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Alkylation with β-funaltrexamine suggests differences between μ-opioid receptor systems in guinea-pig brain and myenteric-plexus

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1 The effects of pre-incubation with β-funaltrexamine (β-FNA) on the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]-DAMGO) to homogenates of guinea-pig brain and myenteric-plexus longitudinal muscle have been studied.

2 β-FNA pretreatment of brain homogenates in Tris-HCl buffer reduced the amount of [^{3}H]-DAMGO binding. This was principally due to a reduction in the maximal number of binding sites measurable. However, approximately 30% of sites labelled by 1 nM [^{3}H]-DAMGO were insensitive to 1 μM β-FNA. Similar findings were obtained when the alkylation was performed in brain homogenates prepared in Krebs solution buffered with HEPES.

3 β-FNA pretreatment of whole myenteric-plexus longitudinal muscle strips caused an increase in the IC_{50} values of μ-agonists, but not of κ-agonists. However, the binding of [^{3}H]-DAMGO to homogenates of myenteric-plexus longitudinal muscle was not altered by pre-incubation with β-FNA in Tris-HCl buffer. On the other hand when the pretreatment was carried out in whole tissue in Krebs solution, or in homogenates in the presence of NaCl and Gpp(NH)p, a marked reduction in [^{3}H]-DAMGO binding was observed.

4 These results suggest that a low affinity form of the μ-opioid receptor is the physiologically relevant site for β-FNA alkylation in the myenteric-plexus and that differences exist between μ-receptor systems in guinea-pig myenteric plexus and brain.

Introduction

β-Funaltrexamine (β-FNA) is an irreversible antagonist at μ-opioid receptors (Portoghese *et al.*, 1980). It is effective in preventing μ-receptor activation by opioids in both *in vitro* bioassay systems (Ward *et al.*, 1982a; 1986; Hayes *et al.*, 1985; Corbett *et al.*, 1985) and *in vivo* (Ward *et al.*, 1982b; Hayes *et al.*, 1986; Takemori & Portoghese, 1987). Ligand binding studies in brain homogenates suggest that this action is due to an irreversible reduction in the number of opioid binding sites (Rothman *et al.*, 1983; Ward *et al.*, 1985; Tam & Liu-Chen, 1986), possibly due to Michael addition with a thiol function close to the ligand-recognition site (Tam & Liu-Chen, 1986).

In homogenates of myenteric-plexus of the guinea-pig ileum, however, pre-incubation with β-FNA apparently does not lead to a reduction in the level of μ-opioid ligand binding. The loss of potency of μ-ligands in intact myenteric-plexus longitudinal muscle preparations following β-FNA treatment has therefore been attributed to an interference with the receptor-effector coupling system in this tissue, rather than a direct blockade of binding sites (Corbett *et al.*, 1985).

To understand this apparent anomaly, we have investigated the action of β-FNA at μ-opioid binding sites, in homogenates of guinea-pig brain and myenteric-plexus longitudinal muscle, as defined by the agonist [D-Ala^2,MePhe^{4}Gly-ol^5]enkephalin (DAMGO). We show that alkylation in the myenteric-plexus longitudinal muscle homogenates depends upon the incubation conditions, which may govern the presence of different affinity states of the μ-opioid receptor.

A preliminary account of this work has appeared (Franklin & Traynor, 1988).

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) (David Hall, Burton-on-Trent) were killed by cervical dislocation. Brains (without cerebellum) and segments of ileum were removed and placed in appropriate buffer solutions, either Tris-HCl (pH 7.4, 50 mM) or Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11 without (Krebs) or with 25 mM HEPES (pH 7.4; Krebs-HEPES).

Binding assays

Homogenates of tissues, either strips of myenteric plexus longitudinal muscle from the ileum or whole brain (without cerebellum), were prepared in Tris buffer (pH 7.4, 50 mM), or Krebs-HEPES, essentially as described by Kosterlitz and colleagues (Gillan *et al.*, 1980; Corbett *et al.*, 1985). Homogenates (20 mg tissue ml^{-1}) were incubated with or without β-FNA (1 μM unless otherwise stated) for 60 min at 37°C in Tris-buffer or Tris-buffer containing NaCl (100 mM) and the guanosine triphosphate (GTP) analogue Gpp(NH)p (50 μM) or Krebs-HEPES. Both control and treated homogenates were washed four times in Tris-HCl buffer and finally resuspended to give a final tissue concentration of 20 mg ml^{-1}.

In a second set of experiments 2 cm strips of whole myenteric plexus longitudinal muscle preparation were treated with β-FNA (1 μM) for 60 min at 37°C in Krebs-HEPES or Tris-HCl buffer. The tissues were then washed for 60 min, homogenized, and resuspended in Tris-buffer at a tissue concentration of 20 mg ml^{-1}.

Binding assays on the above homogenates were performed; [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO) was used to label μ-sites, at 25°C for 40 min in Tris-HCl buffer (pH 7.4 50 mM) (Handa *et al.*, 1981). Specific binding was defined by use of 10 μM naloxone. To label μ-sites [^{3}H]-DAMGO (1 nM) was used in guinea-pig brain homogenates and [^{3}H]-DAMGO (4 nM) used in homogenates of myenteric plexus-longitudinal muscle. This higher concentration was employed to provide optimum specific binding and also because the affinity of [^{3}H]-DAMGO for μ-opioid binding sites in the myenteric plexus is lower than in brain tissue (Corbett *et al.*, 1985). Assuming affinities of [^{3}H]-DAMGO

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for the μ-site in myenteric plexus of 2.27 nM (Corbett *et al.*, 1985) and for the δ- and κ-sites of 407 nM (Cotton *et al.*, 1985) and 4960 (Kosterlitz *et al.*, 1981) respectively and a μ : δ : κ binding site ratio of 25% : 26% : 49% (Corbett *et al.*, 1985), then 97% of the [^{3}H]-DAMGO specifically bound can be calculated to be to μ-sites. A similar calculation for binding in guinea-pig brain homogenates, using an affinity of [^{3}H]-DAMGO for the μ-opioid site of 0.96 nM and a binding site ratio of μ (24): δ (32): κ (44) (Robson *et al.*, 1985), indicates that >99% of the specifically bound [^{3}H]-DAMGO is to μ-sites. IC_{50} values for the displacement of [^{3}H]-DAMGO were determined from Hill Plots of the data and converted to K_i values by the Cheng & Prussoff (1973) equation. Equilibrium dissociation constants (K_D) and binding capacities (B_{max}) were determined from Scatchard (1949) plots of binding isotherms using the programme EBDA (McPherson, 1985).

Bioassays

The myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum was set up in Krebs solution for field stimulation as previously described (Traynor *et al.*, 1987). Tissues were pre-incubated with β-FNA (100 nM) for 60 min and then washed with drug-free Krebs solution for 60 min. The potencies of agonists were obtained from cumulative dose-response curves and dose-ratios were calculated as IC_{50} after β-FNA/IC_{50} before β-FNA.

The equilibrium dissociation constant (K_e) of the κ-antagonist, norbinaltorphimine, was determined against morphine by the single-dose method (Kosterlitz & Watt, 1968). Norbinaltorphimine (0.3 nM) was allowed to equilibrate for 45 min.

Chemicals and drugs

[^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO) (60 Ci mmol^{-1}) was purchased from Amersham International plc and cold DAMGO from Sigma. The drugs used were gifts, as follows: fentanyl citrate (Janssen), methadone hydrochloride (Wellcome), β-funaltrexamine (β-FNA, Glaxo), morphine hydrochloride (Macfarlan Smith), naloxone hydrochloride (Endo Laboratories) and U-50,488H (*trans*-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide, Upjohn Company). 5'-Guanylylimidodiphosphate (Gpp(NH)p) was purchased from Sigma. All other reagents and chemicals were of analytical grade.

Results

Binding of [^{3}H]-DAMGO to homogenates of guinea-pig brain pretreated with β-funaltrexamine

Pre-incubation of homogenates of guinea-pig brain for 60 min, at 37°C in Tris-HCl buffer with different concentrations of β-FNA led to a reduction of specific [^{3}H]-DAMGO binding (Figure 1). Approximately half of the specifically bound [^{3}H]-DAMGO was sensitive to 100 nM β-FNA. The remaining binding sites for [^{3}H]-DAMGO were more resistant and 31.5 ± 4.9% (n = 3) of the specific binding of [^{3}H]-DAMGO was retained, even after pretreatment with 1 μM β-FNA. Use of Krebs-HEPES, rather than Tris-HCl, during the pre-incubation of homogenates with β-FNA did not significantly increase the degree of inactivation afforded (Figure 1).

Analysis of saturation binding isotherms of [^{3}H]-DAMGO binding to both untreated and β-FNA-treated homogenates revealed a single population of saturable high affinity sites (Table 1). The maximum capacity of [^{3}H]-DAMGO binding sites (B_{max}) in the homogenates after pre-incubation with β-FNA (1 μM) for 60 min at 37°C was one-third of that observed in control homogenates. The equilibrium dissociation constant (K_D) of [^{3}H]-DAMGO for these binding sites was not significantly changed in treated homogenates compared with control homogenates.

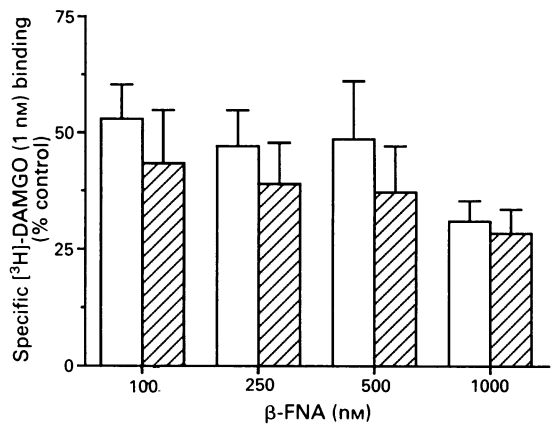

Figure 1 The effects of pre-incubation for 60 min at 37°C with different concentrations of β-funaltrexamine (β-FNA) in Tris-HCl (open columns) or Krebs-HEPES (hatched columns) on the specific binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 1 nM) in homogenates of guinea-pig brain. Each column is the mean of three experiments. Vertical bars represent s.e.mean. All values are significantly different from controls treated in the absence of β-FNA ($P < 0.01$, Student's t test).

The competitive displacement of specific [^{3}H]-DAMGO (1 nM) binding by various μ-opioid ligands was similar in both control homogenates and homogenates pretreated with β-FNA (1 μM) for 60 min at 37°C (Table 2), even though specific binding in the latter homogenates was only 40% of that seen in control homogenates.

Table 1 The effects of pre-incubation with β-funaltrexamine (β-FNA) 1 μM for 60 min on the binding characteristics of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin in homogenates of guinea-pig brain

Pre-incubation	*Binding capacity* (fmol mg^{-1} protein)	*Dissociation constant* (K_D, nM)
Control (Tris-HCl)	96.8 ± 26.8	4.5 ± 0.3
β-FNA	31.5 ± 6.4*	6.8 ± 0.7

The values are means ± s.e.mean from three experiments. Homogenates were incubated for 60 min in the absence or presence of 1 μM β-FNA then washed as described in the methods.
* Significantly different from control $P < 0.01$ (Student's t test).

Table 2 The effects of pre-incubation with β-funaltrexamine (β-FNA) 1 μM for 60 min on the inhibitory effects of opioids on the binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 1 nM) in homogenates of guinea-pig brain

	K_i (nM)	
Competing ligand	*Control*	*After β-FNA*
DAMGO	2.1 ± 0.2	3.3 ± 0.5
Fentanyl	2.3 ± 0.4	2.5 ± 0.4
Morphine	6.3 ± 0.8	11.8 ± 2.5
Naloxone	2.7 ± 0.8	3.2 ± 1.3

The values are means ± s.e.mean from three experiments. Homogenates were incubated in the absence or presence of 1 μM β-FNA for 60 min, then washed as described in the methods.

Table 3 The effects of pre-incubation with β-funaltrexamine (β-FNA) 100 nM on the agonist potencies of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig

	Agonist potency (IC_{50}, nM)		
	Before β-FNA	*After β-FNA*	*Mean dose-ratio*
DAMGO	7.5 ± 0.7	359 ± 86.0*	39.7 ± 15.0
Methadone	23.6 ± 1.7	616 ± 58.7*	26.7 ± 3.4
Morphine	108 ± 16.4	577 ± 24.9*†	7.5 ± 0.7
U-50488H	1.1 ± 0.2	1.7 ± 0.4	1.6 ± 0.1

The values are means ± s.e.mean from three observations. Tissues were pre-incubated with 100 nM β-FNA for 60 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC_{50} after incubation with β-FNA to the IC_{50} before treatment. † Based on a maximum inhibition of 50%.
* $P < 0.01$ compared to control values (Student's t test).

Agonist action of opioids following treatment of the myenteric plexus-longitudinal muscle with β-funaltrexamine

Following pre-incubation of strips of myenteric plexus-longitudinal muscle with β-FNA for 60 min the potency of the μ-agonists DAMGO, methadone and morphine were markedly attenuated with the shift for DAMGO and methadone being much greater than for morphine. In addition, morphine acted as a partial agonist following β-FNA treatment of the tissue and maximally only 50% inhibition of the electrically-induced contractions was observed. The potency of the κ-agonist U-50488H was unaffected (Table 3).

The agonist action of morphine before β-FNA treatment of the myenteric plexus was antagonized by the κ-antagonist norbinaltorphimine affording a K_e value of 2.04 ± 0.48 nM ($n = 3$). Following β-FNA treatment this shifted to a value of 0.10 ± 0.01 nM ($n = 3$) similar to the K_e for norbinaltorphimine of 0.07 ± 0.01 nM ($n = 3$) determined against the selective κ-agonist U-50488H.

Binding of [^{3}H]-DAMGO to homogenates of myenteric-plexus longitudinal muscle of the guinea-pig ileum following pre-incubation with β-funaltrexamine

In contrast to brain tissue, pre-incubation of homogenates of myenteric-plexus longitudinal muscle, even with an increased concentration (1 μM) of β-FNA for 60 min at 37°C in Tris-HCl buffer, caused no reduction in the level of specifically bound [^{3}H]-DAMGO (4 nM). Values of 7.1 ± 0.7 and 8.8 ± 1.4 fmol mg^{-1} protein before and after treatment respectively were obtained. On the other hand the level of specifically bound [^{3}H]-DAMGO (4 nM) in homogenates treated with 1 μM β-FNA in Tris-buffer containing NaCl (100 mM) and Gpp(NH)p (50 μM) was 3.9 ± 0.9 fmol mg^{-1} protein. This represents a reduction of 56% when compared with the control value of 7.9 ± 1.9 fmol mg^{-1} protein (Figure 2).

A similar loss in the level of specific [^{3}H]-DAMGO (4 nM) binding from 9.7 ± 2.2 to 4.4 ± 1.5 fmol mg^{-1} was seen when whole strips of myenteric plexus-longitudinal muscle, in Krebs-HEPES, were incubated with β-FNA (1 μM) prior to homogenization. (Figure 2). The same effect was seen when the pre-incubation was carried out in Tris-HCl buffer, although the amount of specifically bound tritiated ligand was reduced affording values of 4.5 ± 0.5 and 2.5 ± 0.4 fmol mg^{-1} protein ($n = 3$) at 4 nM [^{3}H]-DAMGO in control and β-FNA treated homogenates respectively, presumably due to damage to the tissue as a result of incubation in the very non-physiological medium.

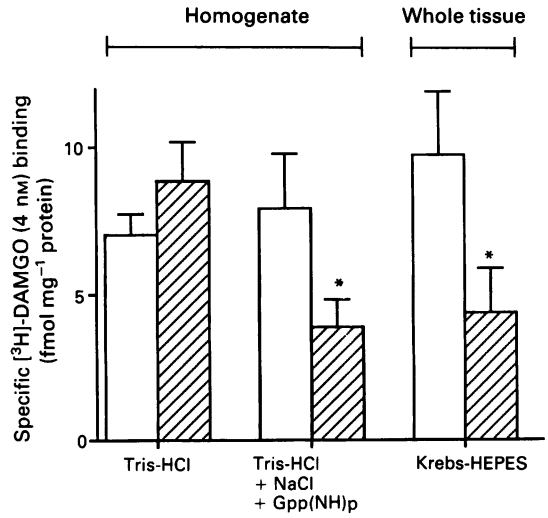

Figure 2 The effects of pre-incubation of guinea-pig myenteric plexus longitudinal muscle without (open columns) or with (hatched columns) 1 μM β-funaltrexamine (β-FNA) for 60 min, on the specific binding of [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin ([^{3}H]-DAMGO, 4 nM). Homogenates of myenteric plexus longitudinal muscle were treated with β-FNA in Tris-HCl (pH 7.4, 50 mM) or in Tris-HCl in the presence of NaCl (100 mM) and Gpp(NH)p (50 μM), then washed as described in the methods. For whole tissue studies 2 cm strips of myenteric plexus-longitudinal muscle were treated with β-FNA as above but in Krebs-HEPES, washed and homogenates prepared as described in the methods. Each column is the mean of three experiments. Vertical bars represent s.e.mean. * $P < 0.01$ vs controls in the absence of β-FNA (Student's t test).

Discussion

The results demonstrate that the binding of [^{3}H]-DAMGO to the μ-opioid binding site in homogenates of myenteric plexus can be irreversibly blocked by pretreatment with β-FNA. The loss of μ-agonist potency of DAMGO, methadone and morphine in the myenteric plexus, following β-FNA treatment is therefore a result of a reduction in the number of functional μ-receptors. The myenteric plexus contains both μ- and κ-receptors (Chavkin & Goldstein, 1981). The results with the κ-antagonist norbinaltorphimine suggest that the lesser shift seen with the alkaloid morphine is due to an action of this compound at κ-receptors, following β-FNA treatment, as a result of its lesser selectivity (Magnan *et al.*, 1982) and efficacy (Smith & Rance, 1983) compared with DAMGO. This finding is in agreement with the results of Ward *et al.* (1986) who obtained a significant increase in the K_e value for naloxone, measured against morphine, but not when measured against the μ-selective peptide RX 78-3030, following β-FNA treatment. Also the reduced response obtained with morphine suggests that at the κ-receptor in the myenteric plexus this ligand acts as a partial agonist.

However, the alkylation by β-FNA of μ-receptors in the plexus differs from that observed in brain homogenates. In the latter homogenates [^{3}H]-DAMGO binding is reduced if pretreatment with β-FNA is carried out in Tris buffer. This reduction in binding is principally due to a reduction in the maximal binding capacity, as observed by others (Rothman *et al.*, 1983; Ward *et al.*, 1985; Tam & Liu-Chen, 1986). Indeed, the results obtained agree with the findings of Tam & Liu-Chen (1986) and those of Ward *et al.* (1985) that irreversible binding of β-FNA occurs in Tris-buffer. However, these groups do report enhancement of alkylation in the presence of Na^+ ions or Krebs-Ringer, whilst in the present study the difference between alkylation in Tris-buffer and Krebs-HEPES did not reach significance. The reasons for this discrepancy are unknown, but could for example relate to the composition of Krebs solution used, since ions are known to have marked effects on binding properties at opioid receptors (Paterson *et al.*, 1986; Kosterlitz *et al.*, 1987). In contrast to results with

brain tissue, [^{3}H]-DAMGO binding in homogenates of myenteric-plexus longitudinal muscle is sensitive to β-FNA only when Na^+ ions and Gpp(NH)p are present, or the alkylation is performed in whole tissue.

In the myenteric plexus therefore the findings suggest that homogenization in Tris buffer alters the μ-opioid binding site to a form different from that found in intact tissue. The μ-opioid receptor is a G-protein coupled receptor (for reviews see Gilman, 1987; Milligan, 1988). Homogenization in Tris buffers generates a tightly coupled high agonist affinity opioid receptor (Wong *et al.*, 1989). Addition of Na^+ ions and Gpp(NH)p restores the receptor to a form susceptible to alkylation by β-FNA and therefore presumably similar to the form present in intact tissue. Several different agonist affinity states of opioid receptors are believed to exist in equilibrium (Werling *et al.*, 1988) and Na^+ ions and guanine nucleotides are known to shift the opioid receptor to a conformational state which is recognised by agonists with low affinity. However antagonist affinity is not reduced (Pert & Snyder, 1974; Simon *et al.*, 1975; Blume, 1978). The results suggest that a low agonist affinity conformation of the μ-opioid receptor represents a predominant state present in intact myenteric plexus. This would explain why opioid antagonists are better able to protect against β-FNA alkylation in whole tissue than opioid agonists and also perhaps why the agonist equivalent of β-FNA, namely β-fluoxymorphamine, does not act irreversibly (Portoghese & Takemori, 1983). Furthermore the results support the suggestion (Carroll *et al.*, 1988) that the low agonist affinity form represents the physiologically relevant state of the μ-opioid receptor.

The different alkylation observed in brain and myenteric plexus must mean that the μ-opioid receptors in the two tissues differ. Certainly it would appear that the high affinity μ-opioid site in guinea-pig brain, that is the binding of [^{3}H]-DAMGO observed in Tris-buffer, is susceptible to alkylation by β-FNA, or that during incubation in the presence of β-FNA the binding site converts to a lower affinity form susceptible to alkylation, whilst high affinity μ-binding in myenteric plexus under these conditions is not susceptible to alkylation. Additionally, the affinity of [^{3}H]-DAMGO measured in brain and myenteric plexus differs slightly (Corbett *et al.*, 1985) and Takemori and colleagues (1974) have suggested a difference between central and peripheral μ-receptors may exist based on responses with an anileridine affinity label. The observed different interactions of β-FNA in brain and myenteric plexus homogenates may reside in the binding site, the efficiency of coupling between receptor and G-protein, the involvement of different G-proteins or the presence of alternative coupling systems.

Any discussion of β-FNA alkylation must consider those receptors, representing some 30% of the μ-binding site population labelled under the experimental conditions, which are resistant to β-FNA. Such β-FNA-insensitive sites have been observed previously (Rothman *et al.*, 1983; 1987). In the competition experiments described in this paper, μ-receptor ligands do not differentiate β-FNA sensitive and insensitive μ-sites. The insensitive sites would thus appear to be a form of the μ-receptor, although it must be realised that the binding studies were performed in Tris buffer which generates a high agonist affinity state. It is interesting to note that 30% of μ-receptors solubilised from 7315c cells are still sensitive to GTP after 18 h treatment of the cells with pertussis toxin (Frey *et al.*, 1989), which may indicate that part of the G-protein associated with the μ-receptor is in some way protected from ADP-ribosylation by the toxin. It is possible then, as discussed by Corbett and colleagues (1985), that it is the coupling system that interacts irreversibly with β-FNA rather than ligand binding domain and that in myenteric plexus, but not brain, this system is only sensitive in the presence of Na^+ ions and guanine nucleotide. Alkylation of G-protein by agents such as N-ethylmaleimide and pertussis toxin generates a low agonist affinity state of the receptor (Smith & Harden, 1984; Spain & Coscia, 1987; Werling *et al.*, 1988). If this is the case with β-FNA alkylation, then the observed reduction in B_{max} could be due to a large reduction in affinity of the [^{3}H]-DAMGO for the μ-opioid receptor, such that binding cannot be measured in a conventional ligand-binding assay, rather than a direct loss of sites due to alkylation of the ligand binding domain by β-FNA.

In conclusion the data presented show that the observed reduction in μ-ligand potency following β-FNA treatment of myenteric-plexus is due to alkylation of a functional μ-receptor. The susceptible form of the receptor is probably a low agonist affinity state, which predominates under physiological conditions. Furthermore β-FNA does uncover differences between μ-opioid receptor sites in brain and myenteric plexus which may suggest differential coupling systems in the two tissues.

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