# μ-Opioid Receptor Superagonists Engage Sodium-Bound Receptor States

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**Abstract**

The simple two-state conformational selection model of G-protein coupled receptor (GPCR) activation suggests that, by binding to a high affinity site, an agonist will shift receptor equilibrium in favor of active state (R*) conformations that recruit heterotrimeric G proteins over inactive state (R) conformations. Agonist binding to the μ-opioid receptor is highly sensitive to $Na^+$ ions which stabilize an inactive receptor state. Higher efficacy opioid agonists, such as DAMGO and fentanyl, are sensitive to $Na^+$ compared to lower efficacy ligands at the μ-opioid receptor. However, the binding of the highly potent oripavine agonists etorphine and dihydroetorphine are less sensitive to $Na^+$ than expected such that the prevailing models fail to explain their pharmacology. To explain this discrepancy, experiments were performed to evaluate the binding properties and G protein activation of the highly potent agonists carfentanil, BU72, etorphine, etonitazene and similarly potent opioid peptidomimetics in comparison to the standard agonists DAMGO, fentanyl, and morphine in the presence or absence of $Na^+$ or $K^+$ ions. Several of the superagonists retained high affinity and potency in both ionic conditions, whereas DAMGO, fentanyl and morphine displayed enhanced binding and signaling in $K^+$, compared to $Na^+$ ions. These functional parameters were used to determine an intrinsic efficacy value, determined as $Log(\frac{Max}{EC50})$. Comparison of affinity shifts with intrinsic

efficacy afforded a negative correlation in which superagonists with the highest intrinsic efficacy are least sensitive to $Na^+$. These data suggest that select μ-opioid receptor superagonists have high affinity for the $Na^+$ bound receptor states (R) and shift these species into active receptor conformations (R*) that efficiently couple to G proteins.

**Significance Statement**

The simple theory of conformational selection suggests the binding affinities of high efficacy μ-opioid receptor ligands, such as fentanyl and DAMGO, are more sensitive to $Na^+$ and guanine nucleotide which stabilize inactive receptor states than lower efficacy agonists and antagonists. Here, we show that ligands with high intrinsic efficacy (superagonists) are much less sensitive to $Na^+$ and guanine nucleotide. This work demonstrates that highly potent ligands can engage a low affinity $Na^+$-bound receptor state that may then convert to a receptor species that efficiently couples to G protein – i.e. a conformational induction.

**Key words:** Opioid, GPCR, Mu-opioid Receptor, Intrinsic Efficacy, Affinity, Conformation Selection Theory

**Abbreviations:** CHO, Chinese hamster ovary; DAMGO, [D-Ala$^2$, NMe-Phe$^4$, Gly-ol$^5$]-enkephalin; GDP, Guanosine 5'-diphosphate; GPCR, G protein-coupled receptor; GTPγ$^{35}$S, (Guanosine-5'-O-(γ-thio) triphosphate-[$^{35}$S]); μOR, mu-opioid receptor.

47 **Introduction**

48 Mu-opioid receptors (μOR) are G protein-coupled receptor (GPCR) proteins that activate heterotrimeric
49 G proteins of the $G\alpha_{i/o}$ family. The mu-opioid receptors, like other GPCRs, exist in an equilibrium of
50 various conformational states that can be grouped into ensembles of inactive (R) and active states (R*);
51 the R* state interacting with the G protein α-subunit. Intrinsic efficacy describes the ability of a ligand to
52 shift the equilibrium from inactive-state conformations to active-state conformations. This parameter
53 distinguishes ligands that prefer the "low affinity" R state (inverse agonists) from those that prefer a "high
54 affinity" R* state (partial agonists and full agonists) and from those that display no preference (neutral
55 antagonists). Intrinsic efficacy, in conjunction with binding affinity and pharmacokinetic properties, are the
56 driving factors that determine the pharmacological activity of a drug for a particular receptor system.[1]

57 Stimulation of GTP$\gamma^{35}$S binding to G proteins is a sensitive indicator of the active R* state and may be
58 used as a predictive measure of the intrinsic efficacy of a ligand.[2] GTP$\gamma^{35}$S binding assays allow for the
59 determination of potency ($EC_{50}$) and relative efficacy as maximal stimulation by a ligand compared to a
60 standard agonist. Determination of a $Log\ (\frac{Max}{EC50})$ from these data is a convenient way to quantify the degree
61 of ligand agonism.[3] One advantage of using the GTP$\gamma^{35}$S binding assay to assess ligand efficacy is that
62 partial agonists are aptly discriminated from full agonists, since G protein activation is directly
63 downstream of ligand-receptor binding and is less susceptible to signal amplification. On the other hand,
64 and like other functional assays that rely on measurement of signal transduction events downstream of the
65 receptor, results are dependent on experimental conditions.[4,5]

66 Agonist-mediated activation of the μOR is negatively modulated by the binding of $Na^+$ ions.[6,7,8] These
67 ions bind deep in the receptor below the orthosteric site and form ionic interactions with transmembrane
68 domains 2, 3, and 7, holding the receptor in an inactive set of conformations.[9] Thus, agonists and $Na^+$
69 ions work in opposition; agonists shift the equilibrium toward active R* ensembles whereas $Na^+$ ions

70 shift the equilibrium toward inactive R ensembles.[9,10,11] This negative cooperativity has been long known
71 at μOR, in which the addition of $Na^+$ ions with guanine nucleotide to uncouple G proteins, decreases the
72 binding affinity ($K_i$) of opioid agonists.[6,12] Higher efficacy opioid agonists show a greater decrease in
73 binding affinity upon the addition of $Na^+$ and guanine nucleotide compared to lower efficacy agonists and
74 antagonists display no shift in affinity.[6,13] There appears to be a positive association between the decrease
75 in the $K_i$ of an agonist by $Na^+$ (i.e. sodium sensitivity) and the intrinsic efficacy of an opioid. However, two
76 very potent opioid agonists, etorphine and dihydroetorphine, demonstrate significantly reduced sodium
77 sensitivity which questions the validity of the observed positive association between sodium sensitivity and
78 intrinsic efficacy at μOR.[13]

79 The effects of sodium on μOR activation cannot be treated as uniform or universal. Sodium may stabilize
80 multiple inactive conformations, but its allosteric influence is probe-dependent and varies with the
81 specific agonist engaged, based largely on agonist efficacy. Different ligands couple to the receptor in
82 distinct ways that either disrupt or accommodate sodium binding.[13,14] For superagonists, it is possible that
83 receptor engagement does not require displacement of the $Na^+$ ion; instead, these ligands may interact
84 with $Na^+$-bound states leading to displacement of $Na^+$ and receptor activation, i.e. conformational
85 induction or induced fit. Alternatively, superagonists bind and stabilize $Na^+$-bound receptor ensembles
86 that are still competent to recruit G protein. Thus, receptor activation may proceed through several parallel
87 trajectories, with some involving sodium displacement and others proceeding through $Na^+$-bound "active-
88 like" states that still facilitate G protein coupling. These observations suggest that the role of $Na^+$ in μOR
89 activation should not be considered strictly inhibitory but instead may include $Na^+$-bound states that
90 remain competent to interact with high-efficacy agonists.

91 Here, we report the determination of intrinsic efficacy for a series of potent opioids including carfentanil,
92 BU72, etorphine and etonitazene, together with a series of highly potent opioid peptidomimetics, and
93 compare them with standard opioids morphine, DAMGO and fentanyl (**Figure 1**). By evaluating the affinity

94 of these ligands in the absence and presence of $Na^+$ and guanine nucleotide and calculating the difference
95 in affinity between the two conditions (i.e. "affinity shift"), we show that for highly potent opioid agonists,
96 reduced $Na^+$ sensitivity correlates significantly with higher intrinsic efficacy. The model of conformational
97 selection would predict the compounds to be neutral antagonists or low efficacy agonists, as they
98 demonstrate small affinity shifts and recognize different affinity states (inactive and active) in a near equal
99 manner. Instead, we suggest a conformational induction model in which highly potent agonists bind to a
100 low-affinity state of μOR in the presence of $Na^+$ ions and shift the receptor into a high-affinity state capable
101 of recruiting G protein.

102 **Methods**

103 ***Materials and Reagents***

104 We have previously reported the synthesis and pharmacological evaluation of novel opioid
105 peptidomimetic compounds.[15,16,17,18,19,20,21] AAH8 (compound 14a) was synthesized by Aubrie A.
106 Harland[16], AFN42 (compound 4d) was synthesized by Anthony F. Nastase[21], AMB46 (compound 10n)
107 and AMB47(Compound 10m) were synthesized by Aaron M. Bender.[15] All chemicals, unless otherwise
108 specified, were purchased from Sigma Aldrich. Radiolabeled [$^3$H]-diprenorphine (DPN) and radiolabeled
109 guanosine-5′-O-(3-[$^{35}$S] thio)-triphosphate (GTPγ$^{35}$S) were purchased from PerkinElmer Life Sciences.
110 All tissue culture media, penicillin-streptomycin, geneticin (G148), trypsin, and fetal bovine serum were
111 purchased from Gibco LifeSciences.

112 ***Cell Lines***

113 The tissue culture and maintenance of C6 rat glioma cells stably transfected with rat μOR was performed
114 as previously described.[21] C6 cells were grown to confluence at 37 °C in 5 % $CO_2$ in Dulbecco's modified
115 Eagle media (DMEM) containing 10 % fetal bovine serum (FBS), 5 % penicillin/streptomycin (P/S), and
116 400 ug/mL G418. CHO cells stably expressing wild-type human μOR were grown to confluence at 37 °C
117 in 5 % $CO_2$ in Dulbecco's modified Eagle media (DMEM) containing 10 % fetal bovine serum (FBS),
118 1 % penicillin/streptomycin (P/S), and 400 ug/mL G418.

119 ***Cell Membrane Preparation***

120 Cell membrane homogenates from C6 and CHO cells were prepared by washing confluent cells three
121 times with phosphate buffered saline (pH 7.4), after which they were then detached using harvesting
122 buffer (20 mM HEPES, 150 mM NaCl, 0.68 mM EDTA, pH7.4) and pelleted by centrifugation at 200 x g
123 for 3 min at room temperature. The pellet was resuspended in ice-cold 50 mM Tris buffer (pH 7.4) and
124 homogenized using a Tissue Tearor (Biospec Products, Inc.). The cell lysate was centrifuged at 20,000 x g
125 at 4 °C for 20 minutes, after which the pellet was then resuspended on ice cold 50 mM Tris (pH7.4),
126 homogenized, and centrifuged once more at 20000 x g at 4 C for 20 minutes. The final pellet was
127 resuspended in 50 mM Tris (pH 7.4) using a glass dounce homogenizer and then dispensed into aliquots
128 and stored at -80 °C. The final protein concentration was determined by performing a BCA protein assay
129 (Thermo Scientific Pierce) using bovine serum albumin as the standard.

130 ***Competition Radioligand Binding Assays***

131 $^{3}$H-diprenorphine competition binding assays were performed in 500µL reactions in 96-well plates using
132 the cell membrane homogenates described above. Briefly, cell membranes [5-20 µg] were incubated in 50
133 mM Tris buffer containing 0.2 nM $^{3}$H-Diprenorphine, and various concentrations of opioid compound for
134 90 minutes in a shaking water bath at room temperature to reach equilibrium. Nonspecific binding was
135 determined using 10 µM of the opioid antagonist, naloxone. The reaction was terminated by vacuum
136 filtration onto glass microfiber GF/C filters using a tissue harvester (Brandel, Gaithersburg, MD) and
137 washed with 50 mM Tris buffer. Filters were dried and following the addition of EcoLume scintillation
138 cocktail, bound radioactivity was quantified in a MicroBeta 2450 Liquid Scintillation and Luminescence
139 Counter (PerkinElmer).

140 Radioligand binding assays in $Na^{+}$ or $K^{+}$ containing buffers were performed in 200 µL reactions in 96-
141 well plates using the cell membrane homogenates described above, Briefly, cell membranes [5-20 µg]
142 were incubated in $Na^{+}$ or $K^{+}$ containing buffers (50 mM Tris, 100 mM NaCl or 100 mM KCl, 5 mM
143 MgCl2, 1 mM EDTA, pH 7.4) or (50 mM Tris, 300 mM NaCl, 5 mM MgCl2, 1 mM EDTA, pH 7.4)

144 containing 10 μM cold GTPγS, and various concentrations of opioid compound for 90 minutes in a
145 shaking water bath at room temperature. Nonspecific binding was determined using 10 μM naloxone. The
146 reaction was terminated by vacuum filtration onto glass microfiber GF/C filters using a tissue harvester
147 and washed with $Na^+$ containing buffer, and the filters were processed as described above.

148 ***Stimulation of GTPγ$^{35}$S Binding Assays***

149 Stimulation of GTPγ$^{35}$S binding experiments were performed in 200 μL reactions in 96-well plates using
150 cell membrane homogenates prepared as described above. Briefly, cell membranes [10-20 μg] were
151 incubated in either $Na^+$ or $K^+$ containing buffer [50 mM Tris, 100 mM NaCl or 100mM KCl, 5 mM
152 MgCl2, 1 mM EDTA, pH 7.4] containing 0.1 nM guanosine-5′-O-(3-[$^{35}$S]thio)triphosphate (GTPγ$^{35}$S)
153 and 30 μM guanosine 5'-diphosphate (GDP) and various concentrations of opioid compound or
154 peptidomimetic for 1 hour in a shaking water bath at 25 °C. Basal stimulation of GTPγ$^{35}$S binding was
155 determined by incubation in the absence of any ligand and maximal stimulation of was defined by using
156 10 μM of the standard mu opioid receptor agonist, DAMGO ([D-Ala$^2$,N-MePhe$^4$,Gly-ol]-enkephalin).
157 The reaction was terminated by vacuum filtration onto glass microfiber GF/C filters using a tissue
158 harvester and washed with $Na^+$ containing buffer, and filters were processed as described above.

159 **Results**

160 **GTPγ$^{35}$S Binding**

161 As evaluated by concentration response curves of GTPγ$^{35}$S binding in C6 μOR membranes, etorphine,
162 BU72, carfentanil and etonitazene were seen to be very potent agonists with $EC_{50}$ values in the low
163 nanomolar and sub-nanomolar range **(Table 1 and Figure 2)**. In addition, we have previously described a
164 series of highly potent peptidomimetics that bind to μOR with exceptional affinity.[15,16,21] These
165 compounds (H-K in **Figure 1**) are at least 70 times more potent than the standard opioid peptide
166 DAMGO, fentanyl, or morphine with potencies that are similar to carfentanil and etonitazene. All
167 compounds demonstrated high relative efficacy, similar to DAMGO, as evaluated by maximal stimulation
168 of GTPγ$^{35}$S binding. The fractional maximal response (i.e. "Max") compared to DAMGO (Max = 1) was
169 equivalent for all ligands.

170 **Agonist Binding Affinities**

171 It has been known for decades that physiologically relevant $Na^+$ ion concentrations decrease the binding
172 affinity of agonists to μOR.[22] Radioligand competition binding assays were performed using membranes
173 from C6 cells expressing the rat μOR in the presence or absence of $Na^+$ ions and guanine nucleotide
174 (GTPγS) to induce R and R* receptor states, respectively. Difference in binding affinities between the two
175 conditions were given as affinity shifts (**Table 1 and Figure 2**). In 50 mM Tris, the superagonists
176 carfentanil, BU72, etorphine and etonitazene bound with approximately 10-fold higher affinity than the
177 classical agonists DAMGO, morphine and fentanyl. The peptidomimetics exhibited similar binding
178 affinities, with AAH8 displaying the highest affinity.

179 Binding affinities of DAMGO, fentanyl, and morphine to μOR decreased in the presence of 100 mM $Na^+$
180 and GTPγS, producing affinity shifts of 131, 52 and 77-fold, respectively (**Table 1**). Of the superagonists,
181 etorphine and etonitazene displayed the greatest affinity shifts of 24 and 33-fold, respectively. Carfentanil,

182 AAH8 and AMB47 all displayed 10-fold decreases in affinity, whereas BU72, AFN42 and AMB47
183 displayed much smaller shifts or no shift.

184 Since the superagonists displayed a less than expected affinity shift in the presence of 100 mM $Na^+$ and
185 GTPγS, we utilized a supraphysiological concentration of $Na^+$ to determine whether the affinity shift for
186 each superagonist was saturated (**Table 1**). In the presence of 300 mM $Na^+$ ions and GTPγS, an affinity
187 shift of 1,240-fold was observed for DAMGO, whereas fentanyl and morphine displayed smaller shifts of
188 105 and 84-fold, respectively. Of the highly potent agonists, only etonitazene produced an affinity shift
189 greater than 100-fold. Etorphine, AAH8 and AMB46 had comparable affinity shifts ranging from 29 to 67-
190 fold. Carfentanil and AMB47 displayed smaller shifts of 14 and 15-fold, respectively. Two compounds
191 exhibited shifts of less than 10-fold, these ligands being BU72 (7-fold) and AFN42 (3-fold).

192 **Comparison of the Effect of $Na^+$ and $K^+$ Ions on Agonist-Mediated GTPγ$^{35}$S Binding and Ligand**
193 **Affinity**

194 Since we compared agonist binding in $Na^+$ and GTPγS to binding in 50 mM Tris-HCl buffer alone, we
195 needed to evaluate the role of ionic strength on the binding and functional measures. To do this, we replaced
196 NaCl with the same concentration of KCl and examined two superagonists, carfentanil and AFN42. The
197 conserved class A GPCR allosteric sodium site is defined by a tight coordination network involving $Asp^{2.50}$
198 and multiple other polar residues. This geometry selectively accommodates $Na^+$ and is not observed with
199 larger monovalent cations, like $K^+$, in solved crystal structures.[23] Therefore, the larger $K^+$ ion would not be
200 expected to efficiently hold the receptor in an inactive conformation. Consequently, in the GTPγ$^{35}$S assay
201 the standard opioid agonist DAMGO exhibited 4-fold greater potency in 100 mM KCl relative to 100 mM
202 NaCl (11 nM (8.4 – 16 nM) vs. 42 nM (39 – 48 nM) respectively), consistent with previous evidence that
203 $Na^+$ stabilizes an inactive receptor (R) conformations and so inhibits G protein coupling (**Figure 3**).[6,7,9] In
204 support, diprenorphine exhibited partial agonist activity in the 100 mM KCl condition. However, the
205 superagonist tested AFN42 demonstrated increased potency in the presence of $Na^+$ compared to $K^+$ (AFN42

206 in NaCl: 0.058 nM (0.043 – 0.081 nM) vs. in KCl: 0.25 nM (0.19 – 0.35 nM) and carfentanil near equivalent
207 potency (carfentanil in NaCl: 0.37 nM (0.28 – 0.49 nM) vs in KCl (0.19 nM (0.15 – 0.25 nM). This suggests
208 that $Na^+$ does not universally prevent agonist activity unlike previously thought to be true.

209 To evaluate the contribution of $Na^+$ ions and GTPγS on ligand-receptor interactions, we compared the above
210 results with radioligand binding assays in the presence of 100 mM KCl and GTPγS. These latter conditions
211 lead to a smaller rightward shift in affinity, consistent with the inability of $K^+$ ions to replace $Na^+$ ions and
212 stabilize a low affinity state (**Table 2**). In contrast, AFN42 displayed a greater rightward shift in 100 mM
213 KCl (18.1-fold) than it did in the presence of 100 mM NaCl (0.7-fold) or even 300 mM NaCl (2.9-fold)
214 (**Table 2**). Similarly, carfentanil displayed no significant change in affinity when examined in 100 mM KCl
215 ($K_i$: 1.2 nM), 100 mM NaCl ($K_i$: 2.1 nM), or 300 mM NaCl ($K_i$: 2.4 nM).

216 These data suggest that $Na^+$ is not always inhibitory to agonist binding and indicates superagonists are
217 capable of binding to a $Na^+$-bound receptor state. It may then convert this receptor species into a $Na^+$-free
218 state that can then efficiently couple to G proteins.

219 **Agonist Binding in CHO Cells Expressing Human μOR**

220 Since the above data was obtained in C6 glioma cells expressing the rat μOR, we performed experiments
221 in CHO cells expressing human μOR (CHO-μOR). Radioligand binding experiments were performed in
222 CHO-μOR cell membranes in the absence or presence of 100 mM $Na^+$ ions and GTPγS to confirm that the
223 affinity shifts observed were not specific to the rat μOR or dependent on the cell type/receptor environment
224 (**Table 3**). Three ligands were examined – DAMGO, BU72 and carfentanil. In the presence of 100 mM $Na^+$
225 and GTPγS, DAMGO showed a substantial decrease in binding affinity, resulting in a shift of 810-fold,
226 larger than the 131-fold shift observed in the C6 μOR cells. Both BU72 and carfentanil exhibited smaller
227 shifts of 3 and 35-fold, respectively, although these shifts were greater than observed in the C6 cells. The
228 differences in affinity shifts observed between ligands and cell types could be due to differences between

229 the human and rat μOR, as there are subtle variations in amino acid sequences which have been shown can
230 lead to differences in ligand binding affinities.[24] Additionally, their cellular environments (hamster ovary
231 versus rat glioma cell) and access to different accessory proteins on the membranes may also be of influence.
232 Nevertheless, the same pattern is observed across the two species of μOR and cell types.

233 **Correlating Intrinsic Efficacy with Affinity Shifts in NaCl Conditions**

234 The level of agonism was determined for each opioid agonist by calculating the $Log\ (\frac{Max}{EC50})$ from GTPγ$^{35}$S
235 binding assays performed in 100 mM NaCl (as described in Kenakin, 2017).[3] The value for AFN42 (10.26)
236 is much greater than that of DAMGO (7.38). Since these parameters are on a logarithmic scale, the
237 difference in agonism between AFN42 and DAMGO (10.26 – 7.38 = 2.88) is a 760-fold difference. Since
238 the superagonists tested give the same maximal response the difference in agonism is attributable to the
239 difference in $EC_{50}$. The agonism value for each compound was then plotted in comparison to the affinity
240 shift seen in the presence of $Na^+$ ions and GTPγS (**Figure 4**). This plot shows that for full agonists in the
241 GTPγ$^{35}$S assay, agonism and affinity shift are negatively correlated in both the 100 mM $Na^+$/GTPγS
242 conditions ($r^2$ = 0.63, p-value < 0.003) and the 300 mM $Na^+$/GTPγS conditions ($r^2$ = 0.45, p-value = 0.02).
243 Thus, both the level of agonism and $Na^+$-dependent affinity shifts serve as complementary and predictive
244 readouts of superagonism at the receptor.

245 **Discussion**

246 In this study, we investigate the potency and binding affinity of a series of highly potent agonists
247 (superagonists) at μOR using GTPγ$^{35}$S assays, and radioligand competition binding assays in the absence
248 or presence of $Na^+$ ions and guanine nucleotide (GTPγS). $Na^+$ ions act as negative allosteric modulators of
249 the receptor, stabilizing the inactive R resting state. Similarly, guanine nucleotides prevent the G protein
250 from stabilizing the active R* state by binding to the Gα subunit of the G protein heterotrimer. Agonists
251 preferentially bind to the active R* receptor state, exhibiting higher affinity for R* than for R. Under the

252 conformational selection model, this binding shifts the receptor population or equilibrium toward the active
253 R* state. However, our data comparing differences in agonist affinities in two ionic conditions with the
254 GTPγ$^{35}$S functional data revealed that superagonists may be far less sensitive to $Na^+$ ions and guanine
255 nucleotide than predicted. Although the conformational selection model predicts the action of most types
256 of ligands (such as endogenous agonists and agonists with similar kinetic properties, inverse agonists,
257 partial agonists and antagonists), our data suggests it falls short in explaining the action of extremely potent
258 opioid agonists, which appear able to function through a conformation induced or induced-fit model,
259 binding to an inactive $Na^+$-bound R states and converting these to active R* states.

260 Binding assays performed in conditions in which $Na^+$ ions were replaced with $K^+$ ions to maintain
261 osmolarity highlighted the difference in behavior seen between superagonists and less potent agonists.
262 DAMGO and morphine bind more tightly to μOR in the presence of 100 mM KCl and GTPγS, consistent
263 with the absence of $Na^+$-mediated allosteric inhibition. However, AFN42 exhibits a paradoxical increase in
264 binding affinity in the presence of 100 mM or even 300 mM $Na^+$ ions and GTPγS compared to its affinity
265 in 100 mM $K^+$ ions with GTPγS. This suggests that $Na^+$ occupancy of its allosteric site on μOR may even
266 favor the binding of some superagonists, supporting a model in which these agonists access a $Na^+$-bound
267 receptor conformation that is far less accessible to conventional, less potent agonists. Thus, $Na^+$ ions do not
268 exhibit a uniform inhibitory effect across all agonists, rather the role of $Na^+$ as a negative allosteric
269 modulator can be in part dependent on agonist affinity. In support of this, at the delta-opioid receptor (δOR),
270 binding of the highly potent agonist BW373U86 has been reported as insensitive to $Na^+$ ions and guanine
271 nucleotide, compared to a peptide agonist.[25] However, high-affinity superagonists are not universally
272 agnostic to the effects of $Na^+$ ions as seen in the aforementioned study with the peptide agonist DSLET and
273 in our own study with etonitazene. It may reflect a specific interaction, or a coordinated interaction network,
274 that mediates this $Na^+$ insensitivity.

275 A recent structural and computational study of μOR partial, full and superagonists demonstrate that G
276 protein activation proceeds through a series of intermediate conformational states that are separated by
277 energetic barriers, and that the differences in agonist efficacy arise from the kinetics of traversing these
278 steps rather than from distinct activation pathways.[26] Superagonists increase receptor dynamics, allowing
279 for a substantially more rapid transition towards active ternary complexes relative to full and partial
280 agonists, where partial agonists stabilize more rigid intermediate conformations that struggle to overcome
281 these energy barriers needed to initiate fully active complexes. Consistent with this, molecular dynamics
282 simulations initiated from the inactive μOR demonstrate that the superagonist lofentanil can drive
283 spontaneous transitions into an active receptor-G protein complex in a subset of trajectories, further
284 indicating that superagonists can bind to inactive receptors and markedly reduce the energetic barrier
285 required for activation.[26]

286 The $Na^+$ ion binding site on class A GPCRs comprising of $Asp^{2.50}$, $Asn^{1.50}$, $Ser^{3.39}$, $Asn^{7.45}$, $Ser^{7.46}$, $Asn^{7.49}$
287 and $Tyr^{7.53}$ is highly conserved across GPCRs.[9] For example, $Asp^{2.50}$, is found in 90% of non-olfactory class
288 A GPCRs, including the opioid receptor family.[9,27,28] Mutating $Asp^{2.50}$ causes different functional outcomes
289 depending on the GPCR. For example, with the rat μOR, a D114(2.50) N mutation reduces binding
290 affinities, potencies and efficacies of both DAMGO and morphine, as well as the binding affinity of the
291 peptide antagonist, CTAP.[29] Mutagenesis studies of this residue in the closely related δOR transform
292 antagonists into potent beta-arrestin-biased agonists, suggesting an important role in mediating the R-R*
293 equilibria.[31] Similar experiments have been performed in a range of Class A GPCRs and lead to a variety
294 of different pharmacological effects, including reduced downstream signaling and receptor internalization,
295 increases in agonist affinity with no change in antagonist affinity, no change in agonist affinity, and changes
296 in antagonist affinity while abolishing G protein signaling.[31-34] However, these effects are not universal.
297 Thus, mutating this residue at the $\beta_1$AR does not lead to significant differences in agonist binding affinity -
298 even in the presence of $Na^+$ - suggesting the activity of this receptor is not modulated by $Na^+$.[35] However
299 consideration should be taken, as the reported experiments were done in insect Sf9 cell membranes and

300 using only one agonist, isoprenaline. Further functional, kinetic, and structural studies will need to be
301 performed on additional GPCRs to determine whether insensitivity to $Na^+$ ions and guanine nucleotide with
302 highly potent agonists is a general phenomenon.

303 The structure of the allosteric $Na^+$ site has been finely tuned to delicately balance the transition between R
304 and R* through stabilizing GPCRs in their ligand-free state. Our results indicate that superagonists of μOR
305 with high intrinsic efficacy may negate this physiological role of $Na^+$ by binding to a $Na^+$-bound receptor
306 state, and facilitating it's transition into an active receptor state that is able to efficiently couple to
307 heterotrimeric G proteins. Alternatively, $Na^+$ ions and guanine nucleotides by promoting the R form of the
308 receptor speed up agonist dissociation. It is possible that very potent agonists hold the receptor in a high
309 affinity active ternary complex due to their slow rates of dissociation from the receptor and markedly slow
310 the ability of $Na^+$ and guanine nucleotides to influence the state of the receptor. Certainly, it is known that
311 carfentanil[36] and BU72[37] dissociate very slowly from the μOR, as does BWU373U86 at δOR[25], and a slow
312 dissociation rate may also explain why it is more difficult for naloxone to reverse respiratory depression
313 caused by highly potent agonists like carfentanil.[38] Furthermore, the rate of dissociation of carfentanil is
314 much slower than that of etonitazene which could explain their different sensitivities to $Na^+$ even when
315 considering their equivalent affinities and functional activity at μOR in this study. Whatever the mechanism,
316 the implication is that a decrease in binding affinity due to the presence of $Na^+$ is not a reliable predictor of
317 efficacy when dealing with very potent compounds in contrast with previous findings.[6,12] Understanding
318 the mechanisms and specific receptor-ligand interactions that mediate $Na^+$ insensitivity may allow for the
319 design of more potent GPCR agonists that are active *in vivo*, in the presence of $Na^+$ and guanine nucleotide.
320 It is important to note that this phenomenon does not occur with existing endogenous opioid peptides, but
321 exclusively with high affinity and intrinsic efficacy ligands.

322 In conclusion, the influence of $Na^+$ ions and guanine nucleotides on GPCR activation should not be viewed
323 as a uniform or obligatory inhibitory mechanism. The allosteric effect of $Na^+$ ions appears to be probe-

dependent, varying with the chemical scaffold of the bound agonist. Different agonists, or in the case reported here, superagonists can bind to low affinity receptor and lead to the collapse of the $Na^+$ binding pocket to generate active states. Alternatively, superagonists may hold the receptor in an active conformation due to their slow dissociation rates or could even stabilize unique $Na^+$-bound intermediate or active-like receptor states that remain competent for G protein engagement. In this context, superagonists may exploit $Na^+$ binding rather than oppose it, thereby promoting receptor activation through $Na^+$-bound states that conventional agonists alone cannot access. It is possible that the role of $Na^+$ in μOR activation may encompass multiple parallel trajectories, some requiring ion displacement and others proceeding through stabilized $Na^+$-bound intermediates that facilitate G protein coupling.

| **C6 μ Cells** | GTPγ$^{35}$S Binding | | | $^{3}$H-Diprenorphine Binding | | | | |
|---|---|---|---|---|---|---|---|---|
| **Compound** | **$EC_{50}$ (nM)** | **Max Stimulation** (% of DAMGO, fractional) | **Agonism** $\text{Log}\frac{\text{Max}}{\text{EC50 (M)}}$ | **$K_i$ (nM)** 50 mM Tris | **$K_i$ (nM)** + 100mM $Na^+$ +10μM GTPγS | **Affinity Shift** $\frac{\text{Ki 100mM Na +}}{\text{Ki 50mM Tris}}$ | **$K_i$ (nM)** + 300mM $Na^+$ +10μM GTPγS | **Affinity Shift** $\frac{\text{Ki 300mM Na +}}{\text{Ki 50mM Tris}}$ |
| DAMGO | 42.2 (39.0-47.9) | 1 | 7.38 | 4.71 (3.63-6.13) | 618 (421-929) | 131 | 5840 (4040-8470) | 1240 |
| Fentanyl | 233 (178-308) | 0.76 (0.72-0.80) | 6.51 | 8.33 (5.74-12.3) | 433 (239-780) | 52.0 | 873 (488-1590) | 105 |
| Morphine | 195 (148-260) | 0.60 (0.57-0.63) | 6.49 | 3.32 (2.47-4.42) | 254 (169-377) | 76.5 | 280 (155-485) | 84.3 |
| Diprenorphine | - | - | - | 0.12 (0.094-0.16) | - | - | 0.10 (0.077-0.13) | 0.83 |
| Etorphine | 0.79 (0.42-1.39) | 0.96 (0.89-1.04) | 9.08 | 0.40 (0.24-0.66) | 9.66 (6.02-15.4) | 24.2 | 26.6 (19.7-35.6) | 66.5 |
| BU72 | 0.35 (0.24-0.51) | 1.02 (0.96-1.08) | 9.46 | 0.20 (0.14-0.29) | 0.50 (0.33-0.77) | 2.5 | 1.48 (0.96-2.33) | 7.4 |
| Carfentanil | 0.37 (0.28-0.49) | 1.06 (1.01-1.11) | 9.46 | 0.17 (0.13-0.23) | 2.05 (1.37-3.03) | 12.1 | 2.36 (1.63-3.40) | 13.9 |
| Etonitazene | 0.63 (0.38-1.03) | 1.0 (0.92-1.08) | 9.20 | 0.18 (0.14-0.22) | 5.94 (4.19-8.46) | 33 | 29.8 (19.3-45.1) | 166 |
| AAH8 | 0.64 (0.43-0.95) | 0.9 (0.85-0.95) | 9.15 | 0.058 (0.043-0.079) | 0.50 (0.37-0.68) | 8.6 | 1.70 (1.25-2.31) | 29.3 |
| AFN42 | 0.058 (0.043-0.081) | 1.06 (1.01-1.11) | 10.26 | 0.093 (0.072-0.12) | 0.067 (0.054-0.083) | 0.72 | 0.27 (0.19-0.36) | 2.9 |
| AMB46 | 0.86 (0.53-1.37) | 0.96 (0.89-1.03) | 9.04 | 0.077 (0.052-0.11) | 0.91 (0.60-1.42) | 11.8 | 3.39 (2.17-5.23) | 44 |
| AMB47 | 0.59 (0.37-0.92) | 0.98 (0.91-1.05) | 9.22 | 0.15 (0.097-0.28) | 0.32 (0.27-0.38) | 2.1 | 2.29 (1.65-3.15) | 15.3 |

**Table 1: Stimulation of GTPγ$^{35}$S Binding and Inhibition of $^{3}$H-Diprenorphine Binding by Opioid Ligands in C6 μ Cells.** Stimulation of GTPγ$^{35}$S binding at the μOR was performed using membranes from C6 cells with various concentrations of opioid agonists. Maximal stimulation of GTPγ$^{35}$S binding was determined by 10 μM of the reference agonist, DAMGO. Effective concentrations ($EC_{50}$) and the fractional maximal response (Max Stimulation) were determined by nonlinear regression analysis of concentration-response curves from GTPγ$^{35}$S binding assays. Agonism (Log [Max/EC50]) was calculated using $EC_{50}$ and max stimulation values for each opioid agonist. $^{3}$H-Diprenorphine binding assays were performed using 0.2 – 0.5 nM of the radioligand in the following three buffers: **1.** 50 mM Tris buffer, **2.** 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ 1 mM EDTA, and 10 μM GTPγS, or **3.** 50 mM Tris, 300 mM NaCl, 5 mM $MgCl_2$ 1 mM EDTA, and 10 μM GTPγS. The affinity shift was determined for 100 mM $Na^+$ and 300 mM $Na^+$ conditions. Data are represented as mean plus 95% confidence intervals and all experiments were performed in duplicate or quadruplicate ($n \geq 3$).

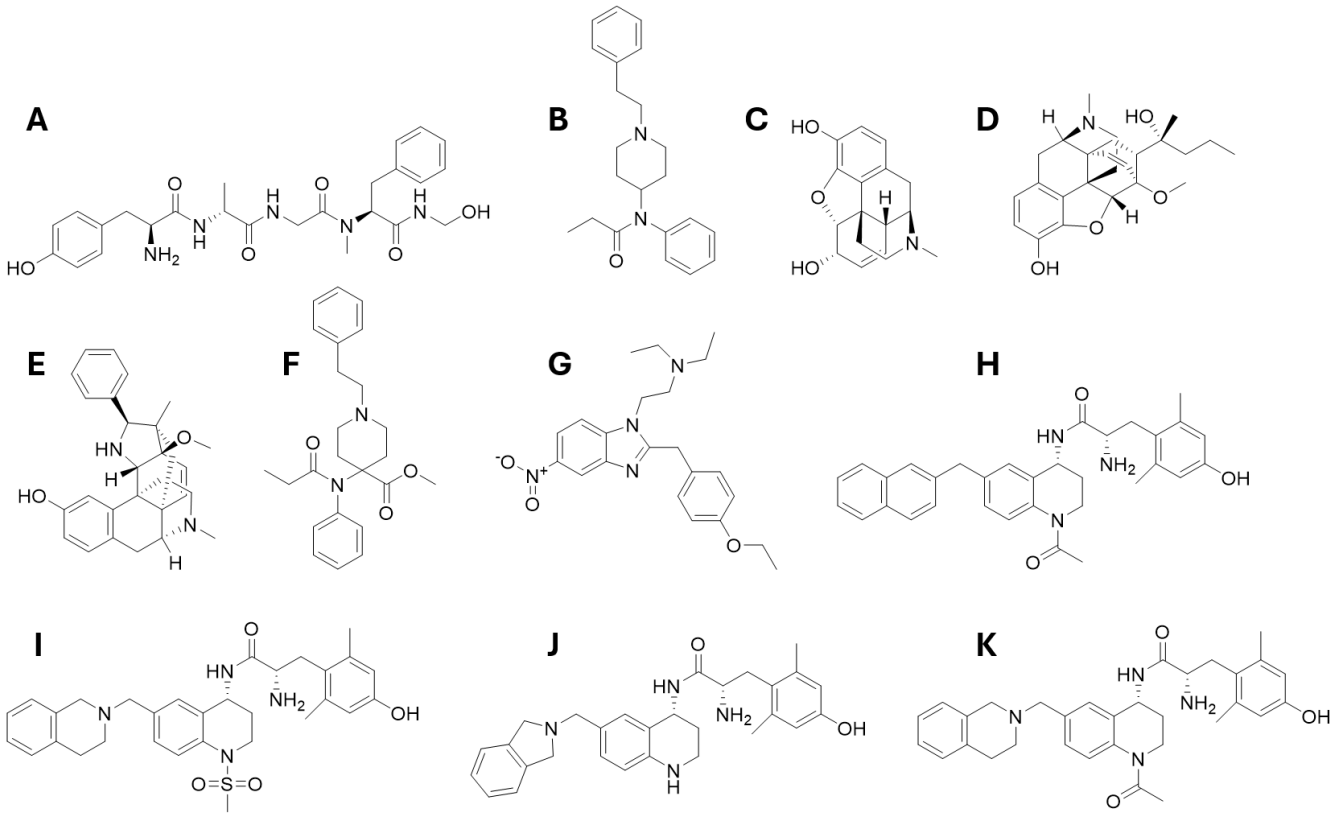


**Figure 1: Chemical Structures of Opioid Agonists and Peptidomimetics.** (A) DAMGO, (B) Fentanyl, (C) Morphine, (D) Etorphine, (E) BU72, (F) Carfentanil, (G) Etonitazene, (H) AAH8, (I) AFN42, (J) AMB46, and (K) AMB47

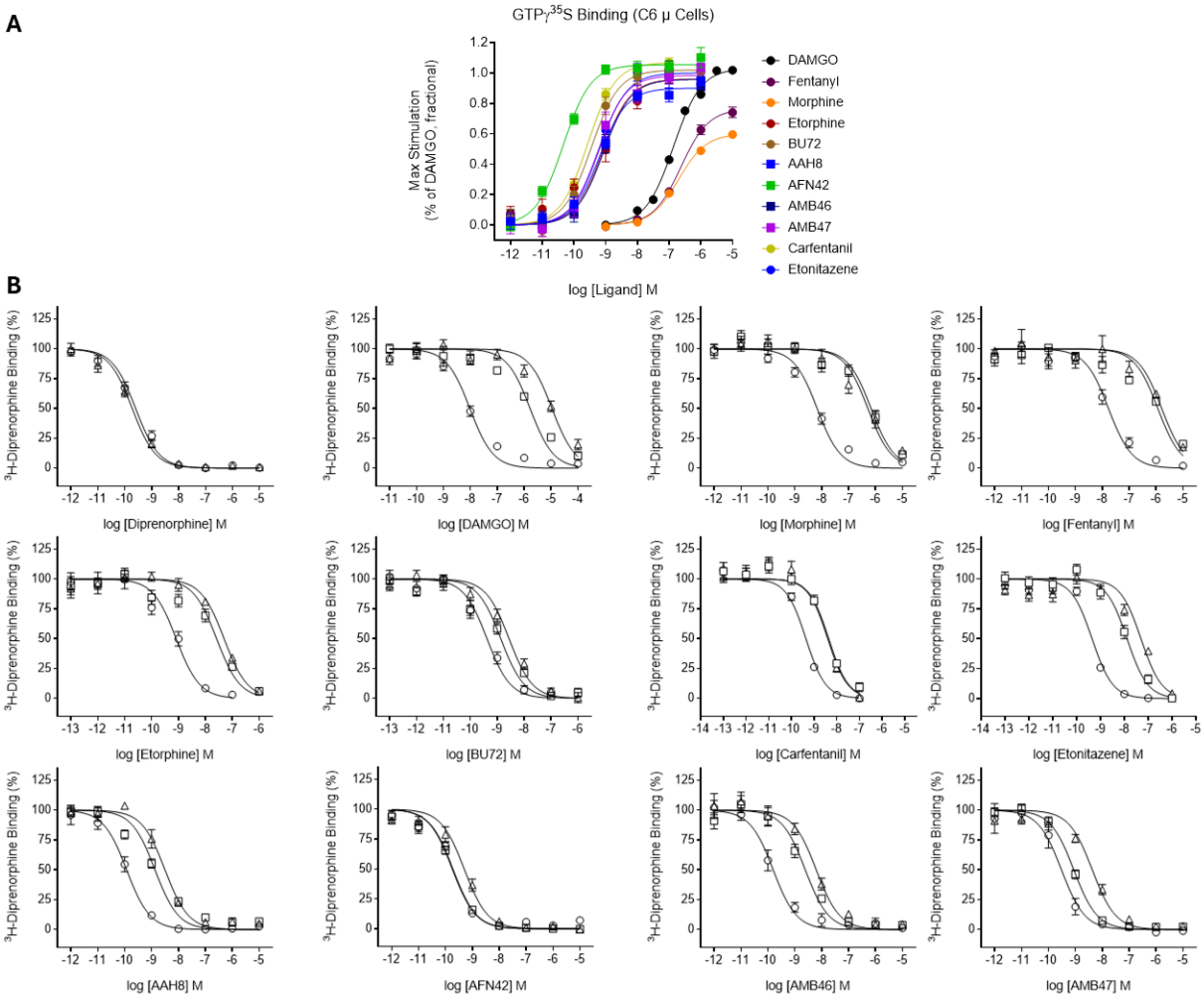


**Figure 2: Stimulation of GTPγ$^{35}$S Binding and Inhibition of $^{3}$H-Diprenorphine Binding Concentration-Response Curves in C6 μ Cells.** (A) GTPγ$^{35}$S binding assays were performed using membranes from C6 cells with various concentrations of opioid agonists. Maximal stimulation of GTPγ$^{35}$S binding was produced by 10 μM of the reference agonist, DAMGO. (B) Radioligand competition binding assays were performed in the following buffers: 50 mM Tris (circles), 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ 1 mM EDTA, 10 μM GTPγS (squares), or 50 mM Tris, 300 mM NaCl, 5 mM $MgCl_2$ 1 mM EDTA, 10 μM GTPγS (triangles). These assays used 20 μg membrane protein from C6 cells, 0.2 – 0.5 nM $^{3}$H-Diprenorphine, and various concentrations of the following opioid ligands: Diprenorphine, DAMGO, Fentanyl, Etorphine, BU72, Carfentanil, Etonitazene, AAH8, AFN42, AMB46, AMB47. All experiments were performed in duplicate or quadruplicate ($n \geq 3$).

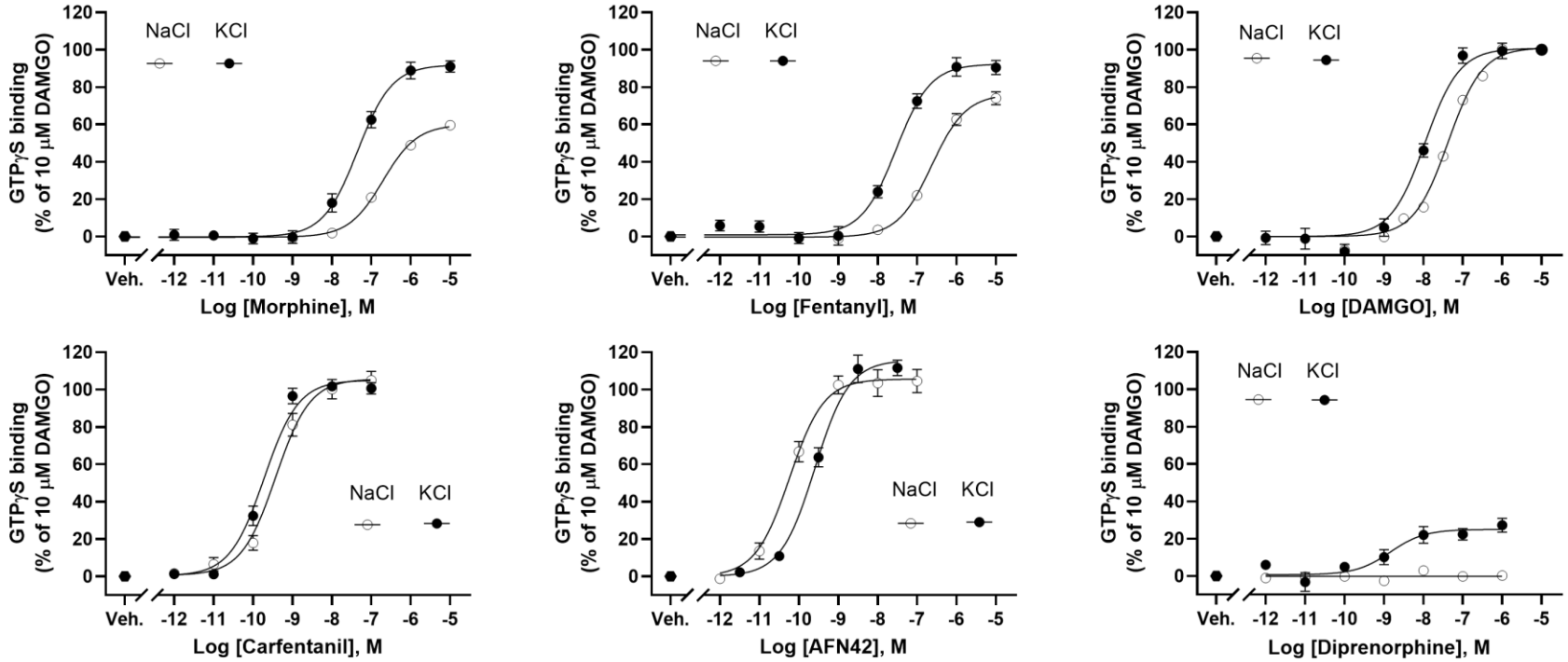


**Figure 3: Stimulation of GTPγ$^{35}$S Binding in 100 mM NaCl and KCl buffer.** Stimulation of GTPγ$^{35}$S binding at the μOR was performed using membranes from C6 cells with increasing concentrations of opioid agonists. Maximal stimulation of GTPγ$^{35}$S binding was determined by 10 μM of the reference agonist, DAMGO. Effective concentrations ($EC_{50}$) were determined by nonlinear regression analysis of concentration-response curves from GTPγ$^{35}$S binding assays. Data are represented as mean plus 95% confidence intervals and all experiments were performed in duplicate ($n \geq 3$).

| **C6** μ Cells | $^3$H-Diprenorphine Binding | | |
|---|---|---|---|
| **Compound** | **$K_i$ (nM)**<br>50 mM Tris | **$K_i$ (nM)**<br>+ 100mM $K^+$<br>+10μM GTPγS | **Affinity Shift**<br>$\frac{\text{Ki 100mM K +}}{\text{Ki 50mM Tris}}$ |
| DAMGO | 4.71<br>(3.63-6.13) | 111<br>(66.8-187) | 23.6 |
| Fentanyl | 8.33<br>(5.74-12.3) | 218<br>(140-344) | 26.2 |
| Morphine | 3.32<br>(2.47-4.42) | 150<br>(93.9-246) | 45.2 |
| Diprenorphine | 0.12<br>(0.094-0.16) | 1.96<br>(1.29-2.94) | 16.3 |
| Carfentanil | 0.17<br>(0.13-0.23) | 1.19<br>(0.81-1.76) | 7 |
| AFN42 | 0.093<br>(0.072-0.12) | 1.68<br>(1.02-2.86) | 18.1 |

**Table 2. Inhibition of $^3$H-Diprenorphine Binding in 100 mM KCl.** Radioligand competition binding assays were performed in 50 mM Tris, 100 mM KCl, 5 mM $MgCl_2$ 1 mM EDTA, 10 μM GTPγS. These assays used 20 μg membrane protein from C6 cells, 0.2 – 0.5 nM $^3$H-Diprenorphine, and various concentrations of opioid ligand. Experiments were performed in duplicate or quadruplicate ($n \geq 3$) and are presented as mean with 95% confidence intervals.

| CHO μ Cells | $^3$H-Diprenorphine Binding | | |
|---|---|---|---|
| **Compound** | **$K_i$ (nM)**<br>50 mM Tris | **$K_i$ (nM)**<br>+ 100mM $Na^+$<br>+10μM GTPγS | **Affinity Shift**<br>$\frac{\text{Ki 100mM Na +}}{\text{Ki 50mM Tris}}$ |
| DAMGO | 0.65<br>(0.40-1.05) | 529<br>(293-946) | 814 |
| BU72 | 0.27<br>(0.18-0.39) | 0.73<br>(0.54-1.01) | 2.7 |
| Carfentanil | 0.06<br>(0.035-0.10) | 2.09<br>(1.56-2.79) | 34.8 |

**Table 3: Inhibition of $^3$H-Diprenorphine Binding by Opioid Ligands in CHO μ Cells.** $^3$H-Diprenorphine binding assays were performed using 0.2 – 0.5 nM of the radioligand in the following two buffers: **1.** 50 mM Tris buffer, and **2.** 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$ 1 mM EDTA, and 10 μM cold GTPγS. The affinity shift was determined for 100 mM $Na^+$ conditions. Data are represented as mean plus 95% confidence intervals and all experiments were performed in duplicate ($n \geq 3$).

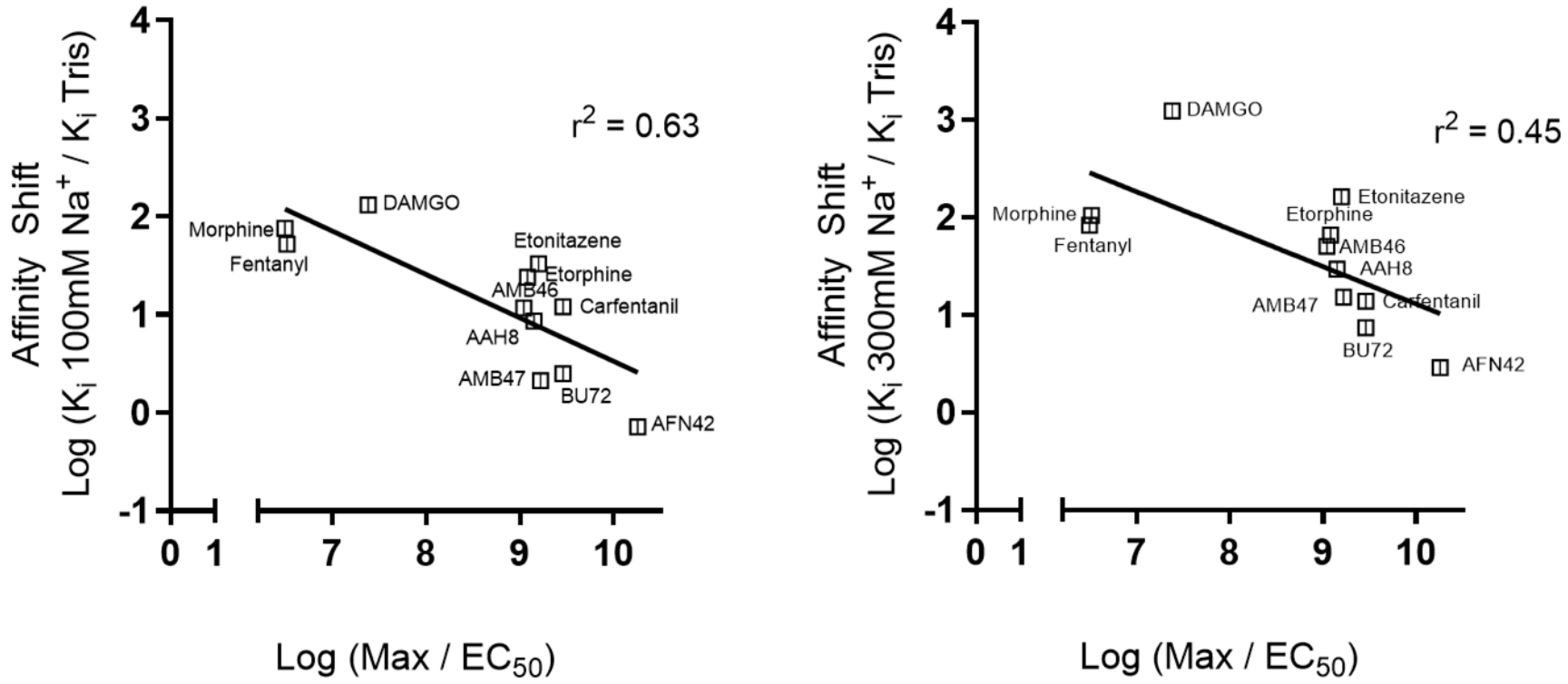


**Figure 4: Intrinsic Efficacy Plots for C6 μ.** Agonism (Log [Max/$EC_{50}$]) was determined from stimulation of GTP$\gamma^{35}$S binding assays and Affinity Shift (Log [Ki, 100 mM $Na^+$ / $K_i$ 50 mM Tris] in **(A)** or Log [Ki, 300 mM $Na^+$ / $K_i$ 50 mM Tris] in **(B)** was determined from $^3$H-Diprenorphine competition binding assays. Data was plotted for each of the following ligands: DAMGO, Fentanyl, Morphine, Etorphine, BU72, Carfentanil, Etonitazene, AFN42, AAH8, AMB46, and AMB47. $r^2 = 0.63$, $p^{**} = 0.003$ **(A)**; $r^2 = 0.45$, $p^* = 0.02$ **(B)**

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