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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 1879–1887

# Sensitivity of neuronal nicotinic acetylcholine receptors to the opiate antagonists naltrexone and naloxone: receptor blockade and up-regulation

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Received 17 November 2003; accepted 7 January 2004

**Abstract**—In HEK293 cells stably expressing α4β2 nAChRs, naltrexone, but not naloxone, blocked α4β2 nAChRs via an open-channel blocking mechanism. In primary hippocampal cultures, naltrexone inhibited α7 nAChRs up-regulated by nicotine, and in organotypic hippocampal cultures naltrexone caused a time-dependent up-regulation of functional α7 nAChRs that was detected after removal of the drug. These results indicate that naltrexone could be used as a smoking cessation aid.


## 1. Introduction

Drug addiction is an illness where drug-seeking behavior dominates the motivation of an individual. The pharmacological actions of different drugs in the central nervous system (CNS) remain the major determinants of addiction. Numerous studies have demonstrated that the addictive properties of many drugs are primarily the result of changes in the rewarding pathways and memory circuits in the brain.[1]

Prototypic addictive drugs are diverse chemicals, including opiates (e.g., morphine and heroin) and nicotine, which interact with distinct receptors in the brain. For instance, the pharmacological effects of opioids are derived from their complex interactions with three opioid receptor types, μ, δ and κ.[2] Likewise, the neurological effects of nicotine, the psychoactive substance in tobacco, arise from its interactions with various nicotinic receptor (nAChR) subtypes in the brain, particularly the α7 and the α4β2 nAChRs.[3] However, the rewarding effects of all of these drugs result from a common change in the CNS, that is, a significant increase in the mesolimbic dopaminergic activity.[4] The craving-seeking behavior induced by addictive drugs, on the other hand, appears to be due to multiple alterations in memory circuits in the prefrontal cortex, amygdala, hippocampus and dorsal striatum, all of which receive innervation from the mesolimbic dopaminergic system.[5]

The chemical and neuronal networks in the brain allow for considerable reciprocal interactions among the various neurotransmitter systems. Consequently, drugs acting on neuromodulatory systems, such as the cholinergic and the opioid systems, modify each other's effects. For instance, the opioid agonist heroin is known to increase the number of cigarettes smoked[6] and nicotine can block morphine-induced analgesia.[7] This potential interaction between the nicotinic and opioid

*Keywords:* Nicotine; Smoking cessation; Naltrexone; Naloxone; Nicotinic receptors; HEK cells; Organotypic cultures.
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doi:10.1016/j.bmcl.2004.01.004

systems and the success and substantial safety of opioid antagonists in the treatment of opioid addiction led to clinical trials designed to investigate the effects of the opioid antagonists naltrexone and naloxone on smoking behavior.

Unfortunately, results from these trials were not consistently positive. For example, Karras and Kane[8] showed a positive correlation between a single subcutaneous naloxone dose and smoking reduction. In contrast, Nemeth-Coslett and Griffiths[9] reported that intramuscular injections of naloxone had no effect in smoking. The different naloxone administration routes could have explained the opposite outcomes. Naltrexone has been more thoroughly investigated in cigarette smoking cessation programs. Three double-blind and placebo controlled studies[10–12] compared nicotine abstinence after naltrexone administration. Wewers and colleagues[10] showed that a number of indicators of nicotine addiction were significantly reduced by naltrexone. Others[11,12] reported that naltrexone did not influence cigarette consumption or withdrawal symptoms after tobacco abstinence. Two other studies investigated co-administration of naltrexone and nicotine replacement therapy.[13,14] Both groups concluded that naltrexone influenced the efficacy of nicotine replacement therapy. However, Hutchison and colleagues[13] reported that concomitant nicotine skin patch and oral administration of naltrexone were additive in reducing smoking urge and decreasing nicotine withdrawal, whereas Brauer et al.[14] found a negative association between naltrexone and skin nicotine patches. Differences in naltrexone dosages could have accounted for the discrepant results.

It is not clear that the usefulness of naltrexone or naloxone in the treatment of nicotine addiction results exclusively from physiological interactions between the opioid and nicotinic cholinergic systems in the brain. Earlier studies have demonstrated that naltrexone can interact directly with muscle nAChRs.[15,16] Recent studies from this laboratory also reported that naltrexone acts as a non-competitive antagonist of α7 nAChRs and causes up-regulation of these receptors in primary hippocampal cultures.[17] The present study was designed to examine the effects of naltrexone and naloxone on α4β2 nAChRs, the most prevalent neuronal nAChR in the brain, and to analyze the time-dependent effects of naltrexone on α7 nAChR activity in hippocampal neurons in primary and organotypic cultures.

## 2. Characterization of α4β2 nAChRs stably transfected into HEK 293 cells

HEK 293 cells stably expressing α4β2 nAChRs were cultured onto polylysine-coated coverslips and used 2–5 days after plating.[18] Agonist-evoked currents were recorded from either isolated cells or small cell aggregates under the whole-cell mode of the patch-clamp technique.[19] Agonist application to cell aggregates resulted in currents that had larger amplitudes than those recorded from isolated cells, thus facilitating measurements of current amplitudes particularly at low agonist concentrations. To reduce receptor desensitization, agonist pulses were applied to the cells every 2 min through a U-tube.[20] The external solution was composed of (in mM): NaCl 165, KCl 5, CaCl 1, Hepes 5, dextrose 10 (pH = 7.3, adjusted with NaOH; 340 mOsm). The muscarinic receptor antagonist atropine (1 μM) was added to the external solution. Electrodes were pulled from 1.5-mm external diameter brosilicate glass and had 3–5 MΩ of series resistance when filled with the following internal solution (in mM): CsCl 80, CsF 80, EGTA 10, CsOH 22.5, Hepes 10 mM (pH = 7.3 adjusted with CsOH, 340 mOsm). Under these conditions, ACh (3 nM–3 mM, 1-s pulse) induced inward currents whose amplitudes increased with increasing ACh concentrations in the ranges of 3 nM to 1 μM and 10 μM to 3 mM (Fig. 1A). The concentration–response relationship for ACh was best fit by a double sigmoid, indicating the existence of a high- and a low-affinity component (Fig. 1B). The $EC_{50}$s for ACh were 211.3 ± 2.6 nM and 54.0 ± 18.6 μM (mean ± S.E.M, $n$ = 3–5 cells). The Hill coefficient (nH) for the high- and low-affinity

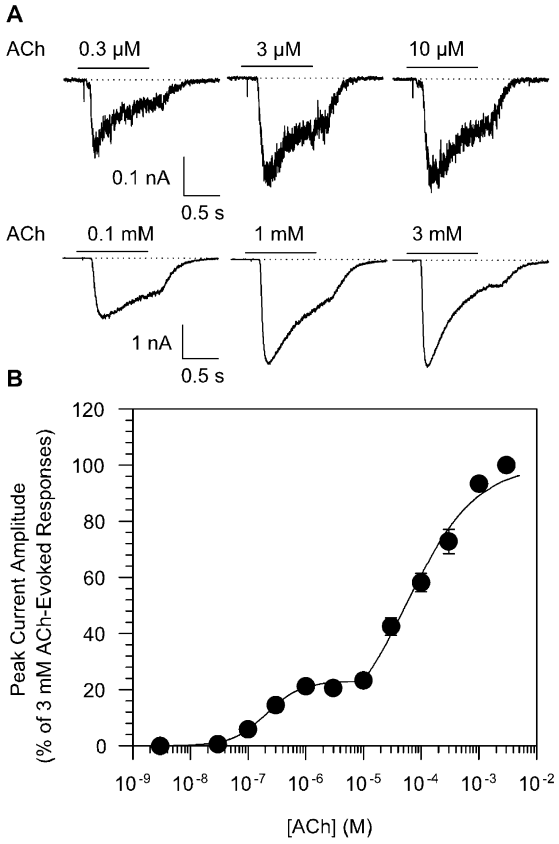


**Figure 1.** Concentration–response relationship for ACh-triggered whole-cell currents in HEK293 cells stably expressing α4β2 nAChRs. (A) Sample recordings of currents evoked by 1-s pulse application of ACh to HEK293 cells at −50 mV. Recordings in each row were taken from a different cell. The dotted line indicates the 0 pA level. (B) The run-down corrected[17] peak amplitudes of currents evoked by 3 mM ACh were taken as 100% and used to normalize the peak amplitudes of currents evoked by any given ACh concentration. Each cell was exposed to 3 mM ACh and two or three other ACh concentrations. Data points and error bars represent mean ± S.E.M. of results obtained from 4–5 cells. The data was best fit by a double sigmoid. When not shown, error bars are smaller than point sizes. Unless indicated otherwise, all chemicals were from Sigma.

components were 1.47±0.23 and 0.72±0.16, respectively (mean±S.E.M, $n=3$–5 cells). At passage 15, the high-affinity component subserved 23±0.84% of the peak of ACh-evoked currents. As the number of passages increased, the relative contribution of the high-affinity component to the ACh response increased. For $<10$ and $>20$ passages, the contributions of the high-affinity component to the total peak current amplitude were 10–20% and 30–40%, respectively (data not shown).

These findings join an ever-growing number of reports of the existence of two affinity components in systems ectopically expressing α4β2 nAChRs.[21–24] The apparent potency for ACh and the proportion of high/low affinity components along with cell passage number presented herein are in good agreement with those reported in previous studies carried in Xenopus oocytes[21] and in another HEK293 cell line.[23,24] The nature of the high- and low-affinity components is not known. They might represent two α4β2 nAChR isoforms that have distinct subunit stoichiometries or a single α4β2 nAChR isoform with distinct posttranslational modifications. Two lines of evidence favor the hypothesis of two α4β2 nAChR isoforms: (i) different ratios of high- and low-affinity components have been obtained after manipulations of α4:β2 proportions in Xenopus oocytes[25] and in another HEK293 cell line,[24] (ii) at least two single-channel conductance levels have been recorded from HEK293 cells and Xenopus oocytes expressing α4β2 nAChRs[24,26,27] and the single-channel amplitudes do not seem to be affected by manipulation of phosphorylation pathways.[28]

Previous studies defined dihydro-β-erythroidine (DHβE) as a competitive antagonist selective for α4β2 nAChRs. Thus, experiments were designed to determine the sensitivity to DHβE of the high- and low-affinity components of ACh responses recorded from the HEK293 cells. In these experiments, ACh was used as the agonist at 300 nM or 3 mM. Responses evoked by 3 mM ACh in the HEK293 cells stably expressing α4β2 nAChRs corresponded to 100% activation of the high- and low-affinity components. In contrast, activation of high- and low-affinity receptors contributed to approximately 83% and 16%, respectively, of the peak amplitudes of currents evoked by 300 nM ACh (calculated as the high-affinity component representing 23% of the 3 mM ACh current). Consequently, 300 nM was selected as an ideal ACh concentration to activate more selectively the high affinity receptors while allowing for recordings of currents with well-defined peaks. DHβE was applied both via the bath and in admixture with ACh. Under these conditions, increasing concentrations of DHβE significantly inhibited currents evoked by 300 nM ACh (Fig. 2A). The concentration–response relationship for DHβE-induced blockade of α4β2 nAChRs was best fitted by a single sigmoid curve; the $IC_{50}$ and nH for DHβE were 3.66±2.1 nM and 0.96±0.36 (mean±S.E.M., $n=4$ cells per concentration), respectively. The source of the large standard error is unknown; however, a plausible explanation is that the high- and low-affinity receptors also recognize DHβE with different affinities. Unfortunately, it was not possible to activate the low-affinity component in isolation (even at low passage number). In addition, the blockade of α4β2 nAChRs by DHβE was rapidly reversed with increasing agonist concentrations; during the agonist pulse the amplitude of currents evoked by 1 mM ACh in the presence of DHβE gradually increased indicating displacement of the antagonist from its binding site (Fig. 2C). Thus, it was not possible to quantify the concentration–response relationship for DHβE when high ACh concentrations were used to fully activate both high- and low-affinity α4β2 nAChRs. Of interest, an $IC_{50}$ of approximately 3 nM has been reported for DHβE-induced blockade of α4β2 nAChRs ectopically expressed

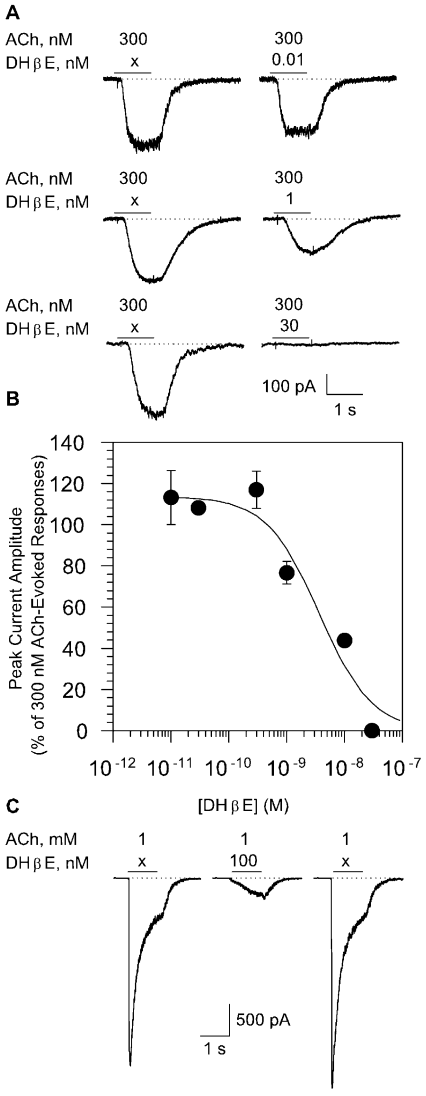


**Figure 2.** DHβE-induced block of α4β2 nAChRs in HEK293 cells. (A) Sample recordings of ACh (300 nM)-triggered currents recorded before and after 10-min exposure of the cells to DHβE. Each row was recorded from a different cell. The dotted line shows the 0 pA level. (B) Concentration–response relationship for DHβE-induced block of ACh (300 nM)-triggered currents. Data points and error bars represent mean and S.E.M., respectively, of results obtained from 4–5 cells. When not shown, error bars are smaller than symbol sizes. (C) Sample recordings of ACh (1 mM)-triggered currents recorded before and after 10-min exposure of the cells to DHβE. The effect of DHβE was completely reversed after 10 min of washing the cells with antagonist-free solution. In all experiments, membrane potential was −50 mV. DHβE was a gift from Merck, Sharp & Dohme.

in systems where only the high-affinity receptors have been detected.[29]

### 3. Naltrexone and naloxone effects on α4β2 nAChRs

A previous study carried out in cultured hippocampal neurons demonstrated that naltrexone blocks non-competitively α7 nAChRs with an $IC_{50}$ of 30 μM.[17] That same study indicated that higher concentrations of naltrexone were needed to block α4β2 nAChRs in hippocampal neurons.[17] Here, the mechanism of inhibition of α4β2 nAChRs by naltrexone was determined using the HEK293 cells. In these experiments, nicotinic currents were evoked by 100 μM ACh. The low- and the high-affinity receptors contributed to ca. 61.7% and 38.3%, respectively, of the peak amplitude of currents evoked by 100 μM ACh. Naltrexone, applied only as an admixture with the agonist, reduced the peak amplitude (Fig. 3A) and accelerated the decay phase (Table 1) of ACh-evoked currents; both effects were concentration dependent. At high naltrexone concentrations (>100 μM), a rebound current appeared after the end of the agonist pulse (see Fig. 3A). This rebound current is interpreted as fast relief of naltrexone-induced receptor block after the end of the agonist pulse with concomitant channel reactivation by the agonist still present around the cell.[30] Support for this hypothesis comes from scaling currents evoked by ACh and ACh-plus-naltrexone to the same maximal peak amplitude and observing that, after the end of the agonist pulse, the amplitudes of both currents are the same (Fig. 3A, insert).

The concentration–response relationship for naltrexone-induced blockade of α4β2 nAChRs was best fit by a single sigmoid ($IC_{50}$ = 139.0 ± 20.3 μM, mean ± S.E.M. of results obtained from 4-5 cells), indicating that naltrexone did not discriminate between the high and low affinity components. This $IC_{50}$ is substantially higher than that reported for naltrexone-induced blockade of α7 receptors in hippocampal primary cultures ($IC_{50}$ = 25 μM) or in acute hippocampal slices ($IC_{50}$ = 31 μM).[17] However, it is equivalent to naltrexone-induced blockade of α4β2 nAChRs in CA1 stratum radiatum interneurons ($IC_{50}$ = 141 μM).[17] To investigate the time dependence of naltrexone-induced blockade of α4β2 nAChRs, ACh-evoked currents were recorded before and 10-min after exposure of the HEK293 cells to the drug (Fig. 3B).

The magnitude of the blockade achieved with 10-min or 1-s exposure of the cells to naltrexone (3, 30 or 100 μM) was similar (Fig. 3B). This result indicates that the equilibrium between naltrexone and α4β2 nAChRs is ≤1 s. In addition, this finding is consistent with an open-channel blocking action of naltrexone, which has been observed in other nAChRs.[17] In this case, opening of the receptor channels by the agonist facilitates the access of the antagonist to its site within the channel.

Naloxone (Fig. 4B, insert) and naltrexone (Fig. 3B, insert) have similar chemical identity with the only difference being cyclization of the propenyl chain in

**Table 1.** Decay-time constant of ACh (100 μM)-evoked currents in the absence or presence of naltrexone

| Drugs, μM | Decay-time constant (ms) |
|---|---|
| ACh, 100 | 547.88 ± 15.78 |
| + Naltrexone, 0.1 | 509.96 ± 81.05 |
| + Naltrexone, 0.3 | 495.98 ± 69.69 |
| + Naltrexone, 1 | 543.11 ± 53.50 |
| + Naltrexone, 3 | 508.74 ± 49.27 |
| + Naltrexone, 10 | 467.84 ± 33.59 |
| + Naltrexone, 30 | 422.02 ± 38.88* |
| + Naltrexone, 100 | 257.74 ± 15.13** |
| + Naltrexone, 300 | 202.53 ± 10.65** |

Data was obtained by fitting the decay phase of ACh-evoked currents with a single-exponential function and are presented as mean ± S.E.M. of results obtained from 4–5 cells. According to the ANOVA followed by Dunnett post-test, results are significantly different from control (ACh, 100 μM), *$p < 0.05$ and **$p < 0.01$.

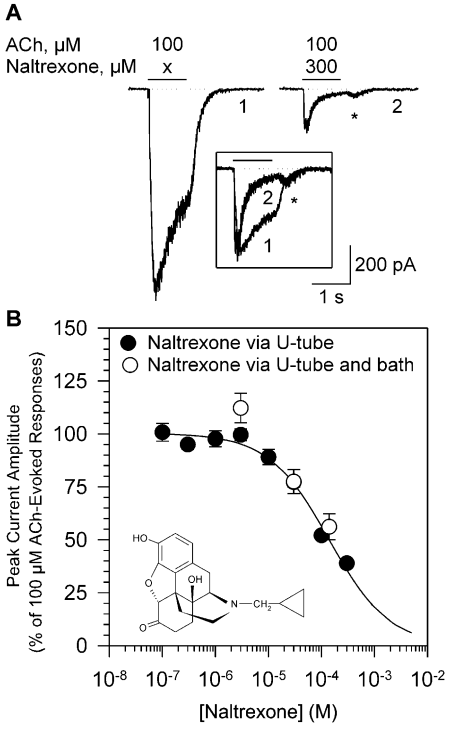


**Figure 3.** Naltrexone blocks α4β2 nAChRs. (A) Sample recordings of ACh (100 μM)-evoked currents in the absence (1) or in the presence of naltrexone (300 μM) (2). With naltrexone concentrations >100 μM, a delayed current peak (*) was observed after the end of the pulse of agonist-plus-antagonist. Inset: Scaling of the traces of currents evoked by ACh (1) and ACh-plus-naltrexone (2) to the same peak amplitude. The dotted line shows the 0 pA level. Membrane potential = −50 mV. (B) Concentration–response relationship for naltrexone-induced blockade of α4β2 nAChRs in HEK293 cells. Any given concentration of the opioid antagonist was applied either exclusively in admixture with ACh (100 μM,●) or in admixture with ACh after a 10-min perfusion of the cells with solution containing that concentration of naltrexone (○). The amplitudes of currents evoked by ACh alone were taken as 100% and used to normalize the amplitudes of currents evoked by ACh-plus-naltrexone. Solid line shows the sigmoid fit of the results. Data points and error bars represent mean and S.E.M., respectively, of results obtained from 4–5 cells. Insert: chemical structure of naltrexone. Naltrexone was purchased from Endo Laboratories.

naltrexone. However, when applied to the HEK293 cells in admixture with ACh (100 μM), naloxone (140 μM) did not alter the amplitude of ACh-evoked currents recorded at various membrane potentials (Fig. 4). Therefore, the propenyl chain hinders the interaction of naloxone with the α4β2 nAChRs. Taken together, these results indicate that (i) α7 nAChRs are approximately 4.6 times more sensitive to naltrexone than α4β2 nAChRs and (ii) α4β2 nAChRs are substantially insensitive to naloxone.

## 4. Naltrexone blocks nicotine induced up-regulation of α7 nAChRs: Consequences for smoking cessation therapy

Up-regulation of nAChRs is considered to be one of the mechanisms involved in initiation and maintenance of nicotine addiction, and craving for smoking during quitting attempts.[31] A previous study from this laboratory demonstrated that a 1-h exposure of primary hippocampal cultures to clinically relevant concentrations of nicotine (10 or 30 nM) causes significant up-regulation of functional α7 nAChRs.[17] Thus, experiments were designed to investigate whether naltrexone could modify expression and/or antagonize nicotine-induced up-regulation of α7 nAChRs. In these experiments, choline was used as a selective full α7 nAChR agonist.

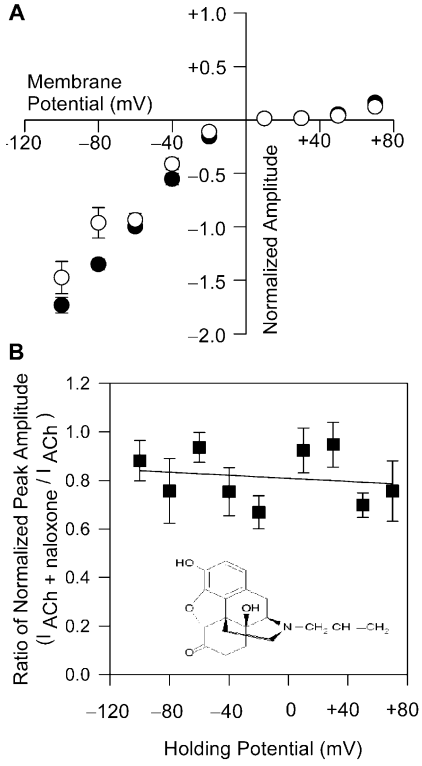


**Figure 4.** Naloxone does not block α4β2 nAChRs. (A) Current-voltage relationship for ACh (100 μM)-evoked currents in the absence (•) and in the presence (○) of naloxone (140 μM). The amplitudes of currents evoked by ACh (100 μM) at −60 mV were taken as 1 and used to normalize the amplitudes of currents evoked by ACh or by ACh-plus-naloxone at all membrane potentials. Each point and error bar represent mean and S.E.M. of results obtained from 4–5 cells. (B) Ratio of the normalized amplitudes of currents evoked by ACh in the presence (IACh + naloxone) or absence (IACh) of naloxone. Data points and error bars represent mean and S.E.M., respectively, of results obtained from 4–5 cells. Solid line is the regression line of the points. Insert: Chemical structure for naloxone. Naloxone was purchased from Endo Laboratories.

In the first experimental protocol, choline (10 mM)-evoked currents were recorded from 10 neurons at −60 mV. Then, following a 1-h perfusion of the culture dishes with external solution containing naltrexone (30 μM), responses evoked by choline in the continuous presence of naltrexone were recorded from another 10 neurons. The average time to obtain 10 patches was 106±11 min (range 49–163 min). The peak amplitudes of currents obtained after exposure of the neurons to naltrexone were normalized to the arithmetic mean of the peak amplitudes of choline-evoked currents recorded prior to exposure to naltrexone. This procedure guaranteed that each culture dish was used as its own control and allowed for comparisons among different culture dishes. Exposure of the neurons for 1 h to naltrexone (30 μM) reduced the peak amplitudes of choline-evoked currents to 55.4±9.1% of control values (Fig. 5A). The magnitude of α7 nAChR inhibition caused by 1-h exposure of hippocampal neurons to 30 μM naltrexone is similar to that reported to occur after a 10-min exposure of the neurons to the same concentration of the drug.[17] Thus, contrary to what has been reported for nicotine,[17] 1-h perfusion of hippocampal neurons with solution containing naltrexone does not result in functional up-regulation of α7 nAChRs.

In the second set of experiments, dishes of primary hippocampal cultures were perfused for 1 h with naltrexone-containing external solution and the peak amplitude of currents evoked by choline (10 mM, 1 s pulses) were recorded from 10 neurons. Subsequently, the dishes were perfused for an additional hour with external solution containing naltrexone (30 μM)-plus-nicotine (1 nM–10 μM). The average peak amplitudes of choline-evoked currents recorded in the presence of naltrexone (30 μM) from a dish were used to normalize the average peak amplitudes of currents evoked by choline in the presence of naltrexone-plus-nicotine in that dish. This protocol allowed for comparisons of results obtained from different cultures. In these experiments, α7 nAChR desensitization caused by 10 μM nicotine was not affected by naltrexone (Fig. 5B). However, up-regulation of functional α7 nAChRs seen when the hippocampal neurons were exposed to 10 or 30 nM nicotine alone was not detected when the neurons were exposed to the admixture of nicotine-plus-naltrexone (Fig. 5B). It was previously reported[17] that significant up-regulation of functional α7 nAChRs can be detected in primary hippocampal cultures exposed for 3 days to nicotine (10 μM) plus naltrexone (30 μM) after 30-min washing with drug-free solution. This up-regulation was not significantly different from that seen in cultures treated for three days with nicotine alone.[17] Thus, the present findings are more likely the result of the naltrexone-induced blockade of the α7 nAChRs functionally up-regulated by nicotine than inhibition of the mechanism underlying nicotine-induced functional up-regulation of α7 nAChRs.

All clinical trials investigating the usefulness of naltrexone in smoking cessation administered the drug at or close to its proposed dosage for treatment of opiod addiction.[10–14,32–34] After 1 h of oral administration of 100 mg naltrexone, the mean nonconjugated naltrexone plasma concentration was 43.6±29.9 μg/l (127.7±87.6 nM); this concentration remained approximately constant for 3–4 h.[35] Because naltrexone is rapidly metabolized (half-life of 8 h), its plasma concentration declined to 2.1±0.5 μg/l (6.2±1.46 nM) within 24 h after a single dose of 100 mg.[35] It should be noted that the brain concentrations of naltrexone are 3.2 times higher than its plasma concentrations.[36] If it is assumed that the actions of naltrexone on α7 and α4β2 nAChRs are important for its beneficial effects on smoking cessation, different plasma concentrations of naltrexone might explain why clinical trials using an in-patient arrangement[10,13,33] reported more positive naltrexone effects on smoking cessation than trials using out-patient configurations.[11,12,14,32,34] The in-patient studies measured the effects of naltrexone on smoking cessation soon after the administration of the drug and more likely sampled the results from a population with higher blood naltrexone levels. On the other hand, out patient studies measured the effects of naltrexone over a longer period of time (weeks) and were more likely to obtain results from a population with much lower blood naltrexone levels. Higher naltrexone levels are expected to translate in blockade of nAChRs up-regulated by nicotine and in returning nicotinic cholinergic activity closer to normal levels. The results reported here predict that higher concentrations of naltrexone and/or prolonged periods of administration would be more efficacious in smoking cessation programs. Indeed, clinical trials with the usual naltrexone clinical dose for opioid detoxification (50 mg once a day), but with continuous use for 4 weeks reported a tendency toward an increase in maintenance of tobacco-free status.[32] However, 6 months after interruption of naltrexone, the effect had subsidized, indicating that prolonged naltrexone administration might be necessary to help smokers maintain a nicotine-free status. Higher doses and prolonged naltrexone administration should not be accompanied by increased toxicity or side effects. Naltrexone has been administered to human populations in doses up to 4 times (200 mg) the usual daily dose without any reported significant side effects[37,38] and high safety margins are expected from primate and non-primate animal toxicity studies.[39] Moreover, once naltrexone treatment was interrupted, no abrupt withdrawal syndrome was reported.[37] Finally, blockade of nAChRs by naltrexone should not be followed by compensatory increases in cigarette smoking, as has been reported by administration of others nicotinic antagonists.[40] This expectation derives from the fact that no clinical trial reported that naltrexone increased cigarette consumption.

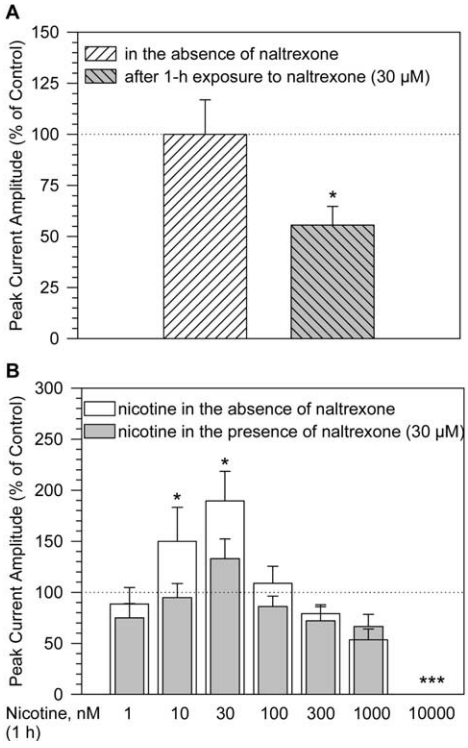


**Figure 5.** Effects of prolonged exposure to naltrexone (30 μM) on α7 nAChR activity and on nicotine-induced up-regulation of α7 nAChRs. (A) Choline (10 mM)-evoked currents were recorded from 10 hippocampal neurons in primary cultures under control conditions and from another 10 neurons after 1-h exposure of the cultures to naltrexone. Amplitudes of currents recorded under control conditions in each dish were taken as 100% and used to normalize the averaged amplitudes of currents recorded from neurons after exposure of that dish to naltrexone. Graph and error bars represent mean and S.E.M. of results obtained from 3 dishes (*, $p<0.05$ according to the paired Student's *t*-test; total of 60 neurons, 30 neurons each for control and naltrexone). (B) In other sets of dishes, choline-evoked currents were recorded from 10 neurons after 1-h exposure to naltrexone (30 μM) or naltrexone (30 μM)-plus-nicotine (1 nM–10 μM) (gray bars). Each dish was exposed to a single concentration of nicotine ($n=353$ neurons). The amplitudes of responses recorded after treatment with naltrexone were taken as 100% and used to normalize the amplitudes of responses recorded after treatment with naltrexone-plus-nicotine. For comparison, we overlaid the results obtained from experiments in which responses were recorded after 1-h perfusion of the neurons to nicotine (reported before by Almeida et al.[17]; white bars). *** $p<0.001$, *, $p<0.05$ according to ANOVA followed by Dunnett post-test. All experiments were performed at −50 mV.

## 5. Naltrexone up-regulates functional nAChRs in hippocampal organotypic cultures

Normal preparation procedures for primary neuronal cultures involve destruction of the physiological synaptic patterns. In addition, the primary cultures used in the present study were obtained from embryonic animals. Thus, organotypic hippocampal cultures were used to investigate the effects of naltrexone in a more physiological preparation. Organotypic hippocampal cultures were obtained using a modification of the procedure of Stoppini et al.[41] Briefly, 8–11-day-old Sprague–Dawley rats, kept in accordance with animal procedures established by the University of Maryland Baltimore in a 12 h light/dark cycle (lights on at 7 AM), were sacrificed under $CO_2$ narcosis and the hippocampi were rapidly removed. Slices that were 300-μm thick were obtained and placed in millicell® membranes (Millipore, USA) pre-coated with a solution of rat tail collagen type I (0.4 mL, 1 mg/mL). Millicell® membranes with hippocampal slices were incubated during

the first 24 h in minimum essential medium enriched with Hepes 10 mM, glucose 28 mM, sodium bicarbonate 7.5%, horse serum 10%, fetal bovine serum 10% and glutamine 1%. After the first 24 h and twice a week thereafter, this medium was substituted for another one of the same composition but without fetal bovine serum. Organotypic cultures were used at 6–8 or 20–22 days after plating. These ages were selected because previous experiments indicated an ontogenic development of α7 nAChRs in CA1 pyramidal neurons with consistent responses at 7 days in vitro and maximal responses at ≥21 days in vitro.[42] Electrophysiological recordings were obtained by the 'blind' technique[43] from CA1 pyramidal neurons and 6-s pulses of choline (10 mM), ACh (3 mM) or γ-aminobutyric acid (GABA, 50 μM) were applied to the neurons via a U-tube. Each patch-clamped neuron was exposed to two pulses of each agonist (3-min interval between pulses) and at least 6 cells were studied in any given slice. To account for the normal variability of amplitudes of agonist-evoked currents in different slices, recordings were obtained from untreated and naltrexone-treated slices in the same culture batch and on the same experimental day. Moreover, the charge (rather than the peak amplitude) of agonist-evoked currents was measured during the 6-s agonist pulse and results from naltrexone-treated slices were normalized to the average current charge recorded from neurons in paired untreated slices. The artificial cerebral spinal fluid (ACSF) bathing the organotypic cultures had the following composition (in mM): NaCl 135, KCl 3, $CaCl_2$ 2, dextrose 16.5, $MgCl_2$ 1, $NaHCO_3$ 26, $NaH_2PO_4$ 1.25 and was continuously bubbled with a mix of 5% $CO_2$ and 95% $O_2$. Tetrodotoxin (300 nM) and atropine (1 μM) were added to the external solution. The internal solution used to fill pipettes contained (in mM): cesium-methanesulfonate 130, CsCl 10, CsOH 20, EGTA 10, Hepes 10, $MgCl_2$ 2 (pH = 7.2, adjusted with CsOH; 330 mOsm). To confirm the pyramidal nature of the sampled cells, biocytin (0.5%) was included in the internal solution for post-hoc image reconstruction.[44]

Photomicrographies of biocytin-filled neurons that were studied electrophysiologically in organotypic hippocampal cultures revealed that these neurons had the morphological features of pyramidal neurons (Fig. 6A). Neuronal cell bodies with a pyramidal shape could be easily distinguished and apical and basal dendrites (up to tertiary ramifications) could be traced (Fig. 6A). Application (6-s pulses) of ACh (3 mM), choline (10 mM) or GABA (50 μM) to the neurons evoked inward currents. Methyllycaconitine (10 nM) and α-bungarotoxin (α-BGT, 100 nM) blocked by 100% and 75%, respectively, fast-decaying currents evoked by ACh or choline, suggesting that the responses were subserved primarily by α7 nAChRs.[45] Surprisingly, 24-h incubation of the organotypic hippocampal cultures with 0.3 μM, but not 30 μM naltrexone induced up-regulation of functional α7 nAChRs that was detected after 30-min washing of the preparations with naltrexone-free solution (Figs 6B and 6C). In 6–8-day-old organotypic hippocampal cultures, naltrexone (0.3 μM) increased the charge carried by choline- and ACh-induced currents by 283% ± 54% and 214% ± 47%, respectively. This effect was specific for the nAChRs, because GABAergic currents were not affected by the treatment with naltrexone (75% ± 9.4% after naltrexone 0.3 μM).

It has been reported that expression of nAChRs in CA1 pyramidal neurons in organotypic hippocampal cultures increases along with time after plating, reaching a peak at 21 days in vitro.[42] Here, choline- and ACh-evoked currents recorded from CA1 pyramidal neurons in 20–22-day-old organotypic cultures were also larger than those recorded from CA1 pyramidal neurons in 6–8-day-old cultures (data not show). However, the ability of naltrexone to increase nAChR activity was not

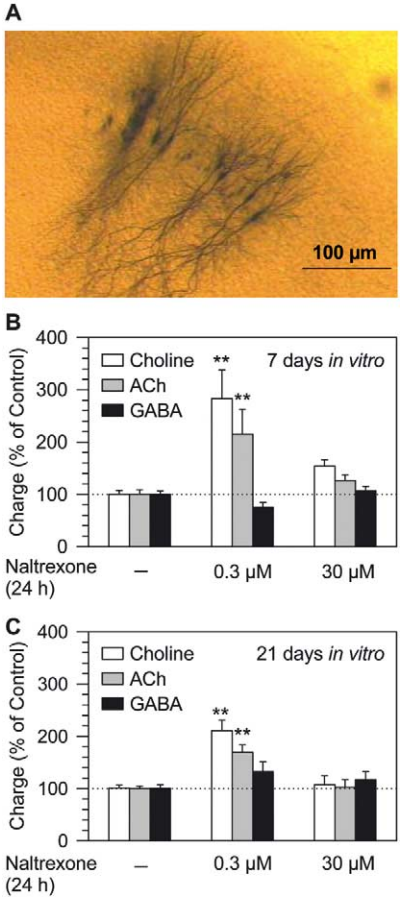


**Figure 6.** Naltrexone-induced up-regulation of functional α7 nAChRs in CA1 pyramidal neurons of organotypic hippocampal cultures. (A) Photomicrographies of biocytin-filled neurons in the CA1 field of a hippocampal slice in a 21-day-old organotypic culture. (B, C) The arithmetic mean of the charge of currents evoked by 6-s pulses of ACh (3 mM), choline (10 mM) or GABA (50 μM) in CA1 pyramidal neurons of untreated cultures was taken as 100% and used to normalize the charge of currents evoked by each agonist in CA1 pyramidal neurons of batch-paired cultures that had been exposed for 24 h to naltrexone (0.3 or 30 μM). At least 6 neurons were studied in each slice and all three agonists were tested on the same cell. Graph and error bars shown in **B** represent mean and S.E.M., respectively, of results obtained from 3–4 organotypic cultures after 6–8 days in vitro (grouped as 7 days in vitro). A total of 37 naltrexone-exposed neurons were studied. In **C**, graph and error bars represent mean and S.E.M., respectively, of results obtained from 3–4 organotypic cultures after 20–22 days of plating (grouped as 21 days in vitro). A total of 51 naltrexone-exposed neurons were studied. ** indicates that results are significantly different from control with $p < 0.01$ (according to the ANOVA with Dunnett post-test). All recordings were obtained at −70 mV.

significantly affected by the age of the cultures (Fig. 6C). Naltrexone (0.3 μM)-induced increase in charge for choline- and ACh-evoked currents in CA1 pyramidal neurons of 20–22-day-old cultures was 210±20% and 169±14%, respectively. The finding that 24-h treatment of organotypic cultures with 0.3 μM (but not 30 μM) naltrexone causes functional up-regulation of α7 nAChRs in pyramidal neurons contrasts the finding that three days treatment of primary hippocampal cultures with 30 μM (but not 0.3 μM) naltrexone up-regulates α7 nAChRs.[17] A few lines of evidence suggest that the different times of treatment with naltrexone (24 h versus 3 days) rather than the different preparations (primary versus organotypic hippocampal cultures) account for these discrepant results. First, the inhibitory effect of short-term exposure (<10 min) to naltrexone on α7 nAChRs is the same regardless whether the receptors are in neurons of primary hippocampal cultures or acute hippocampal slices; no differences in the apparent potency of the drug or in the kinetics of blockade were found.[17] Second, the same applies to the effects of short-term exposure to naltrexone on α4β2 nAChRs present in neurons of primary hippocampal cultures[17] and acute hippocampal slices[17] or ectopically expressed in HEK293 cells (this study). Consequently, it is conceivable that functional α7 nAChR up-regulation induced by 0.3 μM naltrexone is transient, subsiding within three days exposure to the drug, and that the onset of α7 nAChR up-regulation by 30 μM naltrexone requires longer than 24-h exposure to the drug. At this time, however, the possibility that the different preparations account for the discrepant results cannot be completely ruled out.

The functional up-regulation of α7 nAChRs observed after the 24-h treatment of organotypic hippocampal cultures with 0.3 μM naltrexone is likely the result of the interactions of the drug with endogenous opioid receptors altering the cyclic AMP (cAMP) second messenger pathway. Indeed, activity of the cAMP-dependent protein kinase has been shown to influence desensitization, reactivation and expression of several nAChR subtypes[27,46–49] Considering that as a result of the short half-life of naltrexone, concentrations of naltrexone do not plateau for a long period, up-regulation of functional α7 nAChRs induced by the drug may not be clinically relevant.

In summary, the findings that (i) naltrexone, in addition to inhibiting α7 nAChRs,[17] blocks α4β2 nAChRs via an open-channel blocking mechanism and (ii) the continuous presence of naltrexone blocks the α7 nAChRs functionally up-regulated by nicotine indicate that at appropriate dosages, certainly higher than those used to treat opioid addicts, naltrexone is a potential candidate for treatment of nicotine addiction.

## Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Mrs. Barbara Marrow and Mrs. Mabel A. Zelle. This work was supported by grants from USPHS NS41671, University of Maryland School of Medicine Other Tobacco Related Diseases Research Grant and Janssen Pharmaceutical Research Foundation (to EXA).

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