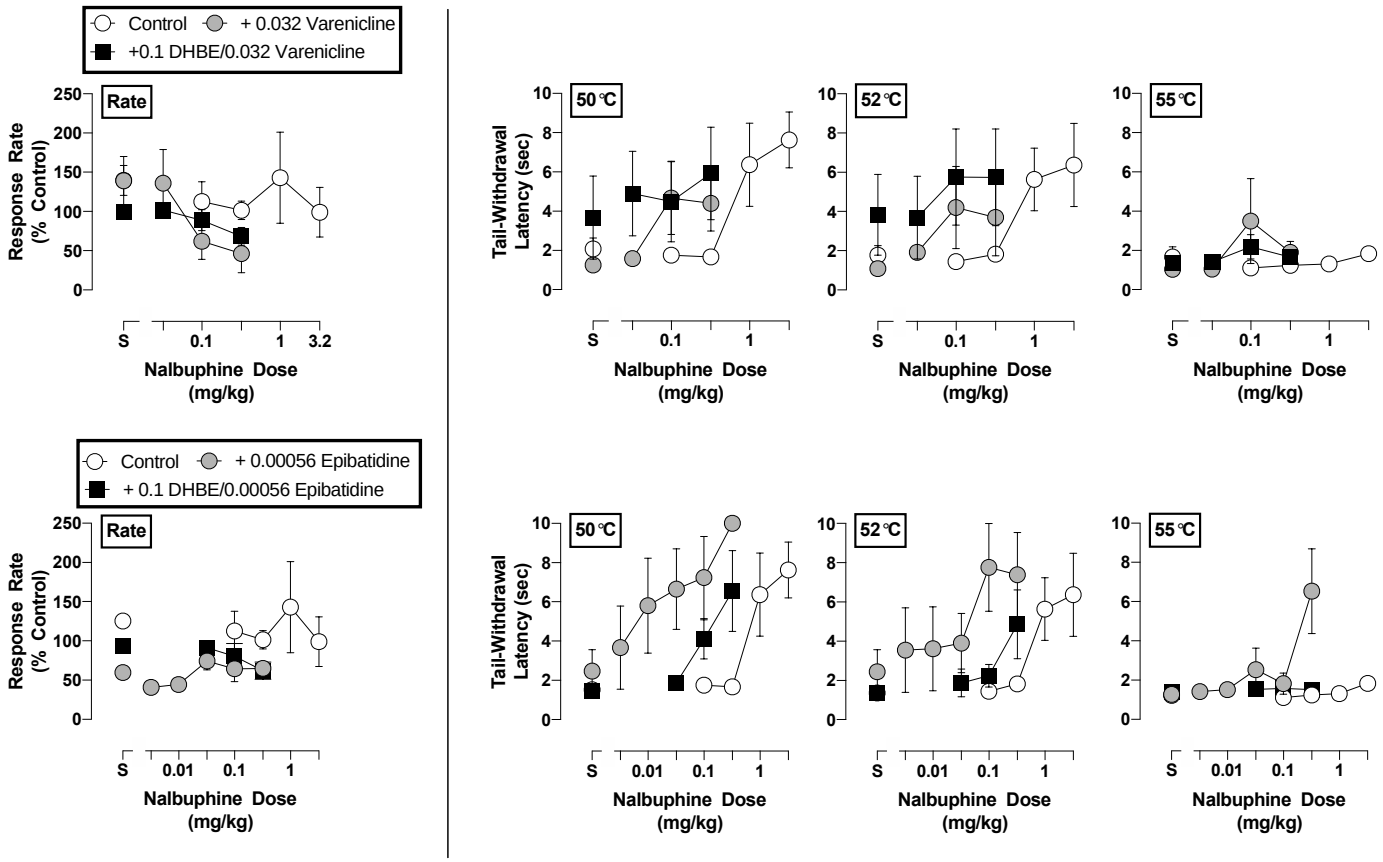

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Figure 4