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Use of Irreversible Antagonists to Determine the Relative Efficacy of μ Opioids in a Pigeon Drug Discrimination Procedure: Comparison of β-funaltrexamine and Clocinnamox

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List of abbreviations:
β-FNA = β-funaltrexamine
C-CAM = clocinnamox
GTPγS = guanosine-5'-O-(3-[^{35}S]thio)triphosphate
S.E.M. = standard error of the mean

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Abstract

The use of irreversible antagonists to assess opioid efficacy has proven fruitful for classifying opioids on the basis of high or low efficacy, but few studies have provided quantitative estimates of efficacy. The purpose of this study was to use β-funaltrexamine (β-FNA) and clocinnamox (C-CAM) in a drug discrimination procedure to examine the efficacy of fentanyl, morphine, l-methadone, sufentanil and etorphine. In pigeons trained to discriminate 0.12 mg/kg fentanyl from water, dose-effect curves were determined for each opioid alone and following pretreatment with β-FNA and C-CAM. Using quantitative analyses according to an extended model of Black and Leff (1983), apparent efficacy (*tau*) and affinity (K_A) of each opioid was determined, as well as the degree of receptor inactivation (q) produced by each dose of each antagonist. β-FNA and C-CAM produced dose- and time-dependent, rightward shifts in the dose-effect curves of each opioid, and analyses based on dose-ratios and *tau* values suggest a rank order of efficacy of etorphine > sufentanil = l-methadone > fentanyl = morphine. Marked differences in the profiles of antagonism produced by β-FNA and C-CAM were also apparent, as C-CAM, but not β-FNA, produced insurmountable antagonism. The q values for each antagonist were consistent with these data in indicating that C-CAM and β-FNA can inactivate nearly 100% and 75% of the receptor population, respectively. In tests conducted in pigeons chronically treated with morphine, doses of β-FNA that produced parallel, rightward shifts in untreated pigeons flattened the morphine dose-effect curve in morphine-treated pigeons. These results indicate that β-FNA and C-CAM can differentiate opioids with high relative efficacy and yield comparable estimates of efficacy for various opioids. There are, however, limitations in the proportion of the receptor population that can by eliminated by β-FNA.

The intrinsic efficacy of an opioid describes the level of receptor occupancy required to produce an effect of a given magnitude (Kenakin, 1997). A related term, efficacy, is equal to the intrinsic efficacy multiplied by the concentration of receptors in a given tissue (Furchgott, 1966), and thus estimates of opioid efficacy vary with the specific response and tissue being studied. In both *in vivo* and *in vitro* preparations, the higher the relative efficacy of the opioid the smaller the proportion of receptors it needs to occupy to produce a maximal effect (Stephenson, 1956; Furchgott, 1966). Consequently, under conditions in which the receptor pool is limited (i.e., smaller receptor reserve), lower efficacy opioids lose their ability to produce a maximal response before higher efficacy opioids.

A number of *in vitro* strategies have been developed to rank opioids along a continuum of relative efficacy. For example, in tissues and cell preparations, opioids can be differentiated by their ability to stimulate signal transduction mechanisms such as G-proteins and potassium channels, with higher levels of stimulation indicative of higher efficacy (Yu et al., 1997; Selley et al., 1998). *In vivo*, opioid efficacy can be determined on the basis of the effectiveness of the opioid across a range of procedural parameters. For example, increases in nociceptive stimulus intensity produce decreases in the maximal antinociceptive effect produced by low but not high efficacy opioids (e.g., Walker et al., 1993). Similarly, low efficacy opioids typically substitute for the discriminative stimulus effects produced by low but not high training doses of fentanyl or morphine (Picker et al., 1993; Zhang et al., 2000). Although these procedures allow for the categorization of opioids on the basis of high or low efficacy, they do not yield quantitative estimates of efficacy.

Irreversible (i.e., noncompetitive) antagonists, such as β-FNA and C-CAM, afford a sensitive tool to study opioid efficacy, and have been successfully employed in assays of drug discrimination (Holtzman, 1997; Morgan and Picker, 1998), antinociception (Zimmerman et al., 1987; Adams et al., 1990; Comer et al., 1992), and self-administration (Zernig et al., 1997). By binding to the receptor in such a way as to prevent reversible interaction with other ligands, these antagonists decrease the functional receptor reserve of the system. As such, increasing doses of

an irreversible antagonist produce decreases in the opioid's potency followed by decreases in its maximal effect. By definition, inactivation of a given proportion of the opioid receptor population will produce greater alterations in the potency and maximal effectiveness of opioids that produce their effects by occupying a large vs. small proportion of the receptor population. Importantly, analyzing changes in agonist potency and maximal effectiveness produced by an irreversible antagonist can yield quantitative estimates of efficacy, a procedure which allows for direct, statistical comparisons among agonists (Furchgott, 1966; Black and Leff, 1983; Zernig et al., 1996b). These quantitative estimates can take into account changes in the agonist potency, slope, and maximal effect, and thus provide a summary of an extensive data set.

Although β-FNA and C-CAM have been used interchangeably as irreversible μ antagonists, differences in their pharmacology warrant comparison. For example, β-FNA exhibits a short-lived, reversible κ agonist profile, whereas C-CAM appears to be devoid of agonist effects (Ward et al., 1982; Aceto et al., 1989). Additionally, binding studies suggest a limit on the fraction of μ receptors than can be labeled by β-FNA (Liu-Chen and Phillips, 1987; Franklin and Traynor, 1991), although no behavioral studies have corroborated this finding. The purpose of the present study was to use a drug discrimination procedure to examine the relative efficacy of fentanyl, morphine, l-methadone, sufentanil and etorphine using β-FNA and C-CAM. These opioids were chosen for study because they display selectivity for the μ opioid receptor and are generally considered to have high relative efficacy (Yoburn et al., 1995; Selley et al., 1998; Walker et al., 1998; Zhang et al., 2000). By comparing the effects produced by both β-FNA and C-CAM against the same opioids, it was also possible to identify similarities and differences in their profiles of antagonism. To extend the generality of the findings, additional tests with β-FNA and C-CAM were conducted in animals treated chronically with morphine. Previous studies indicate that chronic opioid treatment can decrease the functional receptor reserve (Porreca and Burks, 1983), and thus would be expected to increase the relative potency of irreversible antagonists. Finally, to quantify the degree of receptor inactivation (q) as well as apparent efficacy (*tau*) and affinity (K_A) of the agonists, analytical methods developed by

Furchgott (1966) and extended by Black and Leff (1983) and then Zernig et al. (1996b) were used.

Materials and Methods

Subjects. Eleven experimentally naïve, female, White Carneau pigeons maintained at approximately 80 to 85% of their free-feeding weights (400-500g) were used. Each pigeon was housed individually with free access to grit and water in a colony maintained on a 12-h light-dark cycle.

Apparatus. Eight operant conditioning chambers were used. The two operative response keys in each chamber were 2.5 cm in diameter and located 23 cm from the bottom of the front wall, centered approximately 12 cm apart. Three second access to grain was available through an aperture centered below the keys approximately 8 cm from the floor, and was illuminated by a 7-W bulb when the hopper was raised. The chambers also contained a white light for ambient illumination, an exhaust fan for ventilation, and white noise to mask extraneous sounds. Data were collected with a microcomputer using software and interfacing supplied by MED Associates Inc. (Georgia, VT, USA).

Drug Discrimination Training. After key-pecking was established, food delivery became contingent upon a single response (fixed ratio 1: FR 1). The ratio size required for food delivery was increased over several sessions until an FR20 was in effect. At this time the training dose of fentanyl (0.12 mg/kg for 10 pigeons, 0.10 mg/kg for one pigeon) or (distilled) water was administered before each session. For six pigeons, food delivery was contingent upon responding on the right key after drug administration and on the left key after administration of water. The contingencies were reversed for the other five pigeons. During these initial training sessions, the pretreatment time and session length were 15 min. After discriminative control was established, a multiple trial training procedure was initiated. These sessions were comprised of one or two components, with each component consisting of a 15-min time-out period followed by a 5-min response period. At the beginning of the time-out period, either water or the training

dose of fentanyl was administered. After the time-out period, the house light and key lights were illuminated and responding on the injection-appropriate key was reinforced. A pseudorandom sequence of sessions was designed with the stipulation that a distilled water session never followed a drug session and that the number of water and drug sessions were roughly equivalent over a 1 month period. On days in which two drug sessions were scheduled, pigeons received a sham injection at the beginning of the second time-out period. Training sessions were typically conducted 5 days per week. The training conditions described above remained in effect until: (1) the mean percentage of injection-appropriate responses prior to completion of 20 responses on either lever was $\geq$ 80%; and (2) the mean percentage of responses emitted during the entire session on the injection-appropriate lever was $\geq$ 90%, over 10 consecutive sessions. Once these discrimination criteria were met, substitution tests were conducted.

Drug Discrimination Testing. Test sessions were conducted only if the percentage of injection-appropriate responses before the first reinforcer was greater than 80% on the preceding two training days. In addition, tests were frequently conducted with a 4-component water trial and only pigeons that responded exclusively on the water-appropriate key in each component were used in subsequent tests. During test sessions, the completion of the FR20 on either key resulted in food delivery. Using a cumulative dosing procedure, increasing doses of the test drug were administered at the beginning of each time-out such that the total dose administered increased by 0.25 or 0.5 log units. Testing was generally terminated at doses of each agonist that produced full substitution for the fentanyl stimulus. In tests with β-FNA and C-CAM, the doses of β-FNA or C-CAM were administered 2 h before the first component of the test session. At least 4 days intervened between determination of these dose-effect curves and resumption of training sessions. Additional time course tests were conducted with fentanyl and morphine in which selected doses of β-FNA or C-CAM were administered 50 h before determination of the dose-effect curve. To determine whether β-FNA or C-CAM produced fentanyl-like stimulus effects,

these drugs were administered alone, and test sessions were conducted at 15 min, 2 h, 4 h, 6 h and 24 h. A minimum of 9 days intervened between β-FNA or C-CAM administrations.

Chronic Opioid Treatment. Additional tests with β-FNA or C-CAM were conducted prior to and during chronic treatment with morphine. On Day 1 of these tests, a dose-effect curve for morphine was determined, with a training session following on Day 2. On Days 3-8, training sessions were interrupted, and pigeons were injected with a 56 mg/kg dose of morphine at 8:00 am. On Day 9, a cumulative dose-effect curve for morphine (up to 30 mg/kg in all pigeons) was redetermined, and following this test, all pigeons were injected with 26 mg/kg morphine, thus bringing the total dose of morphine for the day to 56 mg/kg. On Day 10, with training still interrupted, pigeons were injected with 56 mg/kg morphine at 8:00 am. On Day 11, pigeons were injected with a dose of β-FNA or C-CAM 2 h before redetermination of a cumulative dose-effect curve for morphine. After this chronic dosing regimen, at least 2 weeks intervened before training was resumed. For this experiment, morphine was selected both as the toleragen and test drug as previous studies indicate that in pigeons, daily injections of morphine result in the rapid development of tolerance (e.g., Walker et al., 1997).

Data Analysis. The percentage of responses on the fentanyl-appropriate key before delivery of the first reinforcer was calculated for each drug and drug combination. Dose-effect curves were generated from these data by expressing the percentage of responses on the fentanyl-appropriate key as a function of the dose of each drug examined. Under conditions in which fentanyl-appropriate responding reached at least 50%, the dose of each drug required to produce a 50% maximal effect (ED_{50}) was derived mathematically (least-squares method) using log-linear interpolation with at least three doses on the ascending limb of the dose-effect curve. To quantify the degree of antagonism produced by the irreversible antagonists, dose ratios were calculated in which the ED_{50} of each agonist-antagonist combination was divided by the ED_{50} of each agonist alone. The rightward shift in the dose-effect curve was considered to be

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significant if the 95% confidence interval of each agonist-antagonist combination did not overlap with that obtained for each agonist alone. Dose-ratios were calculated only under conditions in which the slope of the dose-effect curve for the agonist-antagonist combination was comparable to the slope obtained for the agonist alone. To assess alterations in slope, a test for parallelism (Tallarida and Murray, 1987) was conducted for every dose-effect curve. As such, dose ratios were not reported if the slope of the dose-effect curve was significantly altered or if the maximal level of fentanyl-appropriate responding was less than 50%.

To calculate in vivo apparent affinity (K_A) and efficacy estimates (*tau*), as well as an estimate of the fraction of receptors still available for interaction with an agonist following blockade with a given dose of an irreversible antagonist (q), dose-effect curves for each agonist alone and in combination with β-FNA or C-CAM were analyzed simultaneously according to the model proposed by Black and Leff (1983) and applied to behavioral data according to the methods described by Zernig et al. (1996b). The Black and Leff (1983) model allows for calculation of these parameters according to the following equation:

$$\mathbf{E = E_m/\{[(10^{(\log(K_A) - \log [A])} + 1)/(q*tau_{control})]^n + 1\} + c}$$

where E is the effect (% fentanyl-appropriate responding), E_m is the maximum attainable response, A is the agonist concentration, n is the slope factor of the transducer function and c is the control level of percent drug-appropriate responding; *tau* is represented as ($q*tau_{control}$). All dose-effect curves obtained with a given agonist were fitted to the above equation using a nonlinear fitting program (Efficalc®, Saria Science Consulting) and the mathematical software package Mathematica (Wolfram Research, Champaign, IL; Wolfram 1991). Variance estimates for a given variable were calculated by holding all other fitted curve parameters constant and allowing the parameter under investigation to vary (constrained 95% confidence intervals; see Zernig et al., 1996b). The derived values determined by the mathematical analysis of Black and Leff (1983) can be used in the following equation,

$$\mathbf{ED_{50,control} = K_A/((2+tau^n)^{1/n} - 1)}$$

to determine the control ED_{50} of an agonist. This "back-calculated" ED_{50} value was then compared to the observed ED_{50} value as a measure of internal consistency.

To obtain calculations of efficacy, affinity, and the fraction of available receptors remaining after irreversible antagonist administration, it is first necessary to supply starting values for these parameters, and these can be derived by simple inspection ("eyeballing") of the family of dose-effect curves (Zernig et al., 1996b). Estimates of affinity, which correspond to the midpoint of the dose-effect curve with a decreased maximal effect, can be problematic if the antagonist fails to produce such alterations in the agonist dose-effect curve, such as with the effects of β-FNA in the present experiment. It was, however, possible to supply accurate affinity estimates for each agonist, as C-CAM produced decreases in the maximal effect of four out of the five agonists.

Response rates were calculated as the mean rate of responding obtained during the entire session, and are expressed as responses per sec. Due to limited availability of the various agonists and the potential for toxicity, testing was generally terminated at doses that produced full substitution for the fentanyl stimulus. Thus, in many cases complete dose-effect curves for the rate-decreasing effects were not be obtained, and calculations of the ED_{50}, as well as dose ratios and efficacy estimates, were not possible.

Drugs. The following drugs were used: morphine sulfate, sufentanil citrate, etorphine HCl, l-methadone HCl, fentanyl citrate, β-funaltrexamine HCl (all provided by the National Institute on Drug Abuse), butorphanol tartrate, nalbuphine HCl, U50,488 methanesulfonate, pentobarbital HCl (all from Sigma Chemical Co., St. Louis, Mo., USA), clocinnamox mesylate (Tocris Cookson, Ellisville, Mo., USA), BW373U86 (generously supplied by Burroughs Wellcome Co., Research Triangle Park, N.C., USA). Doses for all drugs are expressed in terms of the salts. All drugs were dissolved in distilled water and injected intramuscularly (IM) in an injection volume of 0.5-1.0 ml/kg.

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Results

Opioids Administered Alone and Control Drugs. Fig. 1 shows the discriminative stimulus effects of fentanyl, morphine, etorphine, sufentanil and l-methadone in pigeons trained to discriminate 0.12 mg/kg fentanyl from water. All opioids produced dose-dependent increases in fentanyl-appropriate responding, with full substitution (≥ 80% fentanyl-appropriate responding) obtained at doses of 0.003 mg/kg etorphine, 0.01 mg/kg sufentanil, 0.12 mg/kg fentanyl, 1.0 mg/kg l-methadone and 3.0 mg/kg morphine. Also shown in Fig. 1 are the findings that the mixed-action opioid butorphanol produced full substitution for the stimulus effects of fentanyl, whereas the mixed-action opioid nalbuphine and the δ agonist BW373U86 produced intermediate levels (< 70%) of fentanyl-appropriate responding when tested up to doses that eliminated responding. In contrast, the κ opioid U50,488 and barbiturate pentobarbital produced predominantly water-appropriate responding up to doses that eliminated responding. B-FNA (5.0 mg/kg) and C-CAM (0.12 mg/kg) also produced predominantly water-appropriate responding when tested 15 min, 2 h, 4 h, 6h and 24 h after administration (data not shown).

Fentanyl and Morphine. Fig. 2 shows the effects of fentanyl and morphine administered alone and in combination with various doses of β-FNA and C-CAM. β-FNA generally produced dose-dependent, parallel rightward shifts in the dose-effect curves of these agonists, with no alteration in the slope of the curve or the maximal effect. Table 1 shows that the 5.0 mg/kg dose of β-FNA, for example, produced a 6.4- and 5.6-fold rightward shift in the dose-effect curves for fentanyl and morphine, respectively. Moreover, tests conducted with higher doses of β-FNA (10 mg/kg) produced no further rightward shifts in the dose-effect curves (Table 1). An additional group of pigeons was administered a 20 mg/kg dose of β-FNA with morphine, and in this group the dose-effect curve was shifted rightward by only 6.5-fold. In contrast to the effects of β-FNA, C-CAM shifted the dose-effect curve for fentanyl and morphine to the right and downward (i.e., decreases in maximal effect). For example, the 0.06 and 0.12 mg/kg doses of C-CAM reduced

the maximal effect produced by fentanyl to 75% and 50%, respectively, and that of morphine to 40% and 3%, respectively.

To determine the time course of antagonist effects, the effects of morphine and fentanyl in combination with 5.0 β-FNA mg/kg and 0.12 C-CAM mg/kg were assessed 50 h after administration of the antagonists. As shown in Table 1, the dose-effect curves for morphine and fentanyl had recovered to their pre-antagonist potency 50 h after administration of both β-FNA and C-CAM.

l-Methadone and Sufentanil. Fig. 3 shows the effects of sufentanil and l-methadone administered alone and in combination with various doses of β-FNA and C-CAM. Similar to the effects observed with fentanyl and morphine, β-FNA produced dose-dependent, parallel rightward shifts in the dose-effect curves of sufentanil and l-methadone, with no alteration in the slope of the curve or the maximal effect. The highest dose of β-FNA tested (5.0 mg/kg), for example, increased the ED_{50} for sufentanil and l-methadone by 3.1- and 5.3-fold, respectively (Table 2). Although low doses of C-CAM also produced dose-dependent, parallel rightward shifts in the dose-effect curves of these agonists, the highest dose tested (0.12 mg/kg) decreased the maximal effect of l-methadone and sufentanil to 23% and 2%, respectively. Indeed, doses of l-methadone 26-fold larger than the ED_{50} alone and doses of sufentanil 164-fold larger than the ED_{50} alone produced predominantly water-appropriate responding.

Etorphine. Fig. 4 shows that both β-FNA and C-CAM produced dose-dependent, parallel rightward shifts in the dose-effect curve of etorphine, and in no case was the slope or maximal effect altered. In contrast to β-FNA, which produced less than a 3-fold rightward shift in the dose-effect curve for etorphine at all doses tested, C-CAM increased the ED_{50} for etorphine by approximately 8.5- and 81-fold at the 0.06 and 0.12 mg/kg doses, respectively (Table 2).

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Effects of β-FNA and C-CAM on Response Rate. Across the range of doses tested, both β-FNA and C-CAM generally produced dose-dependent antagonism of the rate-decreasing effects of all opioids tested, a finding indicative of μ opioid activity. For example, with the highest dose of β-FNA tested (5.0 mg/kg), full substitution was observed at doses of morphine (1.48 ± 0.43 responses/sec), fentanyl (1.84 ± 0.29 responses/sec), l-methadone (1.54 ± 0.38 responses/sec), sufentanil (1.92 ± 0.24 responses/sec) and etorphine (2.16 ± 0.39 responses/sec) that had only minimal effects on rates of responding. In contrast, in tests conducted with C-CAM there were several instances in which rates of responding were eliminated at agonist doses that produced less than full substitution. Fig. 5 shows the effects of all five opioids on response rate when administered alone and in combination with C-CAM. At the 0.12 mg/kg dose of C-CAM, morphine and l-methadone failed to substitute for the fentanyl stimulus and their marked rate-decreasing effects prevented testing higher doses. For these two opioids, however, it was possible to test doses 1.0 log unit higher than those that produced full substitution when administered alone. With fentanyl and sufentanil, testing was terminated at doses 1.5 and 1.75 log units higher than those that produced full substitution when administered alone, respectively, and at these doses only low levels of substitution were obtained.

Comparison between β-FNA and C-CAM. Comparing the dose ratios (Tables 1 and 2) for each agonist in combination with β-FNA and C-CAM indicated that the agonists differed in their sensitivity to antagonism. For example, whereas β-FNA failed to produce a significant rightward shift in the dose-effect curve for etorphine, a dose of 5.0 mg/kg β-FNA produced a 3.1-, 5.3-, 5.6-, and 6.4-fold rightward shift in the dose-effect curves for sufentanil, l-methadone, morphine and fentanyl, respectively. Similar effects were obtained with the C-CAM, and were most apparent at the two highest doses tested. For example, whereas etorphine produced maximal effects when administered in combination with all doses of C-CAM, a dose of 0.12 mg/kg C-CAM decreased the maximal effect (< 80% fentanyl-appropriate responding) of sufentanil and l-methadone, and a dose of 0.06 mg/kg C-CAM decreased the maximal effect of

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fentanyl and morphine. Taken together, these data suggest a rank order of relative efficacy of etorphine > sufentanil = l-methadone > fentanyl = morphine.

Examination of dose-effect data (Fig. 2-4) also indicates differences in the ability of β-FNA and C-CAM to produce insurmountable antagonism of the fentanyl-like stimulus effects of the agonists. In tests with β-FNA, for example, there were no instances in any pigeons in which an agonist failed to produce full substitution for the fentanyl stimulus. In contrast, although C-CAM was relatively ineffective in producing insurmountable antagonism of the stimulus effects of etorphine, dose-dependent insurmountable antagonism of the other agonists was evident. Indeed, at the highest dose of C-CAM tested (0.12 mg/kg) at least 80% of the pigeons tested with l-methadone, sufentanil, fentanyl and morphine failed to display significant levels of fentanyl-appropriate responding (data not shown).

Apparent Affinity and Efficacy Estimates. The data presented in Figs. 2-4 were also analyzed according to the model proposed by Black and Leff (1983) to obtain quantitative estimates for the apparent *in vivo* affinity (K_A), efficacy (*tau*), and the fraction of receptors remaining after antagonist treatment (q) (Furchgott 1966). As shown in Table 3, sufentanil displayed the highest affinity (K_A = 0.14 mg/kg), with etorphine (K_A = 0.39-0.73 mg/kg) and fentanyl (K_A = 0.35-0.45 mg/kg) having somewhat lower affinity, and morphine (K_A = 10.0-25.1 mg/kg) and l-methadone (K_A = 19.9-20.4 mg/kg) having the lowest affinity. The rank order of agonists according to efficacy was somewhat different, with etorphine displaying the highest efficacy estimate (*tau* = 162-582), sufentanil (*tau* = 42.6-51.7) and l-methadone (*tau* = 39.8-52.0) displaying intermediate efficacy estimates, and morphine (*tau* = 11.8-14.3) and fentanyl (*tau* = 12.6-14.2) displaying the lowest efficacy estimates. Importantly, this rank order of relative efficacy, etorphine > sufentanil = l-methadone > fentanyl = morphine, is comparable to the above analyses that rely on dose ratio comparisons to estimate efficacy. As a measure of internal consistency, the derived values determined by mathematical analyses were used to calculate control ED_{50} values for each

agonist administered alone. These "back-calculated" ED_{50} values were similar to the observed ED_{50} values, differing by an average of only 1.1-fold.

q Values for β-FNA and C-CAM. Fig. 6 shows q values for each antagonist dose averaged across the agonists with which they were administered. C-CAM produced dose-dependent decreases in q values such that the 0.01, 0.03, 0.06 and 0.12 mg/kg doses reduced, on average, the receptor population by approximately 23%, 46%, 89% and 99.9%, respectively. Although β-FNA produced dose-dependent decreases in q values, the magnitude of this decrease was less than that observed for C-CAM, and there appeared to be a limit on the fraction of receptors that β-FNA could inactivate. For example, although 1.25, 2.5 and 5.0 mg/kg β-FNA reduced the receptor population by approximately 46%, 64% and 73%, respectively, additional tests conducted with 10 and 20 mg/kg β-FNA in combination with fentanyl and morphine indicated that these doses reduced the receptor population by only 56% and 60%, respectively.

Chronic Morphine Administration. Fig. 7 shows the effects of morphine administered before and during chronic morphine treatment, as well as in combination with β-FNA and C-CAM during chronic morphine treatment. Chronic administration of 56 mg/kg morphine for 6 days produced an approximate 2.8-fold rightward shift in the dose-effect curve for morphine. In these morphine-treated pigeons, 2.5 mg/kg β-FNA further increased the morphine ED_{50} value by 5.1-fold. Although 0.03 mg/kg C-CAM also produced a further rightward shift in the morphine dose-effect curve, the slope of this dose-effect curve was altered, thus precluding an accurate calculation of the dose ratio. Furthermore, in these morphine-treated pigeons 5.0 mg/kg β-FNA and 0.06 mg/kg C-CAM produced an insurmountable antagonism of the effects of morphine, with doses up to 30 mg/kg producing only water-appropriate responding. It was not possible to probe doses higher than 30 mg/kg morphine, as the combination of morphine and 5.0 mg/kg β-FNA or 0.06 mg/kg C-CAM in morphine-treated pigeons completely eliminated responding.

Discussion

In the present study, etorphine, sufentanil, l-methadone, fentanyl and morphine produced full substitution for the discriminative stimulus effects of fentanyl that was reversed in a dose- and time-dependent manner by both β-FNA and C-CAM. Although these findings are consistent with the μ agonist activity reported for these opioids in other assays (Young et al., 1984; Zimmerman et al., 1987; Pitts et al., 1998), differences in their sensitivity to antagonism by β-FNA and C-CAM suggest that these opioids vary in their relative efficacy. A number of pieces of evidence indicate a rank order of relative efficacy for these opioids of etorphine > sufentanil = l-methadone > fentanyl = morphine. First, dose-ratios calculated on the basis of ED_{50} values for all doses of β-FNA and the lower doses of C-CAM indicated that these irreversible antagonists produced the smallest rightward shifts in the dose-effect curves for etorphine, comparable shifts with sufentanil and l-methadone, and the largest shifts with fentanyl and morphine. Second, these opioids differed in the dose of C-CAM required to produce insurmountable antagonism. For example, whereas the 0.12 mg/kg dose of C-CAM failed to alter the maximal effect of etorphine, this dose markedly decreased the maximal effect produced by sufentanil and l-methadone. In contrast, decreases in maximal effectiveness of morphine and fentanyl were evident at a dose as low as 0.06 mg/kg C-CAM.

Third, by simultaneously analyzing dose-effect curves for β-FNA and C-CAM administered in combination with each agonist, quantitative estimates of efficacy could be obtained. These calculations (Furchgott, 1966; Black and Leff 1983) allow statistical comparisons among agonists, and results indicated that etorphine had the highest relative efficacy (*tau* = 162-584), with l-methadone and sufentanil (*tau* = 39.8-52.0) displaying intermediate relative efficacy, and fentanyl and morphine (*tau* = 11.8-14.3) having the lowest relative efficacy of the five opioids tested. The reciprocal value of the efficacy estimate yields the fraction of receptors necessary for a given agonist to produce half of the maximum response under control conditions (Furchgott, 1966; Zernig et al., 1996b). By this convention, etorphine produced a 50% effect by binding to < 1% of the μ receptors, whereas sufentanil, l-methadone,

fentanyl and morphine produced a 50% effect by binding to 2.1, 2.2, 7.5 and 7.7% of the relevant μ receptor population, respectively. Despite the fact that each of these agonists appeared highly efficacious, they could be distinguished on the basis of their quantitative estimates of efficacy. Further, the rank order of efficacy, etorphine > sufentanil = l-methadone > fentanyl = morphine, was identical using each of these three methods of analysis.

When calculating opioid relative efficacy in any preparation, it is important to demonstrate that the loss of effectiveness (i.e., insurmountable antagonism) of the agonist is not a consequence of the failure to test a sufficient range of doses, behavioral disruptions caused by the agonist, or drug toxicity. This can be especially problematic in drug discrimination procedures, in which the rate-decreasing effects of the agonists can limit the range of doses examined for particular agonists. In the present investigation, there was evidence that the rate-decreasing effects of the agonists did not present a major problem. In particular, with β-FNA full substitution was observed at agonist doses that maintained high rates of responding. Although with C-CAM some of the opioids did reduce rates of responding at doses that produced less than full substitution, it was possible to test doses between 1.0 and 1.75 log units higher than those that produced full substitution when administered alone. Additionally, despite the differences in the rate-decreasing effects associated with the agonists when administered with β-FNA and C-CAM, analyses yielded comparable efficacy estimates.

It has been suggested that apparent efficacy differences between agonists that reflect less than 10-fold numerical differences should be interpreted with caution (Zernig et al., 1996b). Although the differences in efficacy estimates across agonists in the present investigation were frequently less than 10-fold, these differences appeared reliable and reproducible. A number of factors may have contributed to these findings, in particular the use of the large number of dose-effect curves obtained for each agonist in combination with β-FNA and C-CAM. Although increasing the number of antagonist doses does not necessarily affect the variance of the parameters, it does allow for more accurate estimates of the *in vivo* affinity, estimates which are critical for obtaining accurate *tau* values. (Zernig et al., 1996b). Indeed, with increasing doses of

C-CAM the dose-effect curves for the agonists were first shifted rightward, then downward, followed by a flattening of the curve. Under these conditions, assessment of the *in vivo* affinity estimate, which is derived by an assessment of the midpoint of the dose-effect curve with a decreased maximal effect, can prove highly reliable. In instances in which the maximal effect produced by the agonist is not depressed, such as that observed with β-FNA in the present investigation, incorrect estimates for *in vivo* affinity could result in inaccurate assessments of efficacy as well as discrepancies between back-calculated and observed ED_{50} values for the control dose-effect curves. That the back-calculated ED_{50} values differed from the observed ED_{50} values by an average of only 1.1-fold suggests that this was not a major problem. Finally, the use of two irreversible antagonists, which yielded comparable estimates of agonist efficacy, supports further the utility of this curve-fitting procedure for assessing the pharmacodynamic properties of opioids.

That etorphine yielded the highest estimate of efficacy is consistent with prior data obtained using irreversible antagonists to quantify agonist efficacy (Pitts et al., 1998; Walker et al., 1998). Moreover, and the ability of etorphine to stimulate potassium channel conductance and [^{35}S]GTPγS binding, *in vitro* estimates of efficacy, has also been shown to be greater than that of morphine and methadone (Yu et al., 1997; Romero et al., 1999). The quantitative efficacy estimates for sufentanil and l-methadone were also higher than that for morphine, and are in agreement with other reports indicating that in assays of antinociception, sufentanil is more resistant than morphine to irreversible antagonism, chronic opioid administration, and increases in nociceptive stimulus intensity (Mjanger and Yaksh, 1991; Dirig and Yaksh, 1995).

The present finding that the relative efficacy of fentanyl is comparable to morphine contrasts with findings indicating that fentanyl is less sensitive to antagonism by β-FNA and C-CAM than morphine (Adams et al., 1990; Comer et al., 1992; Holtzman, 1997; Morgan and Picker, 1998). Although the results from some [^{35}S]GTPγS binding assays also indicate that fentanyl is slightly more efficacious than morphine (Traynor and Nahorski, 1995; Selley et al., 1998), there are studies using both *in vivo* preparations and [^{35}S]GTPγS binding that suggest that

fentanyl and morphine have comparable efficacy (Zimmerman et al., 1987; Selley et al., 1997). As such, it would appear that if there are differences in the relative efficacy of fentanyl and morphine they are relatively small.

A second purpose of this study was to compare β-FNA and C-CAM as irreversible antagonists, and a number of similarities and differences in their profiles were apparent. For example, these antagonists failed to produce fentanyl-like stimulus effects, yielded similar efficacy (*tau*) and affinity (K_A) estimates for the various opioids tested, and produced a similar time-course of antagonism. In contrast, the magnitude of antagonism, as evidenced by rightward and downward shifts in the agonist dose-effect curves, was markedly greater for C-CAM. In addition, C-CAM functioned as an insurmountable antagonist, producing decreases in the slope and maximal effect of four out of the five opioids tested. However, in no instance did β-FNA decrease the maximal effect produced by an opioid.

β-FNA and C-CAM also differed in terms of the fraction of receptors remaining (q values) for agonist interaction. This parameter is independent of the pharmacodynamic properties of the agonist employed and should thus depend solely on the dose of the antagonist (Furchgott, 1966). Consistent with this notion, C-CAM and β-FNA produced dose-dependent decreases in q values, indicating that increasing doses of each antagonist successively inactivated a greater proportion of the receptor population. However, whereas C-CAM was able to inactivate nearly 100% of the receptor population, the maximal level of receptor inactivation produced by β-FNA was 73%, and this was obtained at an intermediate (5.0 mg/kg) dose. These data are in agreement with prior findings that β-FNA can inactivate only 40-70% of the μ receptor population (Liu-Chen and Phillips, 1987; Franklin and Traynor, 1991), whereas C-CAM can inactivate nearly the entire μ receptor population (Burke et al., 1994; Zernig et al., 1995; Zernig et al., 1996a). The explanation for this discrepancy is unclear, although it has been hypothesized that the alkylation produced by β-FNA is limited because the dissociation of reversible binding occurs at 5 times the rate of irreversible binding (Liu-Chen et al., 1990). Alternatively, it has been proposed that

there is a population of μ opioid receptors that is insensitive to alkylation by β-FNA, but available for agonist binding (Rothman et al., 1987).

The lack of insurmountable antagonism with β-FNA is consistent with other drug discrimination studies, in which β-FNA failed to depress the maximal effect of agonists (Holtzman, 1997; Morgan and Picker, 1998). Studies of μ opioid antinociception, however, have reported that β-FNA can alter the slope and maximal effect of the agonist dose-effect curve (Adams et al., 1990; Mjanger and Yaksh, 1991). Discrepancies between procedures may reflect the higher efficacy requirement (i.e., fraction of receptors that must be occupied to produce a maximal effect) of most antinociceptive assays compared to drug discrimination assays (Bergman et al., 2000). For example, whereas the maximal antinociceptive effect of morphine is decreased after 75% of the receptors have been inactivated by β-FNA, inactivation of approximately 90% of the receptor population failed to appreciably alter the maximal discriminative stimulus effects of morphine (Adams et al., 1990; Holtzman, 1997). Although the efficacy requirement is, in part, dependent on the parameters of a given assay, the doses of agonists used in drug discrimination procedures are typically lower than those that produce significant levels of antinociception. Taken together, these findings indicate that in tasks that have a relatively low efficacy requirement, such as the drug discrimination procedure, there can be limitations associated with the use of β-FNA to gauge opioid relative efficacy.

In addition to irreversible antagonism, chronic opioid administration is another technique that decreases the functional receptor pool (Porreca and Burks, 1983). If the inability of β-FNA to produce insurmountable antagonism is related to its inability to inactivate a sufficient proportion of the receptor population, then it would be predicted that chronic opioid administration would enhance the ability of β-FNA to produce insurmountable antagonism. In morphine-treated pigeons, however, the rate-decreasing effects produced by the combination of both β-FNA and C-CAM with morphine precluded a rigorous test of this hypothesis. Nevertheless, there are some aspects of the data suggestive of an increased effectiveness of the antagonists in morphine-treated pigeons. For example, doses of 5.0 mg/kg β-FNA and 0.06

mg/kg C-CAM produced a complete flattening of the morphine dose-effect curve, an effect not observed under acute conditions (i.e., in pigeons not treated with morphine). Further, had higher doses of morphine produced complete substitution, the rightward shift in the dose-effect curve would have been greater than that observed under acute conditions. Such data are consistent with a recent report in rats trained to discriminate morphine from saline, in which chronic opioid treatment enhanced the potency and effectiveness of C-CAM (Walker and Young, 2002). Thus, when there is a relatively small receptor reserve, such as during chronic morphine treatment, β-FNA and C-CAM display similar profiles of action.

In summary, these data indicate that β-FNA and C-CAM can differentiate the relative efficacy of μ agonists that produce similar discriminative stimulus profiles and are all considered high efficacy agonists. β-FNA and C-CAM showed similar time courses of antagonism, produced comparable affinity and efficacy estimates for the agonists tested, and had a similar profile of activity in morphine-treated pigeons. However, the magnitude of antagonism, ability to produce insurmountable antagonism, and the fraction of the receptor population that could be inactivated were all greater for C-CAM than β-FNA. The present findings confirm studies using receptor binding techniques indicating that there are limitations in the proportion of the receptor population that can by eliminated by β-FNA but not by C-CAM.

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References

Aceto MD, Bowman ER, May EL, Harris LS, Woods JH, Smith CB, Medzihradsky F and Jacobsen AE (1989) Very long-acting narcotic antagonists: the 14β-p-substituted cinnamoylaminomorphinones and their partial μ agonist codeinone relatives. *Arzneim Forsch Drug Res* **39**:570-575.

Adams JU, Paronis CA and Holtzman SG (1990) Assessment of the relative intrinsic activity of *mu*-opioid analgesics *in vivo* by using β-funaltrexamine. *J Pharmacol Exp Ther* **255**:1027-1031.

Bergman J, France CP, Holtzman SG, Katz JL, Koek W and Stephens DN (2000) Agonist efficacy, drug dependence, and medications development: preclinical evaluation of opioid, dopaminergic and $GABA_A$-ergic ligands. *Psychopharmacology* **153**:67-84.

Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc Royal Soc London B Bio* **220**:141-162.

Burke TF, Woods JH, Lewis JL and Medzihradsky F (1994) Irreversible opioid antagonist effects of clocinnamox on opioid analgesia and *mu* receptor binding in mice. *J Pharmacol Exp Ther* **271**:715-721.

Comer SD, Burke TF, Lewis JL and Woods JH (1992) Clocinnamox: a novel, systemically-active, irreversible opioid antagonist. *J Pharmacol Exp Ther* **262**:1051-1056.

Dirig DM and Yaksh TL (1995) Differential right shifts in the dose-response curve for intrathecal morphine and sufentanil as a function of stimulus intensity. *Pain* **62**:321-328.

Franklin TG and Traynor JR (1991) Alkylation with β-funaltrexamine suggests differences between μ-opioid receptor systems in guinea-pig brain and myenteric-plexus. *Br J Pharmacol* **102**:718-722.

Furchgott RF (1966) The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes, in *Advances in Drug Research* (Harper N SA ed) pp 21-55, Academic Press, New York.

Holtzman SG (1997) Antagonism of morphine-like discriminative stimulus effects by β-funaltrexamine. *Pharmacol Biochem Behav* **57**:771-777.

Kenakin TP (1997) *Pharmacologic analysis of drug-receptor interaction*. Raven, New York.

Liu-Chen L-Y, Li S and Tallarida RJ (1990) Studies on the kinetics of [^{3}H]β-funaltrexamine binding to the μ opioid receptor. *Mol Pharmacol* **37**:243-250.

Liu-Chen L-Y and Phillips CA (1987) Covalent labeling of *mu* opioid receptor binding site by [^{3}H]*beta*-funaltrexamine. *Mol Pharmacol* **32**:321-329.

Mjanger E and Yaksh TL (1991) Characteristics of dose-dependent antagonism by β-funaltrexamine of the antinociceptive effects of intrathecal *mu* agonists. *J Pharmacol Exp Ther* **258**:544-550.

Morgan D and Picker MJ (1998) The μ opioid irreversible antagonist beta-funaltrexamine differentiates the discriminative stimulus effects of opioids with high and low efficacy at the μ opioid receptor. *Psychopharmacology* **140**:20-28.

Paronis CA and Holtzman SG (1992) Development of tolerance to the analgesic activity of *mu* agonists after continuous infusion of morphine, meperidine or fentanyl in rats. *J Pharmacol Exp Ther* **262**:1-9.

Paronis CA and Holtzman SG (1994) Sensitization and tolerance to the discriminative stimulus effects of mu-opioid agonists. *Psychopharmacology* **114**:601-610.

Picker MJ, Yarbrough J, Hughes CE, Smith MA, Morgan D and Dykstra LA (1993) Agonist and antagonist effects of mixed action opioids in the pigeon drug discrimination procedure: influence of training dose, intrinsic efficacy and interanimal differences. *J Pharmacol Exp Ther* **266**:756-767.

Pitts RC, Allen RM, Walker EA and Dykstra LA (1998) Clocinnamox antagonism of the antinociceptive effects of *mu* opioids in squirrel monkeys. *J Pharmacol Exp Ther* **285**:1197-1206.

Porreca F and Burks TF (1983) Affinity of normorphine for its pharmacologic receptor in the naive and morphine-tolerant guinea-pig isolated ileum. *J Pharmacol Exp Ther* **225**:688-693.

Romero DV, Partilla JS, Zheng Q-X, Heyliger SO, Ni Q, Rice KC, Lai J and Rothman RB (1999) Opioid peptide receptor studies. 12. Buprenorphine is a potent and selective μ/κ antagonist in the [^{35}S]-GTP-γ-S functional binding assay. *Synapse* **34**:83-94.

Rothman RB, Jacobsen AE, Rice KC and Herkenham M (1987) Autoradiographic evidence for two classes of mu opioid binding sites in rat brain using [^{125}I]FK33824. *Peptides* **8**:1015-1021.

Selley DE, Liu Q and Childers SR (1998) Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [^{35}S]GTPγS binding in mMOR-CHO cells and rat thalamus. *J Pharmacol Exp Ther* **285**:496-505.

Selley DE, Sim LJ, Xiao R, Liu Q and Childers SR (1997) μ-opioid receptor stimulated guanosine-5'-o-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *J Pharmacol Exp Ther* **51**:87-96.

Stephenson RP (1956) A modification of receptor theory. *Br J Pharmacol* **11**:379-393.

Tallarida RJ, Murray RB (1987) Manual of pharmacologic calculations with computer programs. Springer, Berlin Heidelberg New York.

Traynor JR and Nahorski SR (1995) Modulation by μ-opioid agonists of guanosine-5'-O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol* **47**:848-854.

Walker EA, Butelman ER, DeCosta BR and Woods JH (1993) Opioid thermal antinociception in rhesus monkeys: receptor mechanisms and temperature dependency. *J Pharmacol Exp Ther* **267**:280-286.

Walker EA, Richardson TM and Young AM (1997) Tolerance and cross-tolerance to morphine-like stimulus effects of mu opioids in rats. *Psychopharmacology* **133**:17-28.

Walker EA and Young AM (2002) Clocinnamox distinguishes opioid agonists according to relative efficacy in normal and morphine-treated rats trained to discriminate morphine. *J Pharmacol Exp Ther* **302**:101-110.

Walker EA, Zernig G and Young AM (1998) In vivo apparent affinity and efficacy estimates for μ opiates in a rat tail-withdrawal assay. *Psychopharmacology* **136**:15-23.

Ward SJ, Portoghese PS and Takemori AE (1982) Pharmacological characterization *in vivo* of the novel opiate, β-funaltrexamine. *J Pharmacol Exp Ther* **20**:494-498.

Yoburn BC, Shah S, Chan K, Duttaroy A and Davis T (1995) Supersensitivity to opioid analgesics following chronic opioid antagonist treatment: relationship to receptor selectivity. *Pharmacol Biochem Behav* **51**:535-539.

Young AM, Stephens KR, Hein DW and Woods JH (1984) Reinforcing and discriminative stimulus properties of mixed agonist-antagonist opioids. *J Pharmacol Exp Ther* **229**:118-126.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR and Wang JB (1997) μ opioid receptor phosphorylation, desensitization, and ligand efficacy. *J Biol Chem* **272**:28869-28874.

Zernig G, Burke TF, Lewis JL and Woods JH (1996a) Mechanism of clocinnamox blockade of opioid receptors: evidence from in vitro and ex vivo binding and behavioral assays. *J Pharmacol Exp Ther* **279**:23-31.

Zernig G, Issaevitch T, Broadbear JH, Burke TF, Lewis JL, Brine GA and Woods JH (1995) Receptor reserve and affinity of mu opioid agonists in mouse antinociception: correlation with receptor binding. *Life Sci* **57**:2113-2125.

Zernig G, Issaevitch T and Woods JH (1996b) Calculation of agonist efficacy, apparent affinity, and receptor population changes after administration of insurmountable antagonists: comparison of different analytical approaches. *J Pharmacol Tox Meth* **35**:223-237.

Zernig G, Lewis JL and Woods JH (1997) Clocinnamox inhibits the intravenous self-administration of opioid agonists in rhesus monkeys: comparison with effects on opioid agonist-mediated antinociception. *Psychopharmacology* **129**:233-242.

Zhang L, Walker EA, Sutherland II J and Young AM (2000) Discriminative stimulus effects of two doses of fentanyl in rats: pharmacological selectivity and effect of training dose on agonist and antagonist effects of mu opioids. *Psychopharmacology* **148**:136-145.

Zimmerman DM, Leander JD, Reel JK and Hynes MD (1987) Use of β-funaltrexamine to determine mu opioid receptor involvement in the analgesic activity of various opioid ligands. *J Pharmacol Exp Ther* **241**:374-378.

Footnotes

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Figure Legends

Fig 1 Discriminative stimulus effects of fentanyl (n=11), morphine (n=11), l-methadone (n=10), sufentanil (n=10) and etorphine (n=10) (left panel), and butorphanol (n=4), nalbuphine (n=5), BW373U86 (n=5), U50,488 (n=4) and pentobarbital (n=4) (right panel) in pigeons trained to discriminate 0.12 mg/kg fentanyl from water. Data reflect the mean percentage of fentanyl-appropriate responding obtained before the first reinforcer. Vertical lines are the S.E.M.

Fig 2 Discriminative stimulus effects of fentanyl (left panel) and morphine (right panel) administered alone and in combination with C-CAM (top panels) and β-FNA (bottom panels) in pigeons (n=5-6) trained to discriminate 0.12 mg/kg fentanyl from water. Data reflect the mean percentage of fentanyl-appropriate responding obtained before the first reinforcer. Vertical lines are the S.E.M.

Fig 3 Discriminative stimulus effects of l-methadone (left panels) and sufentanil (right panels) administered alone and in combination with C-CAM (top panels) and β-FNA (bottom panels) in pigeons (n=5) trained to discriminate 0.12 mg/kg fentanyl from water. Data reflect the mean percentage of fentanyl-appropriate responding obtained before the first reinforcer. Vertical lines are the S.E.M.

Fig 4 Discriminative stimulus effects of etorphine and administered alone and in combination with C-CAM (top panels) and β-FNA (bottom panels) in pigeons (n=5) trained to discriminate 0.12 mg/kg fentanyl from water. Data reflect the mean percentage of fentanyl-appropriate responding obtained before the first reinforcer. Vertical lines are the S.E.M.

Fig 5 Response rates for fentanyl, morphine, sufentanil, l-methadone and etorphine in combination with various doses of C-CAM in pigeons (n=5-6) trained to discriminate 0.12 mg/kg fentanyl from water. Data reflect the mean rate of responding obtained during the entire session, and are expressed as responses for sec. Vertical lines are the S.E.M.

Fig 6 q values (i.e., the fraction of receptors available for agonist interaction following administration of an irreversible antagonist) obtained for each dose of C-CAM and β-FNA administered 2 h before determination of dose-effect curves for morphine, fentanyl, sufentanil, l-methadone and etorphine. Each point represents the mean q value obtained for each antagonist dose averaged across the five opioids tested. Due to limited β-FNA supply, doses of 10 and 20 mg/kg β-FNA were administered in combination with only fentanyl and morphine, and these data are connected to the dose-effect curve by dotted lines. Vertical lines are the S.E.M.

Fig 7 Discriminative stimulus effects of morphine administered under acute conditions, during chronic treatment with 56 mg/kg morphine for 6 days, and following 2 h pretreatment with β-FNA (left panel) or C-CAM (right panel) in pigeons (n=4-5) treated chronically with morphine. Data obtained for the "chronic" dose-effect curve represent an average from two chronic dosing regimens in groups of pigeons that received 2.5 and 5.0 mg/kg β-FNA, and in groups of pigeons that received 0.03 and 0.06 mg/kg C-CAM. Vertical lines are the S.E.M.

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Table 1. ED_{50} values (95% confidence limits) and dose ratios

[(ED_{50} after antagonist) / (ED_{50} before antagonist)] for the dose-effect curves of fentanyl and morphine administered alone and after 2 h (except where indicated) pretreatment with either β-FNA or C-CAM. Also shown are the q values for each agonist-antagonist combination

Agonist + Antagonist	ED_{50} value	Dose ratio	q value[a]
Fentanyl alone	0.036 (0.024-0.055)		
+ 1.25 β-FNA	0.085 (0.045-0.16)	2.4	0.50
+ 2.5 β-FNA	0.26 (0.15-0.47)	7.3[b]	0.19
+ 5.0 β-FNA	0.23 (0.10-0.53)	6.4[b]	0.23
+ 5.0 β-FNA (50 h)	0.053 (0.028-0.098)	1.5	---[d]
+ 10 β-FNA	0.16 (0.03-0.86)	4.5	0.29
Fentanyl alone	0.033 (0.023-0.048)		
+ 0.01 C-CAM	0.035 (0.024-0.052)	1.1	0.94
+ 0.03 C-CAM	0.10 (0.062-0.17)	3.1[b]	0.74
+ 0.06 C-CAM	0.83 (0.17-4.03)	---[c]	0.06
+ 0.12 C-CAM	---[c]	---[c]	0.01
+ 0.12 C-CAM (50 h)	0.045 (0.032-0.064)	1.4	---[d]
Morphine alone	1.54 (0.81-2.94)		
+ 1.25 β-FNA	2.92 (1.34-6.37)	1.9	0.54
+ 2.5 β-FNA	7.95 (4.33-14.62)	5.2[b]	0.30
+ 5.0 β-FNA	8.61 (4.07-18.24)	5.6[b]	0.28
+ 5.0 β-FNA (50 h)	2.13 (1.30-3.50)	1.7	---[d]
+ 10 β-FNA	4.78 (2.75-8.31)	3.1	0.29
+ 20 β-FNA	11.81 (7.89-17.68)	6.5[b]	0.40
Morphine alone	1.26 (0.66-2.38)		
+ 0.01 C-CAM	1.56 (0.79-3.08)	1.2	0.60
+ 0.03 C-CAM	2.71 (1.70-4.32)	2.2	0.40
+ 0.06 C-CAM	---[c]	---[c]	0.11
+ 0.12 C-CAM	---[c]	---[c]	0.0004
+ 0.12 C-CAM (50 h)	2.04 (1.37-3.05)	1.6	---[d]

[a] An estimate of the fraction of receptors still available for interaction with an agonist following blockade with a given dose of an irreversible antagonist

[b] Significant rightward shift (i.e., 95% confidence limits do not overlap with those obtained for the agonist alone)

[c] ED_{50} and/or dose ratio could not be determined (see Data Analysis)

[d] q vales not determined

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Table 2. ED_{50} values (95% confidence limits) and dose ratios

[(ED_{50} after antagonist) / (ED_{50} before antagonist)] for the dose-effect curves of l-methadone, sufentanil and etorphine administered alone and after 2 h pretreatment with either β-FNA or C-CAM. Also shown are the q values for each agonist-antagonist combination.

Agonist + Antagonist	ED_{50} value	Dose ratio	q value[a]
l-Methadone alone	0.50 (0.26-0.96)		
+ 1.25 β-FNA	0.75 (0.55-1.02)	1.5	0.63
+ 2.5 β-FNA	1.90 (1.33-2.71)	3.8[b]	0.30
+ 5.0 β-FNA	2.64 (1.71-4.06)	5.3[b]	0.24
l-Methadone alone	0.39 (0.27-0.57)		
+ 0.01 C-CAM	0.38 (0.27-0.55)	0.97	1.00
+ 0.03 C-CAM	0.45 (0.25-0.80)	1.2	0.64
+ 0.06 C-CAM	2.52 (1.27-5.01)	6.5[b]	0.16
+ 0.12 C-CAM	---[c]	---[c]	0.007
Sufentanil alone	0.0029 (0.0020-0.0043)		
+ 1.25 β-FNA	0.0057 (0.0036-0.0090)	2.0	0.41
+ 2.5 β-FNA	0.0079 (0.0042-0.015)	2.7	0.51
+ 5.0 β-FNA	0.0089 (0.0060-0.013)	3.1[b]	0.31
Sufentanil alone	0.0034 (0.0023-0.0050)		
+ 0.01 C-CAM	0.0041 (0.0021-0.0080)	1.2	0.77
+ 0.03 C-CAM	0.0084 (0.0056-0.012)	2.5[b]	0.40
+ 0.06 C-CAM	0.035 (0.018-0.068)	10.3[b]	0.13
+ 0.12 C-CAM	---[c]	---[c]	0.001
Etorphine alone	0.0028 (0.0017-0.0045)		
+ 1.25 β-FNA	0.0040 (0.0026-0.0061)	1.4	0.63
+ 2.5 β-FNA	0.0042 (0.0032-0.0057)	1.5	0.52
+ 5.0 β-FNA	0.0064 (0.0037-0.011)	2.3	0.29
Etorphine alone	0.0013 (0.00069-0.0023)		
+ 0.01 C-CAM	0.0025 (0.0013-0.0046)	1.9	0.46
+ 0.03 C-CAM	0.0038 (0.0014-0.0010)	2.9	0.27
+ 0.06 C-CAM	0.011 (0.0041-0.031)	8.5[b]	0.099
+ 0.12 C-CAM	0.105 (0.040-0.273)	80.8[b]	0.014

[a] An estimate of the fraction of receptors still available for interaction with an agonist following blockade with a given dose of an irreversible antagonist

[b] Significant rightward shift (i.e., 95% confidence limits do not overlap with those obtained for the agonist alone)

[c] ED_{50} and/or dose-ratio could not be determined (see Data Analysis)

Table 3. Apparent affinity estimates[a], efficacy estimates[a], and observed vs. calculated ED_{50} estimates[b] for fentanyl, morphine, l-methadone, sufentanil and etorphine in pigeons trained to discriminate 0.12 mg/kg fentanyl vs. water

β-FNA +	Fentanyl	Morphine	l-Methadone	Sufentanil	Etorphine
K_A (log mg/kg)	-0.35 [-0.35 – (-0.34)]	1.40 (1.37 – 1.43)	1.31 (1.30-1.31)	-0.86 [-0.86 – (-0.85)]	-0.41 [-0.41 – (-0.40)]
K_A (mg/kg)	0.45	25.1	20.4	0.14	0.39
tau	14.2 (14.1-14.3)	14.3 (13.7-15.0)	39.8 (30.8-51.5)	51.7 (49.1-53.7)	162 (154-169)
Observed $ED_{50,\ control}$	0.036 (0.024-0.055)	1.54 (0.81-2.94)	0.50 (0.26-0.96)	0.0029 (0.0020-0.0043)	0.0028 (0.0017-0.0045)
Calculated $ED_{50,\ control}$	0.035	1.83	0.52	0.0027	0.0024
C-CAM +	Fentanyl	Morphine	l-Methadone	Sufentanil	Etorphine
K_A (log mg/kg)	-0.45 [-0.46 – (-0.44)]	1.00 (1.00 – 1.01)	1.30 (1.30-1.31)	-0.86 [-0.86 – (-0.85)]	-0.13 [-0.15 – (-0.11)]
K_A (mg/kg)	0.35	10.0	20.0	0.14	0.74
tau	12.6 (12.5-12.7)	11.8 (10.3-13.1)	52.0 (51.9-52.2)	42.6 (42.4-42.8)	584 (549-621)
Observed $ED_{50,\ control}$	0.033 (0.023-0.048)	1.26 (0.66-2.38)	0.39 (0.27-0.57)	0.0034 (0.0023-0.0050)	0.0013 (0.00069-0.0023)
Calculated $ED_{50,\ control}$	0.028	0.93	0.39	0.0033	0.0013

[a] Agonist dose-effect curves in the absence and presence of an irreversible antagonist were simultaneously analyzed for a given agonist using the Black and Leff (1983) model as applied to behavioral data (Zernig et al. 1996b). Variance is expressed as the 95% confidence limits.

[b] ED_{50} values for control dose-effect curves were back-calculated using $ED_{50,control} = K_A/((2+tau^n)^{1/n} - 1)$. See Data Analysis.

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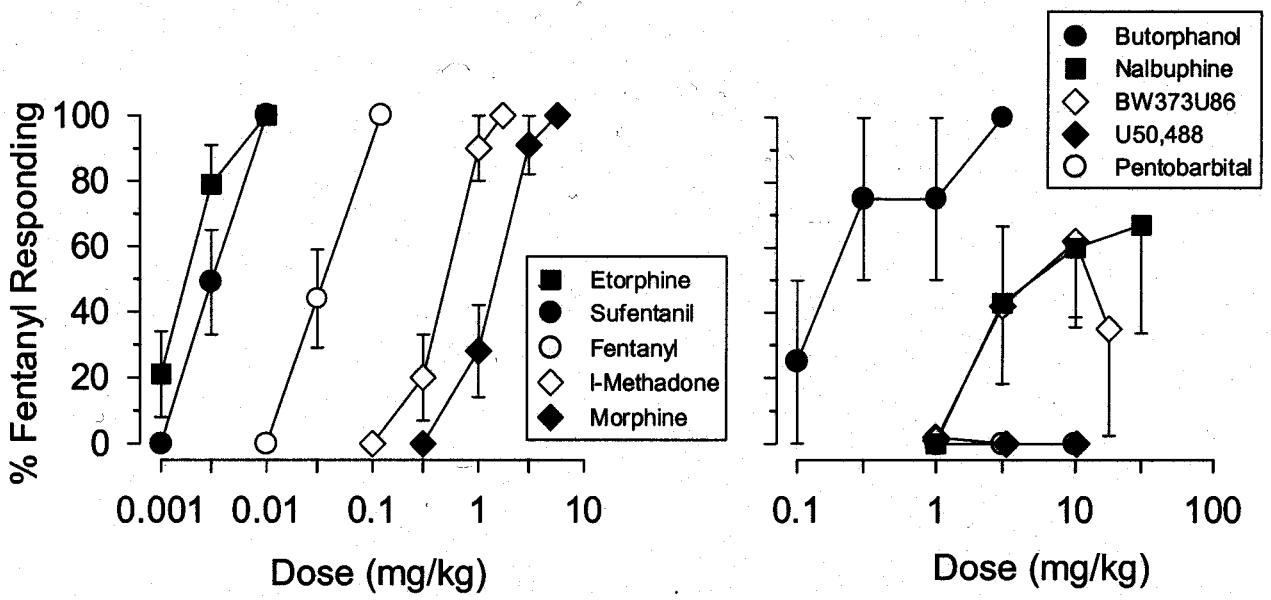

Fig. 1

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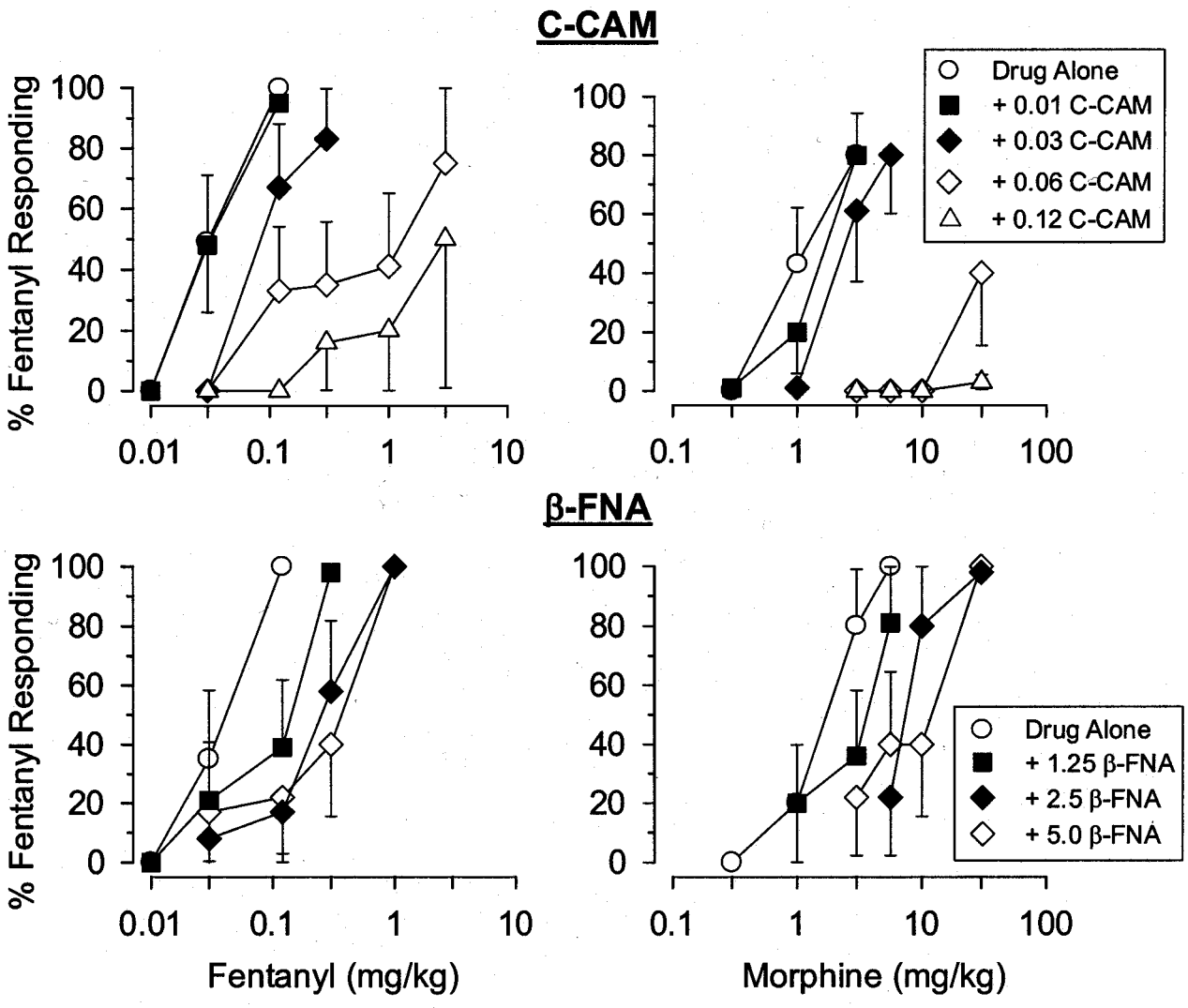

Fig. 2

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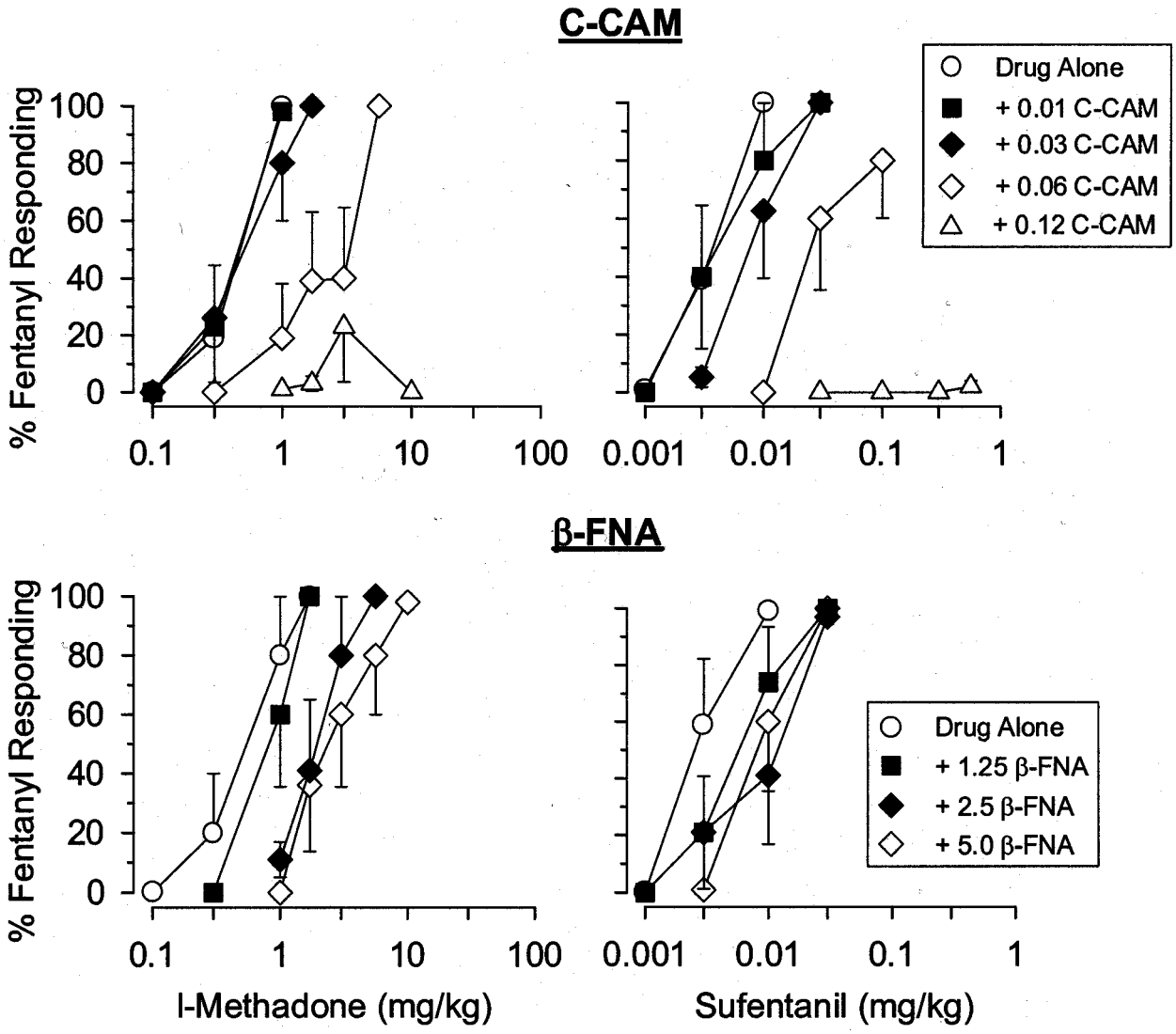

Fig. 3

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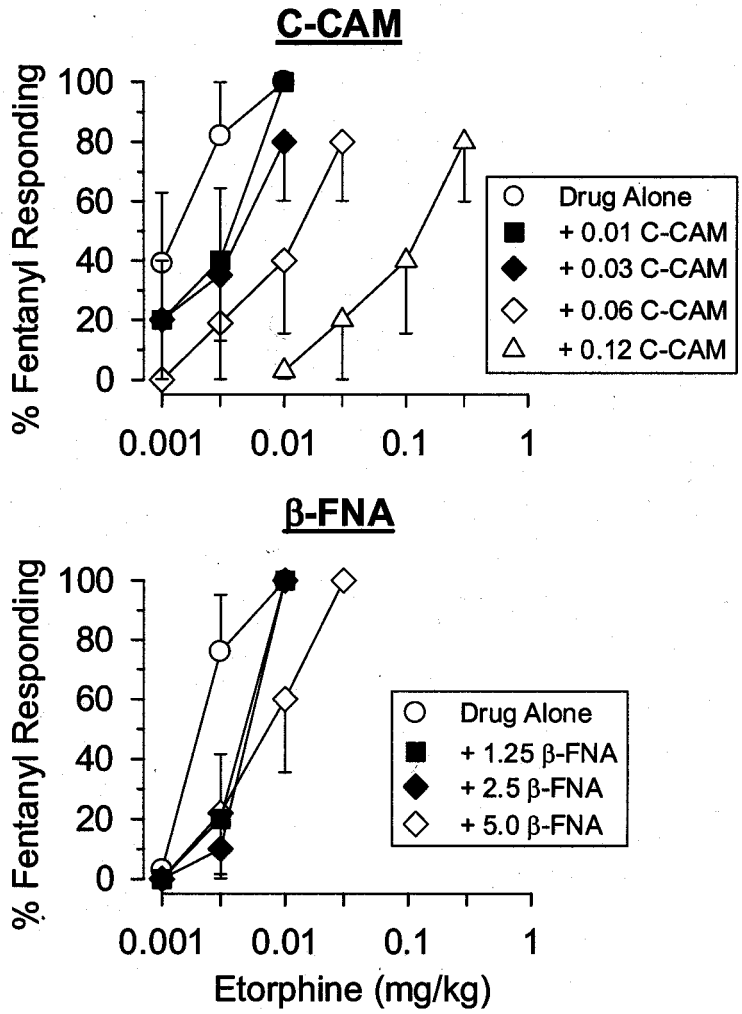

Fig. 4

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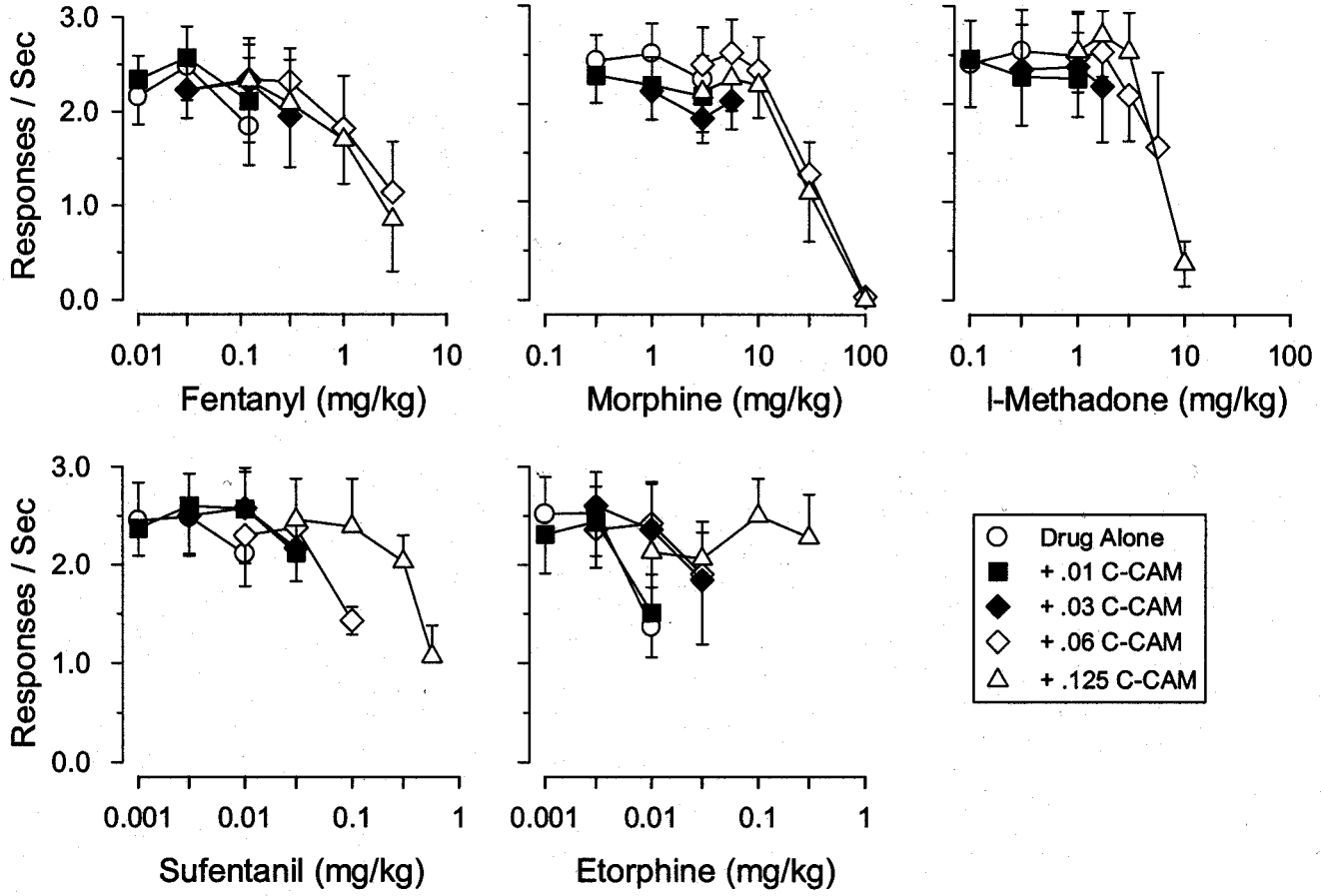

Fig. 5

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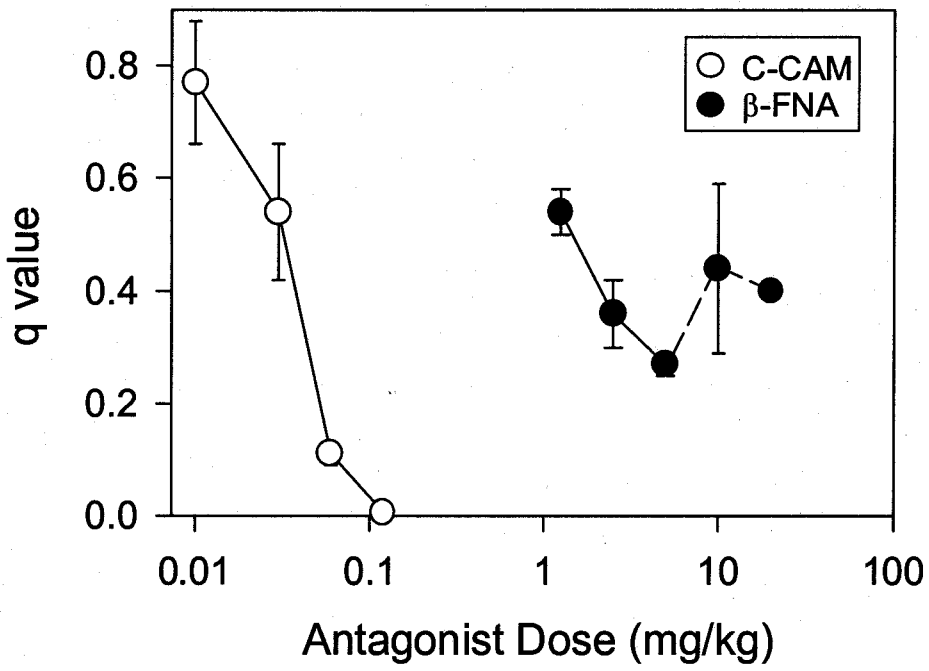

Fig. 6

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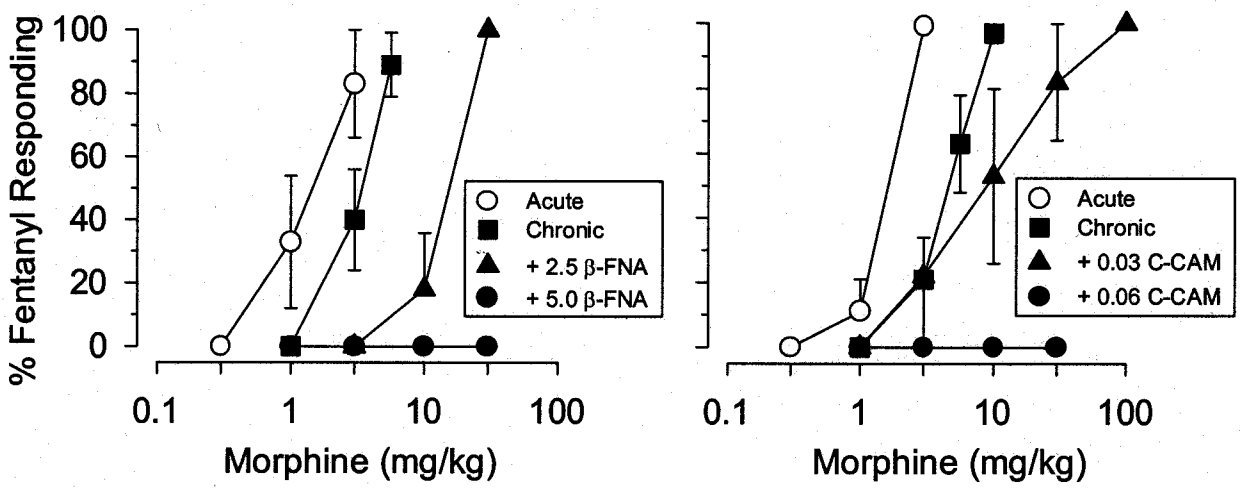

Fig. 7