

IN VIVO LABELING OF DELTA OPIOID RECEPTORS IN MOUSE BRAIN BY
[³H]BENZYLIDENENALTREXONE, A LIGAND SELECTIVE FOR THE DELTA₁ SUBTYPE

Abstract. (E)-7-Benzylidenenaltrexone (BNTX) is a selective ligand for the putative delta₁ (δ₁) opioid receptor. To explore the feasibility of labeling δ₁ sites *in vivo*, we determined the cerebral distribution of radioactivity after systemic administration of [³H]BNTX to CD1 mice. Uptake was highest in striatum and lowest in cerebellum throughout the 4 hr time course. Specific radioligand binding, approximated as the difference in radioactivity concentrations between striatum and cerebellum, peaked at 0.32 percent injected dose/g at 30 min and comprised a modest 23% of total striatal radioactivity. For seven brain regions, radioactivity concentrations correlated with δ site densities known from prior *in vitro* studies ($r_s = 0.79$, $p = 0.03$), and also with the uptake of N1'-([¹¹C]methyl)naltrindole *in vivo* ($r_s = 0.78$, $p = 0.04$) in mice. Specific binding in striatum, olfactory tubercles and cortical regions was saturable by BNTX, and was inhibited stereoselectively by the optical isomers of naloxone. Naltrindole and naltriben (NTB), δ antagonists, blocked 65 -99% of [³H]BNTX specific binding at a dosage of 5.0 μmol/kg. Similar doses of the μ antagonist cyprodime, or the κ agonist U50,488H, did not inhibit binding. Adjusted for the four-fold greater brain penetration of NTB relative to BNTX, dose-response studies suggested that δ₁ selective BNTX (ED₅₀ = 1.51 μmol/kg) was 50% more potent than δ₂ selective NTB (ED₅₀ = 0.56 μmol/kg) in blocking specific [³H]BNTX binding in striatum. In CXBK mice, a strain with functional δ₁ but not δ₂ receptors in antinociceptive assays, radioligand uptake and distribution proved similar to that in CD1 mice. In sum, [³H]BNTX labels murine δ opioid receptors *in vivo* with a low extent of specific binding.

Key Words: delta opioid receptors, benzyldenaltrexone, naltriben

Introduction

Delta (δ) opioid receptors are known to mediate both spinal and supraspinal analgesia, and δ receptor agonists show promise as clinical agents having lower abuse liability and fewer untoward side effects than μ selective agonists such as morphine (1). Further, δ receptor antagonists have been suggested as drugs that might block the tolerance and dependence associated with morphine-like analgesics without loss of antinociception (2). Recent studies have implicated δ receptors in the neurobiology of several other drugs of abuse including amphetamine (3), methamphetamine (4), cocaine (4-6), ethanol (7) and heroin (8). Thus, there is much interest in the development of radioligands for functional assessment of δ receptors (9). For example, we have radiolabeled N1'-methylnaltrindole (MeNTI), a potent δ receptor antagonist (10), with the positron emitting radionuclide carbon-11 (11). [¹¹C]MeNTI labels δ sites *in vivo* in mouse brain (12) and allows positron emission tomographic (PET) studies of δ opioid receptors in human brain (13).

Antinociceptive assays *in vivo*, as well as various biochemical and radioligand binding studies *in vitro*, have provided strong evidence for δ opioid receptor heterogeneity as reviewed by Traynor and Elliott (14). In particular, two semi-synthetic alkaloids, (E)-7-benzylidenenaltrexone (BNTX) and

naltriben (NTB), have been identified as selective δ antagonists that allow differentiation of the subtypes classified as δ_1 and δ_2 , respectively, during antinociceptive tests in mice (14,15). A recent application of this technique suggests that heroin acts as an agonist on supraspinal δ_1 sites while 6-monoacetylmorphine acts on δ_2 sites (8). These elegant studies highlight the potential utility of complementary *in vivo* radioligand binding protocols that would allow additional insight into the functional roles played by δ opioid receptor subtypes. In the present work, we have investigated the feasibility of selectively labeling δ_1 sites *in vivo* in mouse brain with [3 H]BNTX. BNTX is known to display high affinity ($K_i = 0.1$ nM) and good selectivity (>100-fold against μ , δ_2 , and κ) for δ_1 sites *in vitro*, and to be centrally active against antinociception induced by δ_1 selective agonists like [D-Pen^{2,5}]enkephalin (DPDPE) following systemic administration to mice (16).

Materials and Methods

Drugs. Naltriben mesylate (NTB), (E)-7-benzylidenenaltrexone hydrochloride (BNTX), (+)-naloxone hydrochloride, [3 H]BNTX (14.35 Ci/mmol) in toluene / ethanol (7/3), and [3 H]NTB (1.3 Ci/mmol) in ethanol were gifts from the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Naltrindole hydrochloride (NTI), (*trans*)-(\pm)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide mesylate (U50,488H), (-)-naloxone hydrochloride and cyprodime hydrobromide were purchased from Research Biochemicals, Inc. (Natick, MA).

In Vivo Pharmacokinetic Assays. The temporal cerebral distribution of [3 H]BNTX was determined in non-fasted male CD1 mice (22 - 30 g; Charles River, Wilmington, MA). [3 H]BNTX (2 - 4 μ Ci; < 5.5 μ g/kg) in 0.9% saline containing 2% ethanol (0.2 mL) was given by tail vein injection. Sets of 4 mice were killed (cervical dislocation) at intervals from 5 min to 4 hr, and immediately decapitated. Whole brain was removed, and prefrontal cortex, parietal cortex, olfactory tubercles, striatum, hypothalamus, hippocampus, thalamus and cerebellum were dissected on an ice-cold glass plate. These tissue samples, as well as the remainder of the brain, were blotted, weighed in glass scintillation vials, digested with Solvable (1 mL; DuPont NEN Research Products, Boston, MA) overnight, diluted with Formula 989 cocktail (10 mL; DuPont NEN), and then assayed by liquid scintillation spectrometry with an automated beta counter (Packard Instrument Co., Downers Grove, IL). Counts were corrected for quenching, and had an error of 3% or less. The percent injected dose/g (%ID/g) tissue was determined by comparison to standard dilutions of the injected dose. In similar fashion, the cerebral distribution of [3 H]BNTX was determined in non-fasted male CXBK mice (24 - 30 g; Jackson Laboratories).

In Vivo Pharmacologic Assays. Blocking studies followed the protocol described above except saline solutions (0.15 mL) of NTB, BNTX, NTI, (-)-naloxone, (+)-naloxone, cyprodime and U50,488H were given intravenously (i.v.) to male CD1 mice 15 min prior to tail vein injection of radioligand (3 μ Ci). All drugs were used at a dosage of 5.0 μ mol/kg, except for the optical isomers of naloxone that were used at 15.0 μ mol/kg. Saline formulations of NTB included 2% ethanol. Sets of 3 to 5 drug or saline treated control animals were killed 1 hr after [3 H]BNTX administration, and samples of prefrontal cortex, parietal cortex, olfactory tubercles, striatum and cerebellum were dissected and processed as described above. Pharmacologic studies were treated by analysis of variance (ANOVA) using SuperANOVA software (Abacus Concepts). A *post hoc* Dunnett's test was used for comparing control to treatment groups at the $\alpha = 0.05$ significance level. Dose-response data for inhibition of [3 H]BNTX were obtained by subcutaneous (*s.c.*) treatment of groups of 3 - 4 mice with solutions (0.2 mL) of either NTB or BNTX (0.01 - 5.0 μ mol/kg) in saline containing 2% ethanol 30 min prior to i.v. administration of radioligand (3 μ Ci). Analysis of brain tissue radioactivity was conducted 30 min after [3 H]BNTX injection. Log-logit transformation followed by linear regression was used to analyze the data. For determination of NTB and BNTX brain penetration, a sample of [3 H]BNTX was diluted with carrier to match the specific activity of the [3 H]NTB (1.3 Ci/mmol). Subsequently, groups of 4 male CD1 mice (22.5 \pm 4.0 g) were given 2.0

μCi ($0.07 \mu\text{mol/kg}$) of either [^3H]BNTX or [^3H]NTB in saline containing 2% ethanol (0.2 mL) *s.c.* Animals were sacrificed 1 hr after dosing. Brains were removed, weighed and placed in glass vials. Tissue samples were digested, diluted with cocktail, assayed for radioactivity and the %ID in the whole brain was determined as described above.

Results and Discussion

Selected data regarding the temporal distribution of radioactivity in various tissues from CD1 mouse brain after *i.v.* administration of [^3H]BNTX are shown in FIGS. 1 and 2. Throughout the time course, the highest radioactivity concentrations were in brain areas known to have high densities of δ sites; *viz.*, striatum, olfactory tubercles, parietal cortex and prefrontal cortex (17). Intermediate levels of uptake and retention were observed for hippocampus, hypothalamus and thalamus. The lowest levels of radioactivity were consistently noted for cerebellum, a region with few opioid receptors (17,18). Clearance from the cerebellum and striatum followed single exponentials with half-times of 59 min ($r = 0.99$) and 75 min ($r = 1.0$) respectively. Since the density of δ sites in mouse cerebellum is only 4% of that in striatum or cortex (18), specific radioligand binding can be estimated as tissue minus cerebellum radioactivity concentrations. As shown in FIG. 1, striatal specific binding increased to a peak value of 0.32 %ED/g at 30 min, and then fell in linear fashion ($r = 0.99$) with a half-time of 130 min. Over the 30 min to 4 hr time period, specific binding represented a modest 23 - 33% of the total amount of radioactivity in the striatum.

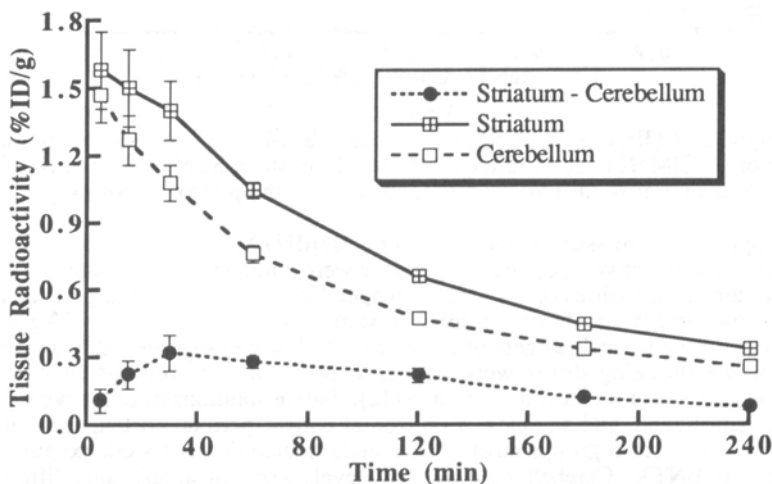


FIG. 1

Cerebral radioactivity distribution after *i.v.* administration of [^3H]BNTX to mice. Values are means \pm SEM, $n = 4 - 7$. Non-visible SEM are within the symbol.

The differential distribution of radioactivity is in accord with δ site densities known for discrete regions of mouse brain from autoradiographic studies with [^{125}I][D-Ala 2]deltorphin-I, a δ selective opioid peptide (17). Comparison of the uptake *in vivo* of [^3H]BNTX with the total binding *in vitro* of the peptide gave good linear ($r = 0.84$) and Spearman rank order ($r_s = 0.79$, $p = 0.03$) correlation for the seven brain regions that were directly comparable between the studies (data not shown). By contrast, no correlation ($r_s = 0.43$, $p = 0.34$) was found with μ site densities determined with [^{125}I]FK33,824 in mouse brain (17). As shown in FIG. 2, the *in vivo* uptake of [^3H]BNTX also displays robust linear ($r = 0.91$, $p = 0.002$) and Spearman rank order ($r_s = 0.78$, $p = 0.04$) correlation with the *in vivo* uptake of [^{11}C]MeNTI in mice that we had previously reported (12). The relative regional distribution of δ_1 as compared to δ_2 sites is not known throughout the mouse brain.

Interestingly, the present study indicates that [³H]BNTX and an analog of NTI display a similar gross anatomical distribution regardless of the particular δ receptor subtype(s) that might be labeled *in vivo*.

Similar results were obtained using male CXBK mice (data not shown, *cf.* FIGS. 1 and 2). This strain was selected for comparison because both δ_1 and δ_2 agonists induce supraspinal antinociception in CD1 mice, while only δ_1 agonists are effective in CXBK mice (19). This suggests that supraspinal δ_1 sites predominate over δ_2 sites in CXBK mice, or that supraspinal δ_1 sites are the only subtype functionally involved in antinociception. Also, CXBK mice are deficient in μ sites. Since the pharmacokinetics, regional distribution and specific binding of [³H]BNTX are nearly identical in CD1 and CXBK strains, we infer that [³H]BNTX has the potential for selective labeling of δ_1 sites *in vivo*.

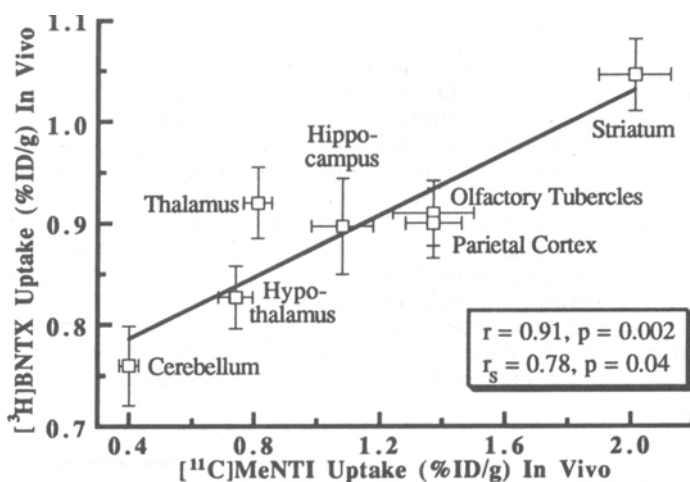


FIG. 2

Total uptake of [³H]BNTX at 60 min (means \pm SEM, n = 7) compared to total uptake of [¹¹C]MeNTI at 60 min (reference 12; means \pm SEM, n = 4) *in vivo* in mice. Cortex refers to the parietal area, and excludes the prefrontal cortex.

Pharmacologic studies to assess the selectivity of [³H]BNTX were conducted in the CD1 strain to ensure that functionally active μ , δ_1 and δ_2 receptors were competing for radioligand *in vivo*. The effect of pretreatment with various opioid drugs on specific [³H]BNTX binding in selected mouse brain tissues is shown in FIG. 3. For the inhibition studies, a dosage of 5.0 μ mol/kg was used for all blockers except for the optical isomers of naloxone which were employed at 15.0 μ mol/kg. The dosage levels of the blocking drugs were based, in part, on our previous *in vivo* studies of [¹¹C]MeNTI binding to 5 opioid receptors in mice (12). Saline solutions of drugs were given *i.v.* 15 min prior to the radioligand, and samples of prefrontal cortex, parietal cortex, striatum, olfactory tubercles and cerebellum from groups of drug or vehicle treated animals were examined 1 hr after administration of [³H]BNTX. Cerebellar radioactivity levels were not significantly different between controls and any of the treatment groups at the $\alpha = 0.05$ level (ANOVA, *post hoc* Dunnett's test). Thus, specific binding was approximated as the difference between radioactivity concentrations in the

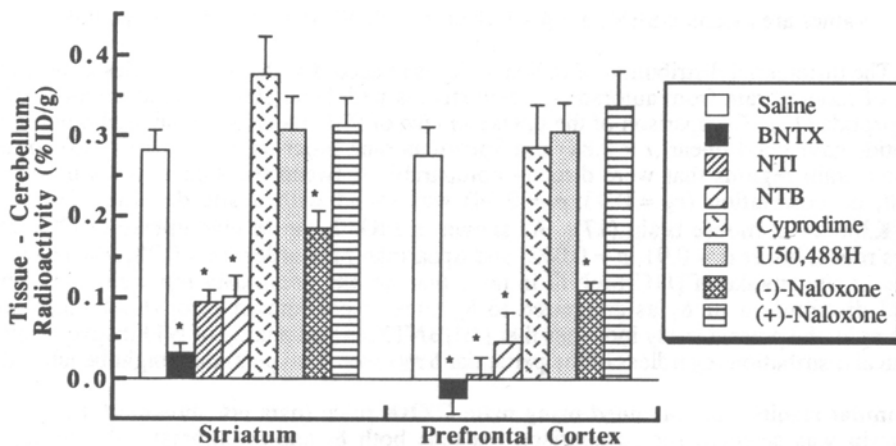


FIG. 3

Effect of drug pretreatments on specific [^3H]BNTX binding in mouse brain tissues 1 hr after *i.v.* administration of radioligand. Blockers were given *i.v.* 15 min prior to [^3H]BNTX at a dosage of 5.0 $\mu\text{mol/kg}$ except for the optical isomers of naloxone (15.0 $\mu\text{mol/kg}$). Values are means \pm SEM, $n = 3 - 8$. * Different from controls at the 0.05 significance level (ANOVA, *post hoc* Dunnett's test).

cerebellum and the various other tissues. For all of the regions, specific binding was saturable by non-radioactive BNTX, and was inhibited stereoselectively by the optical isomers of naloxone. The δ antagonists, NTI and NTB, significantly blocked 65 - 99% of [^3H]BNTX specific binding (*cf.* FIG. 3). Similar doses of the μ antagonist cyprodime, or the κ agonist U50,488H, did not inhibit specific radioligand binding in any region (*cf.* FIG. 3). The blocking studies are consistent with selective *in vivo* labeling of δ opioid receptors in mouse brain by [^3H]BNTX. BNTX (δ_1) proved more effective as an inhibitor than equivalent *i.v.* doses of either NTI (δ_1, δ_2) or NTB (δ_2). This is suggestive of δ_1 selectivity for [^3H]BNTX binding *in vivo*, although blockade of saturable but non-specific binding sites by cold BNTX might also play a role. Further, cross-reactivity of BNTX, NTI and NTB with both δ_1 and δ_2 opioid receptors is likely at the 5.0 $\mu\text{mol/kg}$ dosage level (15).

In an effort to determine which subtype(s) of δ opioid receptors are labeled by [^3H]BNTX *in vivo* in mouse brain, we conducted dose-response inhibition studies using BNTX as a selective δ_1 antagonist and NTB as a selective δ_2 antagonist. Previous studies of the involvement of δ receptor subtypes in supraspinal antinociception by enkephalins in mice established that NTB and BNTX exert maximal effects 30 min after *s.c.* administration, and that 1.2 - 1.3 $\mu\text{mol/kg}$ dosages can be used without loss of selectivity or agonist effects (15). We gave the blockers in doses up to 5.0 $\mu\text{mol/kg}$ *s.c.* 30 min prior to radioligand, and examined striatal tissue after an additional 30 min (FIG. 4).

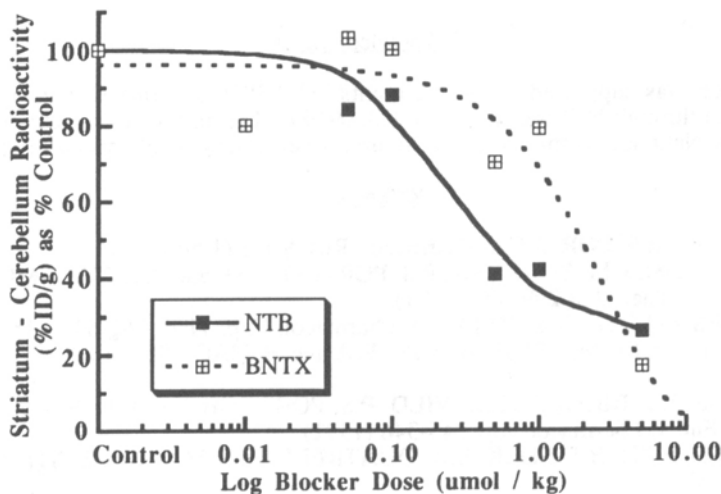


FIG. 4

Effect of NTB and BNTX dosage on specific [^3H]BNTX binding as percent of control in mouse striatum 30 min after i.v. administration of radioligand (means, $n = 3 - 7$). Blockers were given *s.c.* 30 min prior to the [^3H]BNTX injection.

Striatum was selected for analysis because *in vitro* densities of δ_1 and δ_2 subtypes are nearly identical (6.6 - 7.8 $\mu\text{mol}/\text{mg}$ tissue) in striatal slices from Swiss-Webster mice (20). At the highest *s.c.* dosage, BNTX was slightly more effective than NTB as noted upon i.v. administration (FIG. 3). However, linear transformation of the data gave ED_{50} estimates of 0.56 $\mu\text{mol}/\text{kg}$ for NTB and 1.51 $\mu\text{mol}/\text{kg}$ for BNTX. The ED_{50} for BNTX inhibition of [^3H]BNTX binding agrees well with the value (1.34 $\mu\text{mol}/\text{kg}$, *s.c.*) reported for antagonism of antinociception induced by the δ_1 selective agonist DPDPE in mice (16). By contrast, the ED_{50} (0.56 $\mu\text{mol}/\text{kg}$, *s.c.*) for NTB blockade of [^3H]BNTX binding falls below that (1.2 $\mu\text{mol}/\text{kg}$, *s.c.*) associated with δ_1 cross-reactivity during antinociceptive assays in mice (15). Our ED_{50} would be consistent with labeling of δ_1 sites by [^3H]BNTX if the fractional occupancy required for NTB to block half of the radioligand binding to striatal δ_1 sites is less than that required for overall antagonism of the supraspinal analgesia induced by δ_1 selective agonists. Alternatively, noise in our data (*vide infra*) may not allow accurate measurement of the ED_{50} for NTB.

Since the efficacy of NTB and BNTX depend, in part, upon the relative degree of brain penetration, we investigated this parameter explicitly. Using [^3H]BNTX and [^3H]NTB of the same specific radioactivity (1.3 Ci/mmol), we found that a 1.2 $\mu\text{mol}/\text{kg}$ dosage *s.c.* of NTB gave 1.03 ± 0.04 %ID to whole brain after 1 hr, while the same dosage of BNTX gave only 0.26 ± 0.01 %ID (means \pm SEM, $n = 4$). Thus, NTB exhibits four-fold higher brain penetration than BNTX. Taking this into account, the dose-response studies (FIG. 4) suggest that BNTX is approximately 50% more potent than NTB in blocking striatal specific binding of [^3H]BNTX. This interpretation must be viewed with caution since the midpoint of the 95% confidence interval (0.37 - 2.65 $\mu\text{mol}/\text{kg}$) for the ED_{50} of NTB is equivalent to the 1.51 $\mu\text{mol}/\text{kg}$ estimate obtained for the ED_{50} of BNTX. Although the dose-response studies are not conclusive, the weight of all the available evidence favors selective *in vivo* labeling of the δ_1 opioid receptor subtype in mouse brain by [^3H]BNTX.

In summary, [^3H]BNTX labels murine δ opioid receptors *in vivo*, although selectivity for the δ_1 subtype was not conclusively demonstrated. The radioligand should prove useful for certain *in vivo* studies of δ receptors in mice despite a level of specific binding that comprises only 23 - 33% of total binding. This may be partly a consequence of metabolism. On average, 60% of total radioactivity was extractable (two methanol treatments) from whole mouse brain homogenates 30 min after i.v.

administration of [³H]BNTX (70 μCi, n = 2; data not shown). Of this, only 62% was identified as native radioligand by high performance liquid chromatography (HPLC). The [³H]BNTX injected originally had 95% radiochemical purity by HPLC. Thus, development of δ₁ selective radioligands with enhanced metabolic stability might facilitate future studies of this opioid receptor subtype *in vivo*.

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