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Selective in vivo binding of [^{3}H]naltriben to δ-opioid receptors in mouse brain

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Abstract

Naltriben (NTB) is a selective antagonist for the putative δ_2-opioid receptor. We have determined the regional kinetics and pharmacological profile of [^{3}H]naltriben in vivo in mouse brain. After i.v. administration to CD1 mice, [^{3}H]naltriben uptake and retention were high in striatum, cortical regions and olfactory tubercles, and low in superior colliculi and cerebellum. Robust rank order correlation was found between [^{3}H]naltriben uptake in discrete brain regions and prior δ-opioid receptor binding determinations in vitro and in vivo. [^{3}H]Naltriben binding in vivo was saturable, and was blocked by the δ-opioid receptor antagonist naltrindole, but not by the μ-opioid receptor antagonist cyprodime or the κ-opioid receptor agonist (*trans*)-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide mesylate (U50,488H). (*E*)-7-Benzylidenenaltrexone (BNTX), a selective antagonist for the putative δ_1-opioid receptor, was 9.6- to 12.9-fold less potent than naltriben as an inhibitor of [^{3}H]naltriben binding. Thus, the sites labeled by [^{3}H]naltriben in vivo may correspond to the δ_2-opioid receptor subtype. Such assignment is not definitive, particularly considering the 4-fold higher brain uptake of naltriben as compared to (*E*)-7-benzylidenenaltrexone. Moreover, the regional distribution of [^{3}H]naltriben in brains from CXB-7/BY (CXBK) mice, a strain that shows supraspinal δ_1- but not δ_2-opioid receptor agonist effects, was quite similar to that found for CD1 mice.

Keywords: δ-Opioid receptor; Naltriben; Benzylidenenaltrexone; Binding, in vivo; Brain; CD1 mouse; CXBK mouse

1. Introduction

Subtypes of the δ-opioid receptor have been proposed on the basis of differential antagonism of agonist induced antinociception, tolerance and cross-tolerance paradigms, binding assays in vitro, biochemical investigations of second messenger systems, and molecular biology studies as reviewed by Traynor and Elliott (1993), Reisine (1995) and Zaki et al. (1996). The subtypes can be defined as δ_1 and δ_2 using a classification scheme drawn primarily from functional antinociceptive assays in vivo (Jiang et al., 1991; Mattia et al., 1991; Sofuoglu et al., 1991), although as many as four δ-opioid receptor binding sites have been postulated from binding studies in vitro (Xu et al., 1993; Fang et al., 1994; Cha et al., 1995). At the molecular level, the cloned human and mouse δ-opioid receptors are quite similar (Simonin et al., 1994), and display a pharmacological profile conforming to the δ_2-opioid receptor subtype (Reisine, 1995; Zaki et al., 1996). Nonetheless, the existence of a distinct δ_1-opioid receptor is supported by antisense oligodeoxynucleotide studies involving 'knockdown' of δ-opioid binding sites in mice, followed by antinociceptive testing and receptor density determinations (Bilsky et al., 1996).

(*E*)-7-Benzylidenenaltrexone (BNTX; Portoghese et al., 1992) and naltriben (NTB; Portoghese et al., 1988a, 1991), δ-opioid receptor antagonists derived from naltrexone, are viewed as prototypical ligands for distinguishing the putative δ_1- and δ_2-opioid receptor subtypes, respectively. These two ligands have been employed as a pair in a wide variety of studies. For instance, differential antagonism of antinociception by (*E*)-7-benzylidenenaltrexone and naltriben indicates that heroin activates supraspinal δ_1-opioid binding sites, while 6-monoacetylmorphine activates δ_2-opioid binding sites (Rady et al., 1994). Conditioned place preferences induced by cocaine and methamphetamine have been attributed to the δ_2-opioid receptor as a consequence of attenuation by naltriben but not by (*E*)-7-benzylidenenaltrexone (Suzuki et al., 1994). Studies with (*E*)-7-

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benzylidenenaltrexone and naltriben have led to the hypothesis that the rewarding effects of δ_1-opioid receptor agonists involve activation of central dopamine D_1 receptors, while other mechanisms may be more important for δ_2-opioid receptor agonists (Suzuki et al., 1996). (*E*)-7-Benzylidenenaltrexone and naltriben also have been used to investigate coupling of δ-opioid receptor subtypes to adenylyl cyclase in rat brain regions (Búzás et al., 1994; Noble and Cox, 1995; Olianas and Onali, 1995), to study the effect of diabetes on supraspinal antinociception mediated by δ-opioid receptor subtypes in mice (Kamei et al., 1994, 1995), and to explore the δ-opioid receptor subtype selectivity of novel agonists (Tseng et al., 1997). The δ_2-opioid receptor subtype assignments for the cloned human and mouse receptor have been supported, in part, by the higher in vitro affinity of the sites for naltriben as compared to (*E*)-7-benzylidenenaltrexone (Kong et al., 1993; Knapp et al., 1994; Raynor et al., 1994).

Methods for direct in vivo assessment of the putative δ-opioid receptor subtypes would facilitate efforts to understand their respective functional roles. As Zaki et al. (1996) point out, knowledge of the in vivo behavior of δ-opioid receptor subtype selective ligands may provide insight into their relative access to distinct environments that might influence the observed pharmacology. We have identified N1′- ([^{11}C]methyl)naltrindole ([^{11}C]MeNTI) as a radioligand that labels δ-opioid receptors in vivo in mouse brain (Lever et al., 1992), and serves for positron emission tomographic (PET) studies of δ-opioid receptors in human brain (Madar et al., 1996, 1997). However, [^{11}C]MeNTI might not be expected to show pronounced δ-opioid receptor subtype selectivity in vivo because the parent ligand, naltrindole (NTI), binds to the putative δ-opioid receptor subtypes with high, and nearly equal, affinities in vitro (Fang et al., 1994).

Recently, we found that [^{3}H](*E*)-7-benzylidenenaltrexone labels δ-opioid receptor sites in vivo in mouse brain albeit with a low extent of specific binding (Lever et al., 1996). Dose–response inhibition studies using naltriben and (*E*)-7-benzylidenenaltrexone were consistent with, but not conclusive for, a modest degree of δ_1-opioid receptor selectivity. During the course of that work, we established a 4-fold greater brain penetration for [^{3}H]naltriben with respect to [^{3}H](*E*)-7-benzylidenenaltrexone. Explicit studies of [^{3}H]naltriben binding in vivo, however, were precluded by the low specific activity (1.3 Ci/mmol) of the radioligand. [^{3}H]Naltriben of higher specific activity (30 Ci/mmol) is now available. Accordingly, we have investigated the regional kinetics and pharmacological profile of [^{3}H]naltriben binding to δ-opioid receptors in mouse brain. Studies were done using CD1 mice, a normal strain, as well as CXB-7/BY (CXBK) mice, a recombinant inbred strain thought to have functional supraspinal δ_1- but not δ_2-opioid receptors (Raffa et al., 1992). The present work was conducted to ascertain the suitability of [^{3}H]naltriben for labeling δ-opioid receptor sites in vivo, with a view toward selective localization of the putative δ_2-opioid receptor subtype (Lever and Scheffel, 1997).

2. Materials and methods

2.1. Drugs

[^{3}H]Naltriben mesylate (30 Ci/mmol, > 97% purity) in 50% ethanol was obtained from Tocris Cookson (Bristol, UK). Naltriben mesylate and (*E*)-7-benzylidenenaltrexone hydrochloride were gifts from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Naltrindole (NTI) hydrochloride, cyprodime hydrobromide, and (*trans*)-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide mesylate (U50,488H) were obtained from Research Biochemicals (Natick, MA).

2.2. Animals

Male CD1 (Charles River Laboratories, Wilmington, MA) and CXB-7/BY (CXBK) (Jackson Laboratories, Bar Harbor, ME) mice (24–28 g) were housed in rooms maintained at 22°C on a 12 h light/12 h dark cycle, with food and water available ad libitum. Experimentation was done under humane conditions under protocols approved by The Johns Hopkins University Animal Care and Use Committee in compliance with guidelines for the care and use of laboratory animals adopted and promulgated by the National Institutes of Health.

2.3. In vivo pharmacokinetics

The regional cerebral kinetics of [^{3}H]naltriben were evaluated in non-fasted CD1 mice. [^{3}H]Naltriben (1.0 μCi; ≤ 1.5 nmol/kg) in 0.9% saline containing 0.5% ethanol (0.2 ml) was given by tail vein injection. Sets of four mice were killed by cervical dislocation at intervals from 15 min to 16 h, and immediately decapitated. Whole brain was removed, and samples of prefrontal cortex, parietal cortex, olfactory tubercles, striatum, thalamus and cerebellum were dissected on an ice-cold glass plate. Superior colliculi, hippocampus and hypothalamus also were obtained at the 60 min time point. These tissues, as well as the remainder of the brain, were blotted, and then weighed in glass scintillation vials. After digestion (Solvable, 1 ml; DuPont NEN, Boston, MA) and dilution with scintillation cocktail (Formula 989, 10 ml; DuPont NEN), the radioactivity of the samples was measured with an automated β-counter (Packard Instrument, Downers Grove, IL). Standard dilutions of the injected dose (ID) were counted to allow calculation of % injected dose per gram of tissue. The [^{3}H]naltriben distribution was determined by the same method in brain tissues from non-fasted CXBK mice (n = 5) at 15 and 60 min.

2.4. In vivo pharmacology

Mice were pretreated by i.v. injection of 5.0 μmol/kg doses of either naltriben, naltrindole, cyprodime or U50,488H dissolved in saline (0.15 ml). Naltriben formulations included 2% ethanol. Drugs were given 15 min prior to tail vein injection of [^{3}H]naltriben (1.0 μCi). Groups of three to four drug- or saline-treated animals were killed 60 min after [^{3}H]naltriben administration, and brain tissues of interest were processed as described above. Dose–response data for inhibition of [^{3}H]naltriben by naltriben and (*E*)-7-benzylidenenaltrexone (0.01–10.0 μmol/kg) were obtained similarly, but the antagonists were given s.c. 30 min before i.v. [^{3}H]naltriben (1.0 μCi).

2.5. Statistics

Potential differences between control and treatment groups were investigated by analysis of variance (ANOVA) and a post-hoc Dunnett's test at the $\alpha = 0.01$ significance level (SuperANOVA, version 1.11; Abacus Concepts) for data concerning inhibition of [^{3}H]naltriben by compounds used at a single dosage level. ANOVA at the $\alpha = 0.05$ significance level with a post-hoc Bonferroni/Dunn test for all means was used to compare differences between the inhibitors. A two-tailed, unpaired *t*-test was used at the 95% confidence level (StatView, version 4.1; Abacus Concepts) to compare potential differences in [^{3}H]naltriben binding between brain regions of CXBK and CD1 mice. Linear and nonparametric correlations were performed with StatWorks, version 1.1 software (Cricket Software). Dose–response data for (*E*)-7-benzylidenenaltrexone and naltriben inhibition of specific [^{3}H]naltriben binding by 50% (ED_{50} values) were analyzed using a nonlinear regression algorithm (Prism 2.0; GraphPad Software) to minimize the sum of the square of the absolute distances of the points from the curve. Convergence was declared when three consecutive iterations each reduced the sum-of-squares by less than 0.0001%. Fits to one-site and two-site competitive binding models were compared using the *F*-test. Time vs. radioactivity curves also were fitted using Prism 2.0, and the *F*-test was applied to compare single and double exponential decay models.

3. Results

3.1. Regional in vivo kinetics of [^{3}H]naltriben in mouse brain

[^{3}H]Naltriben readily crossed the blood–brain barrier after i.v. administration to CD1 mice. The % injected dose to whole brain fell as a single exponential ($r^2 = 0.98$) from 1.81% at 15 min to 0.30% at 16 h, with a clearance half-time of 87 min (data not shown). Selected regional pharmacokinetic data are given in Fig. 1. Radioligand uptake and retention were highest in striatum, cortical regions and olfactory tubercles, lowest in cerebellum and superior colliculi, and intermediate in hippocampus, hypothalamus and thalamus. The differential distribution was apparent within 15 min, and held over a 4 h time course. At one later time, 16 h, all regions showed a low, uniform level of uptake (data not shown).

Radioactivity accumulated in the striatum, a region rich in δ-opioid receptors, over the first 30 min, plateaued from 30 to 60 min, and then fell gradually as a single exponential ($r^2 = 0.97$) with a half-time of 99 min. By contrast, radioactivity concentrations decreased rapidly in the cerebellum, a region with a low density of δ-opioid receptor sites, and were as low or lower than those of all other regions throughout the study. A single exponential fit ($r^2 = 0.96$) for cerebellar clearance from 15 min to 16 h was noted, with a half-time of 25 min. As a consequence, the striatal to cerebellar radioactivity ratio reached a peak value of 4.3 to 1 at 60 min. Peak ratios also were reached near 60 min for the other regions (Fig. 2). Since the density of δ-opioid receptors is relatively high in many

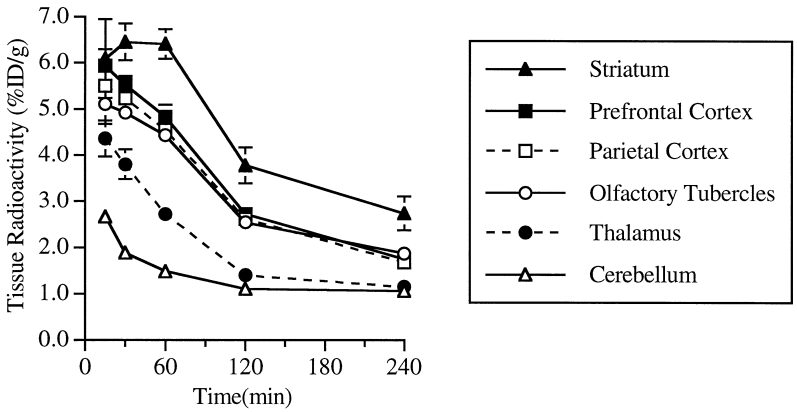

Fig. 1. Temporal cerebral radioactivity distribution after i.v. administration of [^{3}H]naltriben to CD1 mice (mean ± S.D., $n = 4$). Non-visible error bars are contained within the symbol.

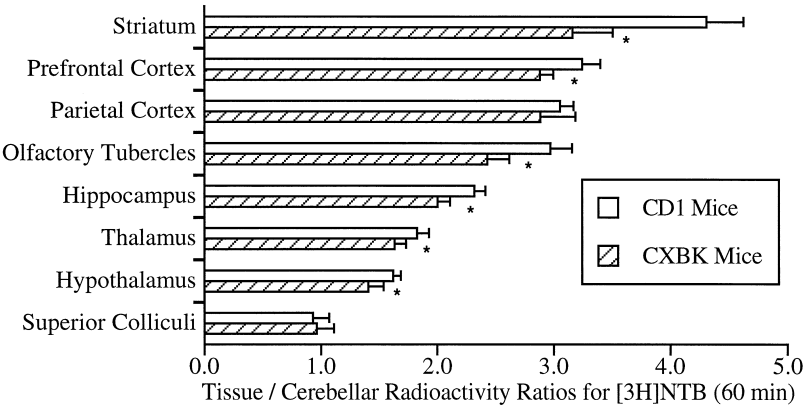

Fig. 2. Tissue to cerebellum radioactivity concentration ratios (mean ± S.D.) determined 60 min after i.v. administration of [^{3}H]naltriben to CD1 ($n = 4$) or CXBK ($n = 5$) mice. ∗Ratios decreased significantly ($P \leq 0.03$) for the CXBK mice.

mouse brain regions as compared to cerebellum (Mansour et al., 1988; Delay-Goyet et al., 1991; Dupin et al., 1991), these region to cerebellar radioactivity ratios serve as an index of specific binding by approximating the total to nonspecific binding ratio. The ratio for the superior colliculi was unity, in accord with the very low density of δ-opioid receptor sites known for this region of CD1 mouse brain (Dupin et al., 1991).

Another index of specific binding to δ-opioid receptors in vivo is the difference between radioactivity concentrations in a given tissue and that in the cerebellum. By this measure, specific [^{3}H]naltriben binding in the striatum of CD1 mice was 70–77% of total uptake between 30 and 120 min, with a maximal value of 4.9 ± 0.3% injected dose per gram at 60 min. Clearance of striatal specific binding from 60 min to 16 h was adequately fit by a single

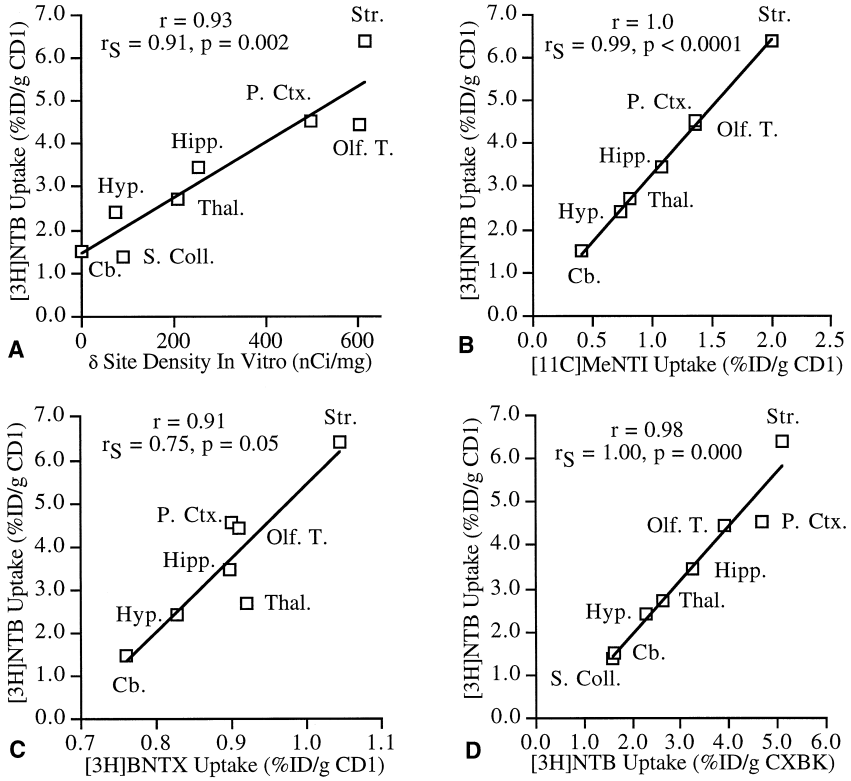

Fig. 3. Uptake of [^{3}H]naltriben in vivo at 60 min in brain regions from CD1 mice (means, $n = 7$) compared to: (A) total binding of [^{125}I][D-Ala^2]deltorphin-I in vitro in CD1 brain sections (Dupin et al., 1991); (B) uptake of [^{11}C]MeNTI in vivo at 60 min in CD1 brain regions (Lever et al., 1992; $n = 4$); (C) uptake of [^{3}H](*E*)-7-benzylidenenaltrexone in vivo at 60 min in CD1 brain regions (Lever et al., 1996; $n = 4$); and (D) uptake of [^{3}H]naltriben in vivo at 60 min in CXBK brain regions (means, $n = 5$). S.D. < 20% of the mean for all in vivo values, error bars omitted for clarity. Str., striatum; Olf. T., olfactory tubercles; P. Ctx., parietal cortex; Thal., thalamus; Hyp., hypothalamus; Hipp., hippocampus; S. Coll., superior colliculi; Cb., cerebellum.

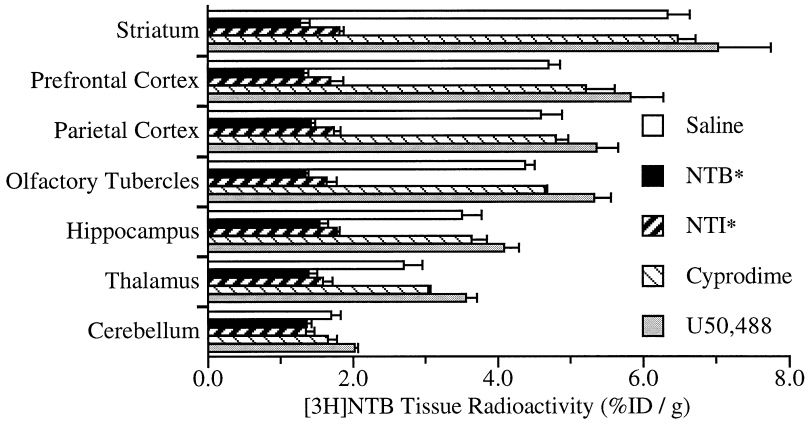

Fig. 4. Effect of drug pretreatments on [^{3}H]naltriben uptake in CD1 mouse brain tissues 60 min after i.v. administration of radioligand (mean ± S.D., $n = 3–4$). Drugs (5.0 μmol/kg) were given i.v. 15 min prior to [^{3}H]naltriben. * Reduced with respect to values for saline-treated controls in all regions at the 0.01 significance level (ANOVA, post-hoc Dunnett's test).

exponential ($r^2 = 0.97$) with a half-time of 96 min. Specific binding also represented the majority (57–69%) of total uptake in the olfactory tubercles and cerebral cortical regions between 30 and 120 min. Specific binding at 60 min was lower for hippocampus (57%), thalamus (45%) and hypothalamus (38%), and was not measurable for the superior colliculi.

The overall distribution of [^{3}H]naltriben in CXBK mouse brain at 15 and 60 min proved similar to that observed at the same times in CD1 mouse brain (Figs. 2 and 3D). However, the striatal to cerebellar radioactivity ratio was significantly ($P = 0.001$) lower by 26% for the CXBK strain (Fig. 2). Ratios for olfactory tubercles, prefrontal cortex, hippocampus, hypothalamus and thalamus also were decreased significantly ($P \leq 0.03$) by 11–18% for CXBK mice. The parietal cortex to cerebellar radioactivity ratio for CXBK mice was only 5% less than that for CD1 mice, and the difference was not significant ($P = 0.3$). Levels of radioactivity in the cerebellum, as well as the superior colliculi, were not different between the two strains ($P \geq 0.25$; Fig. 3D). Compared to the CD1 strain, specific binding for CXBK mice at 60 min tended to be a smaller proportion of total uptake: viz., 68% in striatum, 59% in olfactory tubercles, 65% in cerebral cortical regions, 50% in hippocampus, 39% in thalamus and 29% in hypothalamus. Decrements in [^{3}H]naltriben binding in brain regions from CXBK animals also were observed at the 15 min time point.

3.2. Correlation of the regional distribution of [^{3}H]naltriben in vivo in mouse brain with prior δ-opioid receptor determinations in vitro and in vivo

As shown in Fig. 3A, good linear ($r = 0.93$) and Spearman rank order correlations ($r_S = 0.91$, $P = 0.002$) were observed between [^{3}H]naltriben uptake in vivo and δ-opioid receptor site densities known from in vitro autoradiographic studies of [^{125}I][D-Ala^2]deltorphin-I binding in sections of CD1 mouse brain (Dupin et al., 1991). No correlation ($r_S = -0.24$, $P = 0.57$; data not shown) was found with the μ-opioid receptor site densities determined by Dupin et al. (1991) in vitro using radioiodinated Tyr-D-Ala-Gly-(NMe)Phe-Met(O)-ol ([^{125}I]FK33,824).

Excellent linear ($r = 1.0$) and rank order correlation

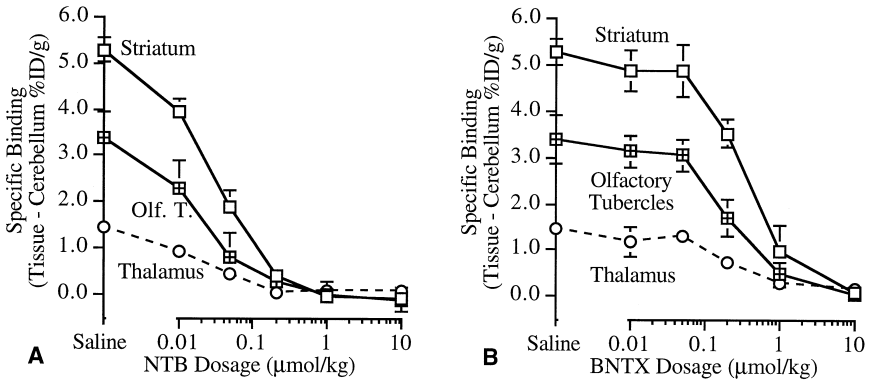

Fig. 5. Dose–response effects of naltriben (A) and (*E*)-7-benzylidenenaltrexone (B) on specific [^{3}H]naltriben binding in CD1 mouse brain tissues 60 min after i.v. radioligand administration (mean ± S.D., $n = 3–8$). Inhibitors were given s.c. 30 min prior to [^{3}H]naltriben. Non-visible error bars are contained within the symbol.

Table 1
Dose–response effects of s.c. NTB and BNTX on specific [^{3}H]NTB binding in vivo at 60 min in discrete regions of CD1 mouse brain[a]

Brain region	ED_{50} values[b] (μmol/kg)		Relative potency (ED_{50} ratio)
	NTB	BNTX	
Striatum	0.030 (0.023–0.038)	0.38 (0.16–0.87)	12.7
Prefrontal cortex	0.027 (0.016–0.046)	0.26 (0.12–0.56)	9.6
Parietal cortex	0.019 (0.013–0.027)	0.24 (0.11–0.51)	12.6
Olfactory tubercles	0.020 (0.015–0.026)	0.24 (0.11–0.51)	12.0
Thalamus	0.017 (0.009–0.032)	0.22 (0.042–1.1)	12.9

[a]Values from nonlinear least squares regression analysis of inhibition data.
[b]95% Confidence interval given in parentheses.

($r_S = 0.99$, $P < 0.0001$; Fig. 3B) was noted between the regional cerebral distribution of [^{3}H]naltriben, and that of N1′-([^{11}C]methyl)naltrindole ([^{11}C]MeNTI) in CD1 mouse brain (Lever et al., 1992). As shown in Fig. 3C, a good linear correlation ($r = 0.91$) but weaker Spearman correlation ($r_S = 0.75$, $P = 0.05$) was apparent between the in vivo distributions of [^{3}H]naltriben and [^{3}H](E)-7-benzylidenenaltrexone (Lever et al., 1996) in CD1 mouse brain. The identical rank order correlation ($r_S = 0.75$, $P = 0.05$) was noted between the [^{3}H]naltriben and [^{3}H](E)-7-benzylidenenaltrexone distributions in CXBK mouse brain (data not shown). These particular rank order correlations improved (e.g., $r_S = 0.94$, $P = 0.005$ for CD1) if the thalamus was not included (data not shown, cf. Fig. 3C). The relative regional [^{3}H]naltriben distribution proved identical in CD1 and CXBK strains, with robust linear ($r = 0.98$) and Spearman rank order ($r_S = 1.00$, $P = 0.000$) correlations (Fig. 3D).

3.3. Pharmacology of [^{3}H]naltriben binding in vivo in CD1 mouse brain

Fig. 4 shows the ability of several opioid receptor ligands, given by i.v. injection at a dosage of 5.0 μmol/kg, to inhibit regional [^{3}H]naltriben binding in CD1 mouse brain at 60 min. The [^{3}H]naltriben uptake was saturable by naltriben, and was blocked by the selective δ-opioid receptor antagonist naltrindole, in all brain regions at the $\alpha = 0.01$ significance level (ANOVA, post-hoc Dunnett's test). Striatum, cortical regions and olfactory tubercles exhibited high levels (70–80%) of specific binding, while thalamus and hippocampus displayed an intermediate level (50–55%). Low, but significant, reduction (ca. 20%) of [^{3}H]naltriben uptake by both naltriben and naltrindole also was detected in the cerebellum. Although naltriben showed a tendency to be a more potent inhibitor than naltrindole in regions rich in δ-opioid receptors (viz., striatum, cortical areas and olfactory tubercles), the differences did not reach statistical significance at the $\alpha = 0.05$ significance level (ANOVA, post-hoc Bonferroni/Dunn test for all means). Cyprodime, a selective μ-opioid receptor antagonist (Schmidhammer et al., 1989), and U50,488H, a selective κ-opioid receptor agonist (Leighton et al., 1988), did not inhibit [^{3}H]naltriben uptake in any region. In fact, U50,488H pretreatment led to significant ($\alpha = 0.01$) elevations in uptake of radioactivity in all brain regions examined except for the striatum.

The dose–response characteristics of naltriben and (E)-7-benzylidenenaltrexone inhibition of [^{3}H]naltriben binding in vivo in CD1 mouse brain were then investigated. (E)-7-Benzylidenenaltrexone and naltriben (0.01–10.0 μmol/kg) were given by s.c. injection to groups of mice 30 min prior to i.v. administration of the radioligand (1.0 μCi). Brain tissues were examined 60 min after [^{3}H]naltriben injection, the time of peak specific binding established by the pharmacokinetic experiments. Tissue minus cerebellum radioactivity concentrations were used as an expedient measure of specific binding, although the use of this index gives a slight underestimation due to the low, but detectable, specific [^{3}H]naltriben binding in the cerebellum. Steep, dose-dependent blockade was observed with both ligands, but naltriben was much more potent than (E)-7-benzylidenenaltrexone in all brain regions (Fig. 5, selected data shown).

Nonlinear regression analysis of the data for inhibition of striatal specific binding gave ED_{50} values of 0.030 μmol/kg ($r^2 = 1.0$) for naltriben and 0.38 μmol/kg ($r^2 = 0.99$) for (E)-7-benzylidenenaltrexone. The relative ED_{50} values for naltriben and (E)-7-benzylidenenaltrexone inhibition of specific [^{3}H]naltriben binding in the other brain regions were near those observed in striatum (Table 1), with coefficients of determination ranging from 0.96 to 1.0. Naltriben proved 9.6- to 12.9-fold more potent than (E)-7-benzylidenenaltrexone as an in vivo inhibitor of [^{3}H]naltriben binding to δ-opioid receptors in the mouse brain regions studied. In each case, a one-site competitive binding curve was a significantly (F-test) better fit for the regional inhibition data than a two-site model.

4. Discussion

In keeping with the high apparent affinity ($K_i = 13$ pM) and selectivity of binding to δ- over μ- or κ-opioid receptors shown for naltriben in vitro (Portoghese et al., 1988a,b, 1991), the present work indicates that [^{3}H]naltriben allows regional localization of cerebral δ-opioid recep-

tors in vivo. The heterogeneous regional distribution observed for [^{3}H]naltriben in CD1 mouse brain mirrors the relative δ-opioid receptor site densities of the regions known from autoradiographic studies in brain sections from the same strain (Dupin et al., 1991). Further, the general rank order for [^{3}H]naltriben uptake and retention (striatum > prefrontal cortex, parietal cortex, olfactory tubercles > hippocampus, thalamus, hypothalamus ≫ superior colliculi, cerebellum) closely parallels that previously established for the in vivo binding of the radioligands [^{11}C]MeNTI (Lever et al., 1992) and [^{3}H] (*E*)-7-benzylidenenaltrexone (Lever et al., 1996) to δ-opioid receptors in CD1 mouse brain. Regional differences in cerebral blood flow are not likely to complicate the interpretation of the data. For instance, measured local blood flow in the mouse brain is equivalent or higher for the cerebellum and superior colliculi with respect to the striatum, cortical regions, thalamus and hypothalamus (Jay et al., 1988).

Considering that naltriben is quite selective as an in vivo antagonist of the effects of δ_2- but not δ_1-opioid receptor agonists, we also examined the regional cerebral distribution of [^{3}H]naltriben in CXBK mice. In this μ-opioid receptor deficient strain, only δ_1-opioid receptor selective agonists induce supraspinal antinociception, while both δ_1- and δ_2-opioid receptor selective agonists are effective in CD1 mice (Raffa et al., 1992). This led to the hypothesis for CXBK mice that either central δ_1-opioid receptors predominate, or that only the δ_1-opioid receptor subtype is able to mediate supraspinal antinociceptive actions of δ-opioid receptor agonists. We found a perfect Spearman rank order correlation for the relative regional [^{3}H]naltriben brain distribution between the CD1 and CXBK strains (Fig. 3D). However, levels of [^{3}H]naltriben binding in discrete brain regions from CXBK mice were 11–26% lower than those from CD1 mice with the notable exception of cerebellum and superior colliculi, the areas known to be poor in δ-opioid receptors. This is exemplified by comparison of the tissue to cerebellar radioactivity ratios for the two strains at 60 min (Fig. 2). Thus, the lower ratios for CXBK mice reflect decreased radioligand uptake in the tissues having appreciable densities of δ-opioid receptor binding sites.

A previous in vitro study using whole brain homogenates found no differences in δ-opioid binding site densities between CXBK mice and a control strain (Reith et al., 1981). However, an autoradiographic study of brain sections at a finer anatomical level noted that strain differences between CXBK and controls could not be simply defined, because levels of δ-opioid receptor binding decreased, were not changed, or actually increased in certain specific areas (Moskowitz and Goodman, 1985). The present work shows that binding sites for putative δ_2-opioid receptor selective ligands, at least the antagonists such as naltriben, exist in the CXBK strain although perhaps to a lesser extent than in control strains of mice.

Interestingly, the rank order correlation between [^{3}H]naltriben and [^{3}H](*E*)-7-benzylidenenaltrexone distributions in vivo in both CD1 and CXBK mouse brain improves significantly if the thalamus is not included. As discussed by Gouardères et al. (1993), in vitro autoradiographic studies in rodent brain show discrepancies in the binding of several δ-opioid receptor radioligands in this region. Methodological considerations, rather than δ-opioid receptor subtype discrimination, were postulated to account for the differences. For the in vivo case, the relative distributions of [^{3}H]naltriben and [^{3}H](*E*)-7-benzylidenenaltrexone match well, particularly considering that the [^{3}H](*E*)-7-benzylidenenaltrexone data is inherently less reliable than the [^{3}H]naltriben data because of the much lower degree of differential uptake and specific binding across the brain regions (cf. Fig. 3C). In addition, a δ-opioid receptor concentration gradient has been noted by autoradiography of the thalamic nuclei (Gouardères et al., 1993) that may have impact on the results obtained using gross anatomical dissection. Nonetheless, the thalamic nuclei appear to be an interesting region for future studies of potential δ-opioid receptor heterogeneity.

Thus, highly significant correlations were found (Fig. 3) upon comparison of three different radioligands that might be expected to show either δ_2-opioid receptor selectivity ([^{3}H]naltriben), δ_1-opioid receptor selectivity ([^{3}H](*E*)-7-benzylidenenaltrexone), or nonselective binding to δ-opioid receptor subtypes ([^{11}C]MeNTI) in vivo in mouse brain. The correlations remained robust regardless of the potential species differences examined, and with respect to detailed autoradiographic studies conducted at a finer anatomical level by others in mouse brain sections in vitro. In aggregate, the data support the hypothesis that [^{3}H]naltriben given i.v. may not be selective for the putative δ_2-opioid receptor subtype. Alternatively, one might infer that differential localization of the putative δ-opioid receptor subtypes requires techniques to be conducted at the cellular biochemical or molecular biological levels (cf. Noble and Cox, 1995; Zaki et al., 1996) as opposed to a gross anatomical approach.

For pharmacological characterization of [^{3}H]naltriben binding in vivo, the CD1 strain was used rather than the CXBK strain so that a full range of opioid receptors would be available. Primarily μ- and δ-opioid receptor sites are competing for the radioligand, since κ-opioid receptor sites represent only 10–20% of the total density of cerebral opioid receptors across several strains of normal mice (Robson et al., 1985; Lahti et al., 1985; Mansour et al., 1988; Bilsky et al., 1996). [^{3}H]Naltriben uptake was not blocked in any brain region by a 5.0 μmol/kg dosage of either the μ-opioid receptor selective antagonist cyprodime (Schmidhammer et al., 1989) or the κ-opioid receptor selective agonist U50,488H (Leighton et al., 1988). Interestingly, pretreatment with U50,488H caused a global increase in the uptake of radioactivity that proved significant in all brain regions except striatum. This may be due

to enhanced delivery of the radioligand, since transient increases in cerebral blood flow has been documented upon administration of κ-opioid receptor agonists during certain protocols depending upon dose, timing and route of administration (cf. Benyo and Wahl, 1996; Upton et al., 1997).

By contrast, [^{3}H]naltriben uptake throughout the brain proved saturable by naltriben, and was inhibited significantly by naltrindole, a δ-opioid receptor antagonist (Portoghese et al., 1988a,b, 1991) that does not display subtype selectivity (Fang et al., 1994), at the equivalent dosage level. Cerebellar specific binding was approximately 7–11% of that noted in striatum or cortex. This finding is in good agreement with in vitro studies that defined the density of δ-opioid receptor sites in mouse cerebellum as 4–6% of that in striatum or cortex (Delay-Goyet et al., 1991). A similar level of specific binding was noted in mouse cerebellum in vivo using [^{11}C]MeNTI (Lever et al., 1992) but not [^{3}H](*E*)-7-benzylidenenaltrexone (Lever et al., 1996).

The pattern of [^{3}H]naltriben inhibition was the same in all brain regions, with a trend for naltriben to be more potent than naltrindole in those areas having high levels of δ-opioid receptors. This is likely to be a consequence of reduced brain penetration by naltrindole with respect to naltriben, rather than an indication of saturable, but non-specific, [^{3}H]naltriben binding. We have observed (unpublished data) that the total uptake (means ± S.D., $n = 4$) in whole CD1 mouse brain 60 min after i.v. administration for [^{3}H]naltrindole (0.14 ± 0.012% injected dose per gram) is a full order of magnitude less than that found for [^{3}H]naltriben (1.4 ± 0.083% injected dose per gram) in the present study.

The regional brain pharmacokinetics and binding inhibition profile determined for [^{3}H]naltriben clearly indicate that the radioligand selectively labels δ-opioid receptors in vivo throughout the CD1 mouse brain. Unequivocal definition of the nature of the potential δ-opioid receptor subtype(s) that might be labeled is more complex. Few ligands that are centrally active after systemic administration are available for in vivo competition experiments that also have selectivity for the putative δ_1- and δ_2-opioid receptor subtypes. Paired studies with (*E*)-7-benzylidenenaltrexone and naltriben are often employed for subtype discrimination in vivo, so we investigated their inhibition of [^{3}H]naltriben binding as a function of dose. The timing and route of inhibitor administration were based on studies of supraspinal enkephalin antinociception in Swiss Webster mice (Takemori and Portoghese, 1993) where peak effects of (*E*)-7-benzylidenenaltrexone and naltriben were observed 30 min after s.c. injection, and 1.2–1.3 μmol/kg dosages could be employed without opioid receptor agonist effects or δ-opioid receptor subtype cross-reactivity. The ED_{50} values for naltriben saturation of specific [^{3}H]naltriben binding in vivo were similar for all the mouse brain regions studied, and ranged from 0.017 to 0.030 μmol/kg. (*E*)-7-Benzylidenenaltrexone was determined to be a weaker inhibitor, with ED_{50} values ranging from 0.22 to 0.38 μmol/kg across the brain regions. The ED_{50} values for inhibition of [^{3}H]naltriben binding by both (*E*)-7-benzylidenenaltrexone and naltriben are 3- to 70-fold lower than the dosage of these ligands associated with δ-opioid receptor subtype cross-reactivity or agonist effects in the antinociceptive assays of Takemori and Portoghese (1993).

Although naltriben proved 9.6- to 12.9-fold more potent than (*E*)-7-benzylidenenaltrexone as an inhibitor of [^{3}H]naltriben binding in vivo, our previous work showed that naltriben has 4-fold higher brain uptake than (*E*)-7-benzylidenenaltrexone 60 min after s.c. dosing (Lever et al., 1996). Differential accessibility to populations of δ-opioid receptors is a consideration that may contribute to the subtype pharmacology observed in vivo for various ligands (Zaki et al., 1996). The enhanced brain penetration of naltriben with respect to (*E*)-7-benzylidenenaltrexone is probably due to the higher lipophilicity expected for naltriben. Using the ClogP computer program (Biobyte Software) that calculates log P values from input of a chemical structure model (Leo, 1993), we estimate a log P value for naltriben that is 0.9 units higher than that for (*E*)-7-benzylidenenaltrexone. Adjusting for the delivery factor suggests a more modest 2.4- to 3.2-fold higher innate in vivo potency of naltriben over (*E*)-7-benzylidenenaltrexone for inhibition of specific [^{3}H]naltriben binding. Considering the low mass (≤ 1.5 nmol/kg) of [^{3}H]naltriben used in the present studies, and the higher inhibitory potency of naltriben as compared to (*E*)-7-benzylidenenaltrexone, it is quite possible that the δ-opioid receptors labeled in vivo by [^{3}H]naltriben correspond to those defined as the δ_2-opioid receptor subtype in functional studies. Although the findings are consistent with selective labeling of the putative δ_2-opioid receptor subtype in vivo by [^{3}H]naltriben, the evidence is not compelling. In this regard, note that naltriben and (*E*)-7-benzylidenenaltrexone are both more potent in vivo against [^{3}H]naltriben than they are against [^{3}H](*E*)-7-benzylidenenaltrexone (Lever et al., 1996). The in vivo binding data obtained so far with [^{3}H]naltriben and [^{3}H](*E*)-7-benzylidenenaltrexone in CD1 and CXBK mouse brain could represent simply the different binding potentials of the two radiotracers for a single δ-opioid receptor recognition site.

The in vitro binding studies reported to date do not help clarify the nature of the δ-opioid receptors labeled in vivo by [^{3}H]naltriben, or support the δ-opioid receptor subtype selectivity clearly shown by naltriben in numerous prior functional paradigms. Assays that may be taken as representative of selective in vitro binding to δ_1- or δ_2-opioid receptors have been reported in mouse brain homogenates using ^{3}H-labeled versions of the prototypical peptide agonists that have been employed for pharmacological definition of the putative subtypes (Sofuoglu et al., 1992; Chakrabarti et al., 1993; Búzás et al., 1994). Naltriben

exhibited high, and nearly equal, apparent affinities (K_i values = 0.4–0.6 nM) for all of the agonist binding sites despite their potential assignments as δ_1- or δ_2-opioid receptors (Sofuoglu et al., 1992; Búzás et al., 1994). Moreover, naltriben is at least 20-fold more potent than (*E*)-7-benzylidenenaltrexone when tested against [^{3}H]-labeled agonists selective for either δ_1- or δ_2-opioid binding sites using either whole brain or striatal membranes (Búzás et al., 1994). Homogenate assays may not be conclusive, since the integrity of ligand binding to the putative subtypes seems better retained in tissue slices, a medium that more closely resembles the in vivo milieu (Chakrabarti et al., 1993). Finally, limited data is available regarding relative densities of δ_1- and δ_2-opioid binding sites in discrete brain regions. Their populations seem nearly identical in striatal slices from Swiss–Webster mice (Chakrabarti et al., 1993), while δ_2-opioid binding sites may predominate by about 1.4-fold in membranes prepared from whole mouse brain minus cerebellum (Sofuoglu et al., 1992). Thus, correlations of in vitro data with in vivo distributions are not likely to aid in δ-opioid receptor subtype discrimination.

In conclusion, the present work shows that [^{3}H]naltriben is a useful radioligand for selective regional localization of murine cerebral δ-opioid receptors in vivo. Definitive assignment of radioligand binding to the putative δ_2-opioid receptor subtype, however, is not possible. Such delineation would be facilitated by the identification of a wider range of ligands having δ-opioid receptor subtype selectivity, and by a knowledge of the detailed in vitro binding properties of [^{3}H]naltriben and [^{3}H](*E*)-7-benzylidenenaltrexone in brain tissue homogenates and sections. Of note, [^{3}H]naltriben exhibits a greater degree of contrast between regions known to be rich and those known to be poor in δ-opioid binding sites than either [^{11}C]MeNTI or [^{3}H](*E*)-7-benzylidenenaltrexone (cf. Fig. 3B,C). For illustration, consider striatal minus cerebellar radioactivity 60 min after i.v. administration as a measure of specific binding for the three radioligands in CD1 mouse brain. The value for [^{3}H]naltriben (4.9% injected dose per gram) proved 3-fold higher than for [^{11}C]MeNTI (1.6% injected dose per gram; Lever et al., 1992), and 17-fold higher than for [^{3}H](*E*)-7-benzylidenenaltrexone (0.28% injected dose per gram; Lever et al., 1996). Striatal specific binding represented 77% of total uptake at 60 min, similar to the value reported for [^{11}C]MeNTI (79%; Lever et al., 1992) and much greater than the value determined for [^{3}H](*E*)-7-benzylidenenaltrexone (26%; Lever et al., 1996). Since [^{11}C]MeNTI allows localization of δ-opioid receptors in human brain by positron emission tomography (Madar et al., 1996, 1997), the present work suggests that a stronger ‘δ signal’ and improved image quality would be possible with appropriately radiolabeled naltriben analogues. In fact, a recent abstract described a synthesis, albeit in low yield, of [^{11}C]naltriben (Sajjad et al., 1995).

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