

# Vivitrex<sup>®</sup>, an Injectable, Extended-Release Formulation of Naltrexone, Provides Pharmacokinetic and Pharmacodynamic Evidence of Efficacy for 1 Month in Rats

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While oral naltrexone is effective in treating alcohol and opiate dependencies, poor patient adherence and widely fluctuating plasma levels limit its efficacy. To overcome these problems, an extended-release formulation of naltrexone (Vivitrex<sup>®</sup>) was developed by encapsulating naltrexone into injectable, biodegradable polymer microspheres. Pharmacokinetic studies in rats demonstrated that this formulation produced stable, pharmacologically relevant plasma levels of naltrexone for approximately 1 month following either subcutaneous or intramuscular injections. While rats receiving placebo microspheres demonstrated a pronounced analgesic response to morphine in the hot-plate test, morphine analgesia was completely blocked in rats treated with extended-release naltrexone. This antagonism began on day 1 following administration and lasted for 28 days. Rats reinjected with extended-release naltrexone 34 days after the initial dose and tested for another 35 days showed consistent suppression of morphine analgesia for an additional 28 days.  $\mu$ -Opioid receptor density, as measured by [<sup>3</sup>H]DAMGO autoradiography, increased up to two-fold following a single injection of extended-release naltrexone. Saturation binding assays using [<sup>3</sup>H]DAMGO showed changes in the midbrain and striatum at 1 week after extended-release naltrexone administration, and after 1 month in the neocortex. These receptor increases persisted for 2–4 weeks after dissipation of the morphine antagonist actions of naltrexone. These data suggest that therapeutically relevant plasma levels of naltrexone can be maintained using monthly injections of an extended-release microsphere formulation, and that changes in  $\mu$ -opioid receptor density do not impact its efficacy in suppressing morphine-induced analgesia in the rat. Clinical trials of extended release naltrexone for treating alcohol and opiate dependency are currently ongoing.

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## INTRODUCTION

Alcohol and opiate dependence represent serious medical problems in the United States and throughout the world. In the US alone, it is estimated that 10–14 million people are alcohol-dependent, while 1 million people suffer from opiate addiction (Grant, 1997). Evidence implicating the functional involvement of the endogenous opioid system in mediating many of the reinforcing aspects of drug consumption (Spanagel *et al*, 1992; Herz, 1997) has led to the use of opioid receptor antagonists as a pharmacotherapeutic intervention for treating drug dependency syndromes (Martin *et al*, 1973; Litten and Allen, 1998; Garbutt *et al*, 1999). The opiate antagonist naltrexone (Resnick *et al*,

1974) is effective and approved by the FDA for treating opiate and alcohol dependence (Food and Drug Administration NDA 18-932/S-010, 1994). Despite its utility, the efficacy of oral naltrexone in treating drug dependencies is limited by at least two deficiencies. The first involves poor adherence to the prescribed daily dosing schedule (Volpicelli *et al*, 1997). Although adherence is important in achieving efficacy with all drugs, it is particularly important in treating alcohol and opiate dependence. In these syndromes, the compulsion to self-administer drugs directly conflicts with the need to remain abstinent. This daily conflict further exacerbates problems of poor adherence, contributing to the 'spiral towards relapse' common to both maladies. Indeed, a recent study of alcoholics (Volpicelli *et al*, 1997) found that patients who were highly adherent benefited significantly from naltrexone, while nonadherent patients responded no differently than those receiving placebo. Moreover, it is not uncommon for less than 30% of the opioid-dependent patients to continue with antagonist therapy for more than 6 months (Greenstein *et al*, 1984; Capone *et al*, 1986; D'Ippoliti *et al*, 1998). These data

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argue that the efficacy of naltrexone might be significantly enhanced if adherence could be improved. A second deficiency with oral naltrexone involves the widely fluctuating plasma levels that occur with daily oral dosing (Verebey, 1980). These daily fluctuations may cause side effects during peak plasma levels (Croop *et al*, 1997; King *et al*, 1997), while compromising efficacy during the nadir in plasma concentration (Verebey, 1980).

One approach for improving upon the effectiveness of naltrexone would be to develop an injectable formulation that would maintain plasma levels within the therapeutic range for several weeks, but below levels that induce serious side effects. Polymers such as poly-lactide co-glycolide (PLG, (Shive and Anderson, 1997)) can be formulated into small-diameter (<100  $\mu\text{m}$ ), injectable microspheres that incorporate active moieties to provide extended release for several weeks (Lewis, 1990). Such an extended-release formulation of naltrexone might provide several advantages over oral naltrexone, including a significant reduction in daily high plasma peaks and a decrease in the gastrointestinal exposure and first-pass hepatic metabolism associated with oral delivery (Kranzler *et al*, 1998). These improvements could reduce the incidence of adverse effects associated with oral naltrexone administration. Similarly, the ability to maintain therapeutically relevant plasma levels of naltrexone continuously should provide for a more uniform occupation of opioid receptors, and therefore, a more consistent pharmacodynamic response throughout the course of treatment. Finally, adherence would be assured for an entire month after each injection, providing yet another important advantage.

Owing to these potential advantages, we formulated naltrexone into PLG-microspheres using Alkermes' proprietary Medisorb<sup>®</sup> technology to provide a product intended to deliver therapeutic levels of naltrexone for a full month following each injection. The following experiments describe preclinical pharmacokinetic and pharmacodynamic results from such an extended-release microsphere formulation of naltrexone (ie Vivitrex<sup>®</sup>), demonstrating that it provides plasma concentrations of naltrexone that are consistently elevated above therapeutic levels for approximately a 1-month period following each injection. Moreover, the plasma levels of naltrexone show a close, temporal correlation with a sustained pharmacodynamic response (morphine-induced analgesia as measured in the hot-plate test) that reflects an effective opioid receptor blockade.

## MATERIALS AND METHODS

### Subjects

Male Sprague-Dawley rats (450  $\pm$  50 g; Taconic Farms, Germantown, NY) were used in all studies. Rats were pair-housed in polypropylene cages with free access to food and water. The vivarium was maintained on a 12 h light:dark cycle with a room temperature of 22  $\pm$  1°C and relative humidity level of 50  $\pm$  5%. All studies were approved by Alkermes Institutional Animal Care and Use Committee and were conducted in adherence with the NIH 'Guide for the Care and Use of Laboratory Animals, 1996'.

## Drug Treatments and Experimental Design

Naltrexone-containing microspheres (Vivitrex<sup>®</sup>) were fabricated from the PLG polymer using a proprietary process (Lewis, 1990) to provide loading densities of approximately 35% (w/w) naltrexone base. Placebo (nonloaded) microspheres were prepared in an identical manner, except that naltrexone was omitted.

Microspheres were suspended in 1 ml of an aqueous diluent (0.9% saline, 0.1% Tween-20 and 3.0% low-viscosity carboxymethylcellulose) and injected using a 22 G needle to provide a total of 50 mg/kg naltrexone, or a comparable mass of placebo microspheres. In the first series of experiments, animals received either a subcutaneous (s.c.) or intramuscular (i.m.) injection of placebo or naltrexone-loaded microspheres. Animals received an intraperitoneal (i.p.) injection of morphine (1 mg/kg) or saline 1, 3, 7, 14, 21, 28, and 35 days after microsphere administration and were retested on the hot plate 30 min after each morphine injection.

Rats receiving i.m. injections were also used in studies further exploring the relationships between extended-release naltrexone, morphine antagonism, and changes in brain  $\mu$ -opioid receptor binding and immunoreactivity. Half of the animals receiving extended-release naltrexone i.m. were killed 36 days after injection, a time when the behavioral effects of naltrexone were diminished. The placebo and remaining extended-release naltrexone-treated animals received a second, identical microsphere injection 34 days after the first. These animals were retested on the hot plate 1, 7, 14, 21, 28, 30, 32, 34, and 36 days later and were sacrificed on day 37, a time when the pharmacodynamic effects of naltrexone had completely disappeared.

Injections of extended-release naltrexone or placebo were well tolerated by the rats, independent of the route or number of injections. This was evidenced by the absence of local site reactions (redness, swelling, exudation, or skin scratching) upon clinical examination, both *in vivo* and *ex vivo*. Body weights of rats receiving extended release naltrexone and placebo microspheres were measured weekly for 35 days. The body weight of extended-release naltrexone-treated rats was 2.8  $\pm$  0.27% lower than placebo-treated rats over this period ( $P < 0.01$ , two-way ANOVA).

## Quantitation of Plasma Levels of Naltrexone

Blood samples were collected from all animals immediately after each behavioral test (see below). Animals were briefly anesthetized with 1–2% isoflurane and blood samples (approximately 500  $\mu\text{l}$  of whole blood) were collected via a lateral tail vein into tubes containing EDTA. The tubes were centrifuged for 10 min at 1000 g to separate plasma, which was subsequently stored at  $-70^\circ\text{C}$  until levels of naltrexone were determined by LC-MS (Naidong *et al*, 2002). The lower limit of quantitation (LOQ) for these studies was 1.0 ng/ml, and the coefficient of variation for the assay was <4.4%.

## Hot-Plate Testing

Morphine-induced (1 mg/kg) analgesia was used to determine the ability of extended-release naltrexone to block opioid receptors in the central nervous system. Analgesia

was monitored using a commercially available hot-plate apparatus (Columbus Instruments, Columbus, OH). Rats were individually placed on the hot plate (surface temperature = 48°C) and the latency (60 s maximum) to lick either hind paw was recorded. Animals received two baseline trials on the hot-plate test and were then randomly assigned to treatment groups. The effect of placebo or extended-release naltrexone formulations on morphine-induced analgesia was assessed on the indicated days after injection by administering morphine, starting at 1000 h. Each rat was tested on the hot plate 20 min after morphine injection, and then returned to its cage. The testing order was randomized over the course of the investigation.

### **$\mu$ -Opioid Receptor Changes Following Administration of Extended-Release Naltrexone to Rats**

Increases in  $\mu$ -opioid receptor density are commonly observed in response to antagonist administration (Lahti and Collins, 1978; Zukin *et al*, 1982). Therefore, the status of  $\mu$ -opioid receptor density following the administration of an extended-release naltrexone preparation was investigated as a biochemical measure of pharmacodynamic efficacy. The time course of the changes in  $\mu$ -opioid receptor density and expression following the administration of extended-release naltrexone microspheres was investigated using two different radioligand binding assays and immunohistochemical techniques. For the saturation radioligand binding assays, male Sprague–Dawley rats were injected once i.m. with 79.2 mg of microspheres (placebo or naltrexone, approximately 50 mg/kg naltrexone) suspended in 0.75 ml of diluent. The rats were then killed 3, 5, 7, 28, 30, 32, 36, or 40 days after naltrexone, and 3, 7, 38, or 40 days after placebo administration. The brains were rapidly removed, placed in isotonic sucrose (0–4°C), the cortex, midbrain (from approximately bregma –5 to –10 mm), and striatum dissected free on ice, rapidly frozen on dry ice, and stored at –80°C until use. For autoradiographic and immunohistochemical studies, rats were injected twice with extended-release naltrexone (see above).

**$\mu$ -Opioid receptor binding.** [<sup>3</sup>H]DAMGO (D-ala<sup>2</sup>, N-methyl-phe<sup>4</sup>, glycol<sup>5</sup>) enkephalin; specific activity = 55.0 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL) binding to  $\mu$ -opioid receptors in the midbrain, striatum, and cortex was performed using a modification of a previously described technique (Goldstein and Naidu, 1989). At the time of the assay, these regions were homogenized using a probe sonicator in 10 vol of 50 mM Tris-HCl, 1 mM EDTA, and 280 mM sucrose buffer, pH 7.4, then centrifuged at 20 000 g for 20 min. The pellet was retained and resuspended in 50 mM Tris-HCl pH 7.4 buffer alone and then recentrifuged. The latter step was repeated a total of four times.

The equilibrium binding constants ( $K_d$  and  $B_{max}$ ) for [<sup>3</sup>H]DAMGO binding to  $\mu$  receptors in brain homogenates were determined using saturation binding assays consisting of 0.5–10 nM concentrations of [<sup>3</sup>H]DAMGO, tissue (0.08–0.7 mg protein), 10  $\mu$ M naltrexone (for determination of nonspecific binding), and sufficient 50 mM Tris-HCl buffer to yield a final volume of 250  $\mu$ l. The assays were performed in duplicate in polystyrene 96-well plates incubated at 25°C

for 1 h, and terminated by filtration. The  $B_{max}$  and  $K_d$  values were determined by nonlinear regression fitting of saturation isotherms to the data (Prism, GraphPad Software, San Diego, CA).

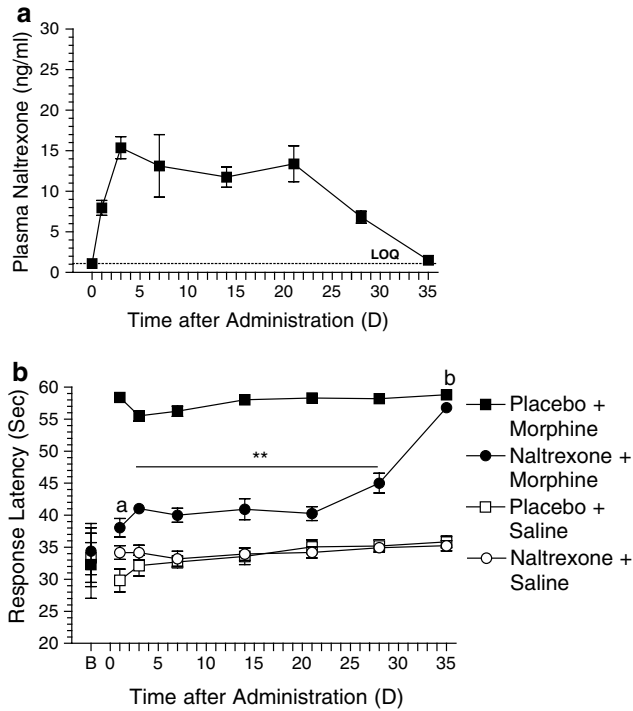
**$\mu$ -Opioid receptor autoradiography.** At the conclusion of behavioral testing (see above), animals were killed, their brains removed, flash frozen, and stored at –80°C. Frozen brains were cut into 20  $\mu$ m thick sections using a cryostat, the sections thaw-mounted onto glass slides, and stored at –80°C until they were used for quantitative autoradiography of  $\mu$ -opioid receptor binding (Morris *et al*, 2001). Brain sections were prewashed in 120 mM NaCl and 50 mM Tris-HCl buffer, then incubated in a solution containing 5 nM [<sup>3</sup>H]DAMGO and 120 mM NaCl in Tris-HCl buffer (pH 7.4, 1 h, 25°C). Nonspecific binding was determined using 1  $\mu$ M DAMGO. At the end of the incubation period, the sections were washed 5  $\times$  1 min in Tris buffer (pH 7.4, 0–4°C) with a final rinse in distilled H<sub>2</sub>O (0–4°C), then dried under a cool stream of air. The sections were apposed to film (Hyperfilm-<sup>3</sup>H; Amersham Pharmacia Biotech), together with a tritium standard calibration slide (American Radiolabeled Chemicals, St Louis, MO) and nonspecific control sections. The films were stored at –80°C and developed 9 weeks later. The optical density of autoradiographic exposures was quantified using an MCID 4 image analysis system (Imaging Research, St Catherines, Ontario, Canada).

**$\mu$ -Opioid receptor immunoreactivity.** Brain sections adjacent to those used for receptor binding were processed for  $\mu$ -opioid receptor immunoreactivity (Unterwald *et al*, 1998). Sections were immersion fixed in 6% paraformaldehyde, 20% sucrose, 20% ethanol, 20% ethylene glycol, and 10% glycerol in 0.05 M phosphate buffer (Jones *et al*, 1992), then washed in phosphate-buffered saline. Subsequently, the sections were treated to suppress endogenous peroxidase activity and the nonspecific sites were blocked. The slides were then incubated with the primary antibody to  $\mu$ -opioid receptors (Ab-1, Oncogene Research Products, Cambridge, MA; 1:2500) in a humidifying chamber for 24 h. The next day, the slides were washed and incubated for 2 h with the secondary antibody (I<sup>125</sup> anti-rabbit IgG; Amersham Biosciences, Piscataway, NJ, 1:100) in the humidifying chamber. The sections were then washed and dried under a stream of cool air. Nonspecific immunoreactivity was assessed by deleting either the primary antibody or using sections treated with antibody preabsorbed to the antigenic peptide (Opioid  $\mu$ -Receptor Control Peptide, Oncogene). These sections, together with an I<sup>125</sup> microscale standard (Amersham Biosciences), were apposed to film (Hyperfilm- $\beta$ max, Amersham Biosciences) for 7 days. The films were developed and the autoradiographs analyzed using the MCID 4 image analysis system.

## **RESULTS**

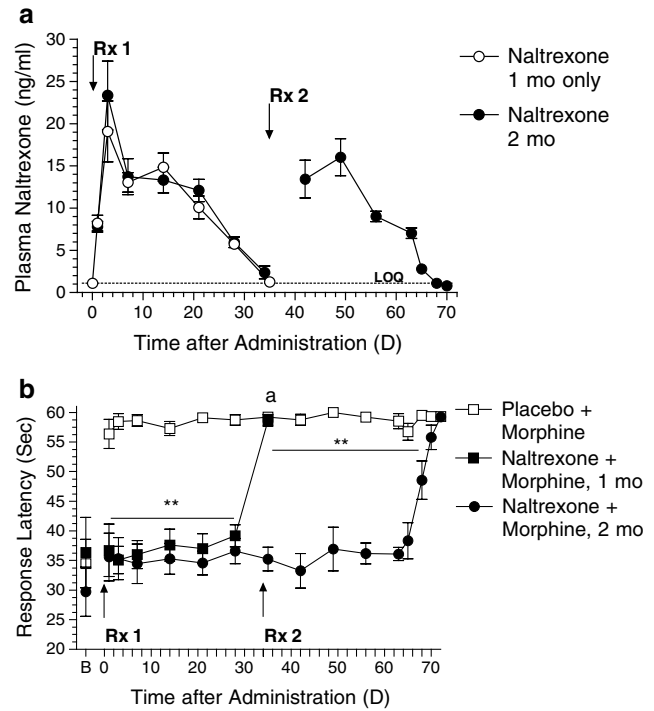
### **Quantitation of Plasma Levels of Naltrexone in Rats**

Plasma naltrexone levels were below the LOQ in all rats tested prior to treatment. The route of administration (i.m. or s.c.) had no significant effect on either the plasma



**Figure 1** (a) Plasma levels of naltrexone in rats following a single, s.c. injection of extended-release naltrexone microspheres (50 mg/kg naltrexone). Blood was sampled from the tail vein immediately after the hot-plate test. Note that the plasma levels of naltrexone were maintained for 21 days and were above the LOQs for at least 28 days. Each point represents the mean  $\pm$  SEM of plasma naltrexone concentrations (ng/ml) from eight rats. (b) Pharmacodynamic effects of a single s.c. injection of extended-release naltrexone microspheres. This was assessed by testing naltrexone (circles) or placebo formulation (squares)-treated rats on the hot plate 30 min after an injection of morphine (1 mg/kg i.p., closed symbols) or saline (open symbols). Rats receiving placebo microspheres manifested a pronounced analgesic response to morphine that consistently approached the maximum latency of 60 s. In contrast, animals pretreated with naltrexone microspheres showed substantial block of morphine analgesia, responding at or near the level of saline-treated animals. Data represent the mean  $\pm$  SEM of the latency to lick a hind paw measured in eight rats. \*\*Performance of the naltrexone + morphine group is significantly different from placebo + morphine, placebo + saline, and naltrexone + saline groups,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. (a) Performance of the naltrexone + morphine group is significantly different from placebo + morphine and placebo + saline groups,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. (b) Performance of the naltrexone + morphine group is significantly different from placebo + saline and naltrexone + Saline GROUPS,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. B: baseline conditions.

naltrexone levels ( $P = 0.72$ , two-way ANOVA, Figures 1a, 2a), or the area under the curves (332 vs 360 ng day/ml, i.m. vs s.c., respectively). However, plasma naltrexone concentrations changed significantly with time ( $P < 0.01$ , Two-way ANOVA), increasing to approximately one-half of the maximum within 24 h of injection, with maximum levels ( $15 \pm 1.4$  and  $19 \pm 3.6$  ng/ml, s.c. and i.m., respectively) observed by 3 days. Plasma concentrations of naltrexone did not differ significantly from each other between 3 and 14 days (i.m.) or 21 days (s.c.) postinjection, with detectable levels of naltrexone maintained to 35 days. A similar pattern was observed in animals that received a second i.m. injection of extended-release naltrexone microspheres (Figure 2a, Rx 2). There were no significant differences in



**Figure 2** (a) Plasma levels of naltrexone in rats following either a single i.m. injection of extended-release microspheres (open circles) or a second, identical injection 34 days following the first treatment (closed circles). Plasma levels of naltrexone were maintained for approximately 21 days following a single injection, an effect that was repeated with a second injection after an additional 34 days. Each point represents the mean  $\pm$  SEM naltrexone plasma concentration (ng/ml) from nine rats. LOQ: lower limit of quantitation ( $< 1$  ng/ml). (b) Analgesic actions of morphine (1 mg/kg i.p.) following either one or two i.m. injections of extended-release naltrexone microspheres in rats as tested on the hot plate. Naltrexone, whether administered for 1 or 2 months i.m., was capable of antagonizing morphine-induced analgesia, with responses equivalent to those observed under baseline conditions. The analgesic actions of morphine in rats treated once with naltrexone increased to levels observed in the placebo + morphine by 41 days. In contrast, two naltrexone treatments consistently suppressed morphine-induced analgesia for a total of 68 days. Data represent the mean  $\pm$  SEM of latency to lick a hind paw by nine rats. \*\*Performance of the naltrexone + morphine, 1 or 2 months groups, are significantly different from the placebo + morphine group,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. (a) Performance of the naltrexone + morphine 1 month group is significantly different from the naltrexone + morphine 2 months group,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. (b) Performance of the naltrexone + morphine 1 month group is significantly different from the naltrexone + morphine 2 months group,  $P < 0.01$ , two-way ANOVA followed by Tukey's *post hoc* analysis. B: Baseline conditions. Rx 1, 2: Times of first and second naltrexone injections.

plasma naltrexone levels during the plateau phase between the first and second injections. Moreover, naltrexone was no longer quantifiable in the plasma ( $< 1$  ng/ml) 35 days after the second injection.

### Hot-Plate Testing

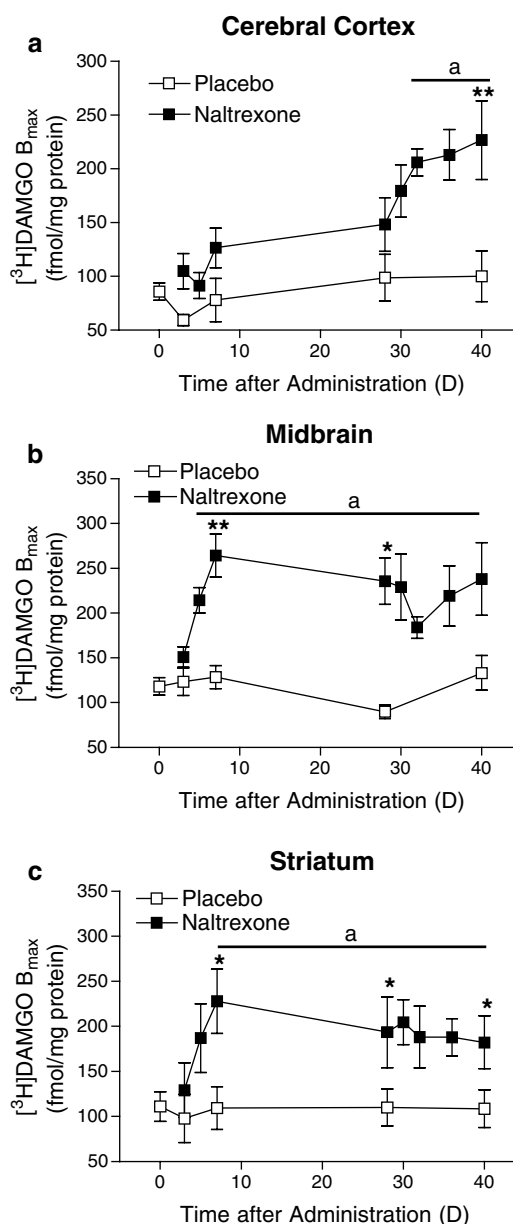
The pharmacodynamic effects of extended-release naltrexone corresponded well with the pharmacokinetic profile derived from the same animals. Extended-release naltrexone significantly suppressed the analgesia produced by morphine, independent of the route of microsphere administration (Treatment Effect,  $P < 0.01$ , Route Effect,  $P = 0.35$ , Multiway ANOVA, Figures 1b, 2b). Morphine

induced a profound analgesia in rats receiving placebo microspheres, as evidenced by hot-plate times approaching the maximum duration ( $57 \pm 0.60$  s). In contrast, the analgesic effects of morphine were suppressed in rats previously administered extended-release naltrexone (Figures 1b, 2b). Over a 21-day period, the rats receiving extended-release naltrexone + morphine showed hot-plate response times ( $40 \pm 0.54$  s) that were approximately 70% of the level of those that received extended release placebo + saline ( $34 \pm 0.80$  s). After 28 days, the morphine-associated response latencies of the extended-release naltrexone-treated rats increased to the level of those rats receiving placebo microspheres ( $59 \pm 0.84$  vs  $57 \pm 1.8$  s, placebo + morphine vs naltrexone + morphine). Rats receiving extended-release naltrexone i.m. had hot-plate response times after morphine treatment ( $36 \pm 0.40$  s) that were statistically indistinguishable from saline-treated animals (eg placebo + saline =  $31 \pm 5.1$  s; naltrexone + saline =  $34 \pm 3.5$  s). Animals receiving a second i.m. injection of extended-release naltrexone on day 34 continued to exhibit complete antagonism of the analgesic effects of morphine throughout the second month (36 s, Figure 2b). The analgesic effects of morphine on naltrexone-treated rats reverted to the level of the placebo-treated rats ( $59 \pm 0.48$  vs  $59 \pm 0.58$  s, placebo + morphine vs naltrexone + morphine) by 35 days after treatment (Figure 2b).

### Opioid Receptor Changes Following Extended-Release Naltrexone in Rats

**Radioligand binding assays.** Saturation binding assays revealed that the  $B_{\max}$  for [<sup>3</sup>H]DAMGO binding to the midbrain and the striatum was significantly increased (110 and 110% vs placebo treatment,  $P < 0.01$ , 0.05, respectively, ANOVA followed by Tukey's *post hoc* comparison test) by 1 week after administration of extended-release naltrexone (Figures 3b, c). Evidence of increased  $\mu$ -opioid receptor density was observed as early as 5 days after administration. These increases in receptor density were sustained throughout the subsequent 33 days, at least 1 week after the significant decline in pharmacodynamic effectiveness of the extended-release preparation. Interestingly, the density of cortical  $\mu$ -opioid receptors did not begin to increase until 30 days after extended-release naltrexone administration (Figure 3a), reaching significance at 40 days (120% increase vs placebo,  $P < 0.01$ , ANOVA followed by Tukey's *post hoc* comparison test). No significant changes in radioligand affinity for the receptors were observed in any brain region at any time, regardless of treatment (cortex:  $7.1 \pm 0.40$ ; midbrain:  $4.2 \pm 0.30$ ; striatum:  $3.1 \pm 0.10$  nM).

Similar results, albeit with higher regional resolution, were obtained using radioligand binding autoradiography. In this study, rats received either one or two injections of extended-release naltrexone spaced 34 days apart, and the changes in  $\mu$ -opioid receptor density quantified at 1 month and 24 h after the antagonism of morphine's analgesic effects had dissipated (ie 2 months from initial injection). Autoradiography revealed that radioligand binding to  $\mu$ -opioid receptors was significantly increased above control in all brain regions examined, ranging from 90% in the habenular nucleus to 160% in the dorsal raphe nucleus (Table 1, Figure 4) after 1 month. In most regions, these



**Figure 3** Regional changes in the  $B_{\max}$  of [<sup>3</sup>H]DAMGO binding to  $\mu$ -opioid receptors in the brain following a single i.m. dose of extended-release naltrexone. Data represent the mean  $\pm$  SEM of data from eight rats (panels a, b) or eight sets of striata pooled from 16 rats (panel c). Significant increases in the density of  $\mu$  receptors in the cerebral cortex were not observed until 32 (relative to  $t_0$  control) to 40 (relative to contemporaneous placebo control) days after naltrexone administration (panel a). In contrast,  $\mu$ -opioid receptor density in the midbrain (panel b) and striatum (panel c) was significantly increased by 7 days (relative to contemporaneous placebo) after naltrexone administration. (a) Significantly different from  $t_0$  control,  $P < 0.05$ ; \* \*\*significantly different from contemporaneous placebo control,  $P < 0.05$ , 0.01, two-way ANOVA followed by Tukey's *post hoc* comparison matrix.

densities continued to increase at 2 months, from 100% in the subiculum to 220% in the dorsal raphe.

**Immunohistochemistry.** Immunohistochemistry using brain sections adjacent to those used in the radioligand autoradiography also revealed significantly increased  $\mu$ -opioid receptor immunoreactivity (Table 2, Figure 5). After

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