μ-Opioid Receptor Superagonists Engage a Sodium-Bound Active State

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Abstract

The 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 sodium 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 mu-opioid receptor (μOR). 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. In an attempt 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^+. In agreement with prior studies on etorphine, several of the superagonists showed minimal affinity shifts and retained high potency in both ionic conditions, whereas conventional agonists DAMGO, fentanyl and morphine displayed enhanced

binding and signaling only in K^+. These functional parameters were used to determine an intrinsic efficacy value, determined as $Log(\frac{Max}{EC50})$. Comparison of affinity shifts with intrinsic efficacy gives 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 (R) and shift these species into active receptor conformations (R*) that efficiently couple to G proteins.

Significance Statement

The prevailing theory of conformational selection is that high efficacy ligands, such as fentanyl and DAMGO, are more sensitive to Na^+ and guanine nucleotide compared to lower efficacy agonists. Here, instead of a positive correlation and what has previously thought to be true, there is a negative correlation in which the ligands with the highest intrinsic efficacy (colloquially termed as superagonists) are the least sensitive to Na^+ and guanine nucleotide and thus show a smaller affinity shift. Thus, demonstrating that these highly potent ligands are capable of engaging a Na^+-bound receptor that may then efficiently couple to G protein. This work has broad implications for GPCR pharmacology – challenging foundational models of class A GPCR activation and providing framework for designing next-generation GPCR therapeutics that retain high affinity and potency under physiological ionic conditions.

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.

53 **Introduction**

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

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

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

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

85 From first principles, the effects of sodium on μOR activation cannot be treated as uniform or universal.
86 Sodium may stabilize multiple inactive conformations, but its allosteric influence should be considered
87 probe-dependent and vary with the specific agonist or allosteric modulator engaged. Different ligands
88 may couple to the receptor in distinct ways that either disrupt or accommodate sodium binding. For
89 superagonists, it is possible that receptor activation does not require complete displacement of the sodium
90 ion; instead, these ligands may stabilize sodium-bound intermediate receptor ensembles that are still
91 competent to recruit G proteins. Thus, receptor activation may proceed through several parallel
92 trajectories, with some involving sodium displacement and others proceeding through Na^+-bound "active-
93 like" states that facilitate G protein coupling. These observations suggest that sodium's role in GPCR
94 activation should not be considered strictly inhibitory, but rather as part of an ensemble of conformations
95 whose functional relevance depends on the ligand's mode of allosteric engagement.

96 Here, we report the determination of intrinsic efficacy for a series potent opioids, including carfentanil,
97 BU72, etorphine and etonitazene, together with a series of highly potent opioid peptidomimetics and
98 compare them with standard opioids morphine, DAMGO and fentanyl (**Figure 1**). By evaluating the affinity
99 of these ligands in the absence and presence of Na^+ and calculating the difference in affinity between the

100 two conditions (i.e. "affinity shift"), we confirm that for highly potent opioid agonists, reduced Na^+
101 sensitivity correlates significantly with higher intrinsic efficacy. The prevailing model would predict the
102 compounds to be neutral antagonists or low efficacy agonists, as they demonstrate little affinity shifts and
103 recognize different affinity states in a near equal manner. Instead, we develop a model that shows how
104 highly potent agonists bind to a low-affinity state of μOR, even in the presence of Na^+ ions, and shift the
105 receptor into a high-affinity G protein-coupled state, explaining the exceptional potency of these
106 compounds. Present results suggest that a simple conformational selection model of agonist receptor
107 interaction controlled by Na^+ ions may not be adequate to explain the receptor kinetics of highly potent
108 opioid agonists.

109 **Methods**

110 ***Materials and Reagents***

111 We have previously reported the synthesis and pharmacological evaluation of novel opioid
112 peptidomimetic compounds.[14,15,16,17,18,19,20] AAH8 was synthesized by Aubrie A. Harland, AFN42 was
113 synthesized by Anthony F. Nastase, AMB46 and AMB47 were synthesized by Aaron M. Bender.[14,15,20]
114 All chemicals, unless otherwise specified, were purchased from Sigma Aldrich. Radiolabeled [^{3}H]-
115 diprenorphine (DPN) and radiolabeled guanosine-5′-O-(3-[^{35}S] thio)-triphosphate (GTPγ^{35}S) were
116 purchased from PerkinElmer Life Sciences. All tissue culture media, penicillin-streptomycin, geneticin
117 (G148), trypsin, and fetal bovine serum were purchased from Gibco LifeSciences.

118 ***Cell Lines***

119 The tissue culture and maintenance of C6 rat glioma cells stably transfected with rat μOR was performed
120 as previously described.[21] C6 cells were grown to confluence at 37 °C in 5 % CO_2 in Dulbecco's modified
121 Eagle media (DMEM) containing 10 % fetal bovine serum (FBS), 5 % penicillin/streptomycin (P/S), and
122 400 ug/mL G418. CHO cells stably expressing wild-type human μOR were grown to confluence at 37 °C
123 in 5 % CO_2 in Dulbecco's modified Eagle media (DMEM) containing 10 % fetal bovine serum (FBS),
124 1 % penicillin/streptomycin (P/S), and 400 ug/mL G418.

125 ***Cell Membrane Preparation***

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

136 ***Competition Radioligand Binding Assays***

137 ^{3}H-diprenorphine competition binding assays were performed in 500µL reactions in 96-well plates using
138 the cell membrane homogenates described above. Briefly, cell membranes [5-20 µg] were incubated in 50
139 mM Tris buffer containing 0.2 nM ^{3}H-Diprenorphine, and various concentrations of opioid compound for
140 90 minutes in a shaking water bath at room temperature to reach equilibrium. Nonspecific binding was
141 determined using 10 µM of the opioid antagonist, naloxone. The reaction was terminated by vacuum
142 filtration onto glass microfiber GF/C filters using a tissue harvester (Brandel, Gaithersburg, MD) and
143 washed with 50 mM Tris buffer. Filters were dried and following the addition of EcoLume scintillation
144 cocktail, bound radioactivity was quantified in a MicroBeta 2450 Liquid Scintillation and Luminescence
145 Counter (PerkinElmer).

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

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

154 ***Stimulation of GTPγ^{35}S Binding Assays***

155 Stimulation of GTPγ^{35}S binding experiments were performed in 200 μL reactions in 96-well plates using
156 cell membrane homogenates prepared as described above. Briefly, cell membranes [10-20 μg] were
157 incubated in either Na^+ or K^+ containing buffer [50 mM Tris, 100 mM NaCl or 100mM KCl, 5 mM
158 MgCl2, 1 mM EDTA, pH 7.4] containing 0.1 nM guanosine-5′-O-(3-[^{35}S]thio)triphosphate (GTPγ^{35}S)
159 and 30 μM guanosine 5'-diphosphate (GDP) and various concentrations of opioid compound or
160 peptidomimetic for 1 hour in a shaking water bath at 25 °C. Basal stimulation of GTPγ^{35}S binding was
161 determined by incubation in the absence of any ligand and maximal stimulation of was defined by using
162 10 μM of the standard mu opioid receptor agonist, DAMGO ([D-Ala^2,N-MePhe^4,Gly-ol]-enkephalin).
163 The reaction was terminated by vacuum filtration onto glass microfiber GF/C filters using a tissue
164 harvester and washed with Na^+ containing buffer, and filters were processed as described above.

165 **Results**

166 **GTPγ^{35}S Binding in Sodium**

167 As evaluated by concentration response curves of GTPγ^{35}S binding in C6 μOR membranes, etorphine,
168 BU72, carfentanil and etonitazene are very potent with EC_{50} values in the low nanomolar and sub-
169 nanomolar range **(Table 1 and Figure 2)**. In addition, we have previously described a series of highly
170 potent peptidomimetics that bind to μOR with exceptional affinity.[20,22] They are at least 70 times more
171 potent than the standard opioid peptide DAMGO, fentanyl, or morphine with potencies that are similar to
172 that of the highly potent opioid agonists, etorphine, BU72, carfentanil and etonitazene. All compounds
173 demonstrated a high relative efficacy, as evaluated by maximal stimulation of GTPγ^{35}S binding. The
174 fractional maximal response (i.e. "Max") compared to DAMGO (Max = 1) was nearly equivalent for all
175 ligands, except for carfentanil and AFN42, which demonstrate supramaximal μOR stimulation.

176 **Binding Affinities in 50 mM Tris, 100 mM Na^+ and Guanine Nucleotide, or 300 mM Na^+ and Guanine**
177 **Nucleotide**

178 It has been known for decades that physiologically relevant Na^+ concentrations decrease the binding affinity
179 of agonists to μOR.[23] Radioligand competition binding assays were performed using C6 μOR cell
180 membranes in the absence or presence of Na^+ ions and guanine nucleotide to induce R and R* receptor
181 states. Affinity shifts were then determined **(Table 1 and Figure 2**). In 50 mM Tris, the superagonists
182 carfentanil, BU72, etorphine and etonitazene bound with roughly 10-fold higher affinity than the classical
183 agonists DAMGO, morphine and fentanyl. The peptidomimetics also exhibited binding affinities similar to
184 etorphine, BU72, carfentanil and etonitazene, with AAH8 displaying the highest affinity.

185 μOR binding affinities of DAMGO, fentanyl, and morphine decreased in the presence of 100 mM Na^+ and
186 guanine nucleotide, producing affinity shifts of 131, 52 and 77-fold, respectively (**Table 1**). Of the
187 superagonists, etorphine and etonitazene displayed the greatest affinity shifts of 24 and 33-fold,

188 respectively. Carfentanil, AAH8 and AMB47 all displayed 10-fold decreases in affinity, whereas BU72,
189 AFN42 and AMB47 displayed little shift.

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

198 **Effect of Ionic Composition on GTPγ^{35}S Binding and Ligand Affinity**

199 Since we compared Na^+ to Tris, we needed to evaluate the role of ionic composition in functional receptor
200 activation with GTPγ^{35}S assays either in the presence of 100 mM KCl or 100 mM NaCl. The larger K^+ ion
201 does not fit well into the allosteric Na^+ binding pocket of MOR and so would not hold the receptor in an
202 inactive conformation. Subsequently, the standard opioid agonists like DAMGO exhibited 4-fold greater
203 potency in 100 mM KCl relative to 100 mM NaCl (11 nM (8.4 – 16 nM) vs. 42 nM (39 – 48 nM)), consistent
204 with previous evidence of Na^+ stabilizing inactive receptor (R) conformations that prevent G protein
205 signaling (**Figure 3**).[6,7,9] However, the two superagonists tested – AFN42 and carfentanil – demonstrate
206 increased potency (AFN42 in NaCl: 0.058 nM (0.043 – 0.081 nM) vs. in KCl: 0.25 nM (0.19 – 0.35 nM)) or
207 near equivalent potency (carfentanil in NaCl: 0.37 nM (0.28 – 0.49 nM) vs in KCl (0.19 nM (0.15 – 0.25
208 nM) in the presence of Na^+ relative to K^+. This suggests that Na^+ does not universally prevent agonist
209 activity but may instead promote a distinct active Na^+-occupied receptor state that may then still efficiently
210 couple to G proteins when bound to these highly potent ligands.

211 To evaluate the contribution of Na^+ ions on to ligand-receptor interactions, we performed radioligand
212 binding assays in the presence of 100 mM KCl and guanine nucleotide. Our conventional opioid agonists,
213 DAMGO and morphine, exhibited higher affinity in 100 mM KCl, or a smaller rightward shift in binding
214 affinity compared to 100 mM NaCl (**Table 2**). This is consistent with reduced binding affinity due to Na^+-
215 mediated stabilization of inactive (R) receptor states. In contrast, AFN42 displayed a greater rightward shift
216 in 100 mM KCl relative to 100 mM NaCl (1.68 nM vs. 0.07 nM), and this effect is still seen in 300 mM
217 NaCl conditions (**Table 2**). Supporting this narrative, Carfentanil displayed no statistically significant
218 change in affinity between 100 mM KCl, 100 mM NaCl and 300 mM NaCl conditions. These binding data
219 suggest that Na^+ is not inhibitory to binding – rather it may enhance binding affinity in the case of AFN42,
220 further supporting the existence of a Na^+-bound receptor state that may then efficiently couple to G proteins
221 when stabilized by these superagonists.

222 **Radioligand Binding in CHO-μOR Cells with 50 mM Tris or 100 mM Na^+ and Guanine Nucleotide**

223 Additional radioligand binding experiments were performed in CHO-μOR cell membranes in the absence
224 or presence of 100 mM Na^+ and guanine nucleotide to confirm the affinity shifts were cell type/environment
225 specific (**Table 3**). Of the three ligands tested – DAMGO, BU72 and carfentanil all displayed sub-
226 nanomolar binding affinities in 50 mM Tris. In the presence of 100 mM Na^+ and guanine nucleotide, only
227 DAMGO showed a large decrease in binding affinity, resulting in a shift of 810-fold. Both BU72 and
228 carfentanil exhibited smaller shifts of 3 and 35-fold, respectively. The differences in affinity shifts observed
229 between ligands and cell types could be due to differences between the human and rat μOR, as there are
230 subtle variations in amino acid sequences which have been shown can lead to differences in ligand binding
231 affinities.[24] Additionally, their cellular environments (hamster ovary versus rat glioma cell), level of
232 receptor expression and access to different accessory proteins on the membranes may also be of influence.
233 Nevertheless, the same pattern can be observed between cell types.

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

235 The level of agonism was then determined for each opioid agonist by calculating the $Log\ (\frac{Max}{EC50})$ from
236 GTPγ^{35}S binding assays performed in 100 mM NaCl (as described in Kenakin, 2017).[3] The value for
237 AFN42 (10.26) is much greater than that of DAMGO (7.38). Since these parameters are on a logarithmic
238 scale, the difference in agonism between AFN42 and DAMGO (10.26 – 7.38 = 2.88) represents a 760-fold
239 difference. Since AFN42 and DAMGO are full agonists (Max ≥ 1), as well as the other three highly potent
240 ligands, the difference in agonism is attributable to the difference in EC_{50}. The agonism value for each
241 compound was then plotted in comparison to the affinity shift due to Na^+ and guanine nucleotide (**Figure**
242 **4**). This correlation plot shows that agonism and affinity shift are negatively correlated to both the 100 mM
243 Na^+ conditions ($r^2 = 0.63$, p-value < 0.003) and the 300 mM Na^+ conditions ($r^2 = 0.45$, p-value = 0.02). This
244 correlation confirms that these experimental methods are both predictive measures of intrinsic efficacy.
245 Moreover, etorphine, BU72, carfentanil, etonitazene, and the opioid peptidomimetics demonstrate a
246 remarkable degree of agonism and a significantly decreased sensitivity to Na^+ (100 mM or 300 mM) and
247 free nucleotide (10 μM cold GTPγS). AFN42 was the most potent compound examined with roughly 1,000
248 to 3,000-fold higher potency than DAMGO, fentanyl, and morphine. AFN42 also is the least sensitive
249 opioid compound to Na^+ and guanine nucleotide, and AFN42 has the same affinity for the inactive R state
250 and active R* state receptor.

251 **Discussion**

252 In this study, we investigate the potency and binding affinity of a series of highly potent agonists at μOR
253 using GTPγ^{35}S assays, and radioligand competition binding assays in the presence of Na^+ and guanine
254 nucleotide. Correlation plots utilizing our functional data and affinity shifts revealed that these
255 superagonists are far less sensitive to Na^+ and guanine nucleotide than predicted, supporting an inadequacy
256 of the conformational selection model to explain their pharmacology. The conformational selection model
257 of receptor activation suggests that, by binding to high affinity states of the receptor, an agonist will shift
258 the equilibrium in favor of an active state (R*) conformations. Although this model predicts the action of

259 most types of agonists (e.g. inverse agonists, partial agonists, antagonists), the current data shows it falls
260 short in explaining the mechanism of action of extremely potent opioid agonists. It is generally accepted
261 that agonists have a greater affinity for R* compared to R and are sensitive to Na^+ and guanine nucleotide,
262 because these act as negative allosteric modulators and stabilize the low affinity R conformational state.[8]
263 In contrast to this idea, the very potent and high efficacy opioid agonists appear to be much less sensitive,
264 even insensitive to Na^+ or may even prefer the Na^+-bound state. Indeed, these compounds bind with a
265 similar high affinity to both active (R*) and inactive state (R) conformations. Binding assays performed in
266 Na^+-free conditions reinforce this difference in behavior seen between conventional agonists and
267 superagonists. DAMGO and morphine bind more tightly in 100 mM KCl conditions, consistent with the
268 absence of Na^+-mediated allosteric inhibition. However, AFN42 exhibits a paradoxical decrease in affinity
269 in KCl, with greater binding affinity in 100 mM NaCl and little to no further shift in 300 mM NaCl
270 conditions. This suggests that Na^+ occupancy of its allosteric site may even favor the binding conformation
271 of superagonists. The opposing shifts in KCl buffer between ligand classes reveal that the influence of
272 sodium ions on receptor conformation, and therefore ligand affinity, is not universal and ligand-dependent,
273 further supporting a model in which superagonists access a Na^+-bound receptor that is not accessible to
274 conventional agonists.

275 Additionally, our functional data suggests that Na^+ does not exhibit a uniform inhibitory effect across all
276 agonists. While the conventional opioid agonists such as fentanyl show reduced potency in Na^+ relative to
277 K^+ conditions, consistent with the established models of Na^+-mediated negative allosteric modulation,
278 superagonists like AFN42 display positive cooperativity with Na^+ in GTPγ^{35}S assays, maintaining or even
279 enhancing their potency in Na^+ conditions. These findings provide the functional support for the existence
280 of ligands that interact with a Na^+-bound state of μOR and drive it to couple to G proteins; where
281 superagonists not only tolerate Na^+ binding to its conserved allosteric site but may actively exploit it to
282 promote receptor activation. These findings challenge the traditional two-state model of GPCR activation
283 and suggest that the role of Na^+ as a negative allosteric modulator is ligand-dependent. Together with our

284 binding data, these results support a model in which superagonists stabilize a distinct active-like
285 conformation that is permissive to Na^+ binding.

286 The β_1AR is the only currently known GPCR to not be modulated by Na^+ ions.[26] Na^+ interacts with a highly
287 conserved residue, $Asp^{2.50}$, which is found in 98% of GPCRs, including the β_1AR and opioid receptor
288 family.[27,28] Mutating this residue causes different functional outcomes depending on the GPCR. In the rat
289 μOR, a D114(2.50) N mutation reduces binding affinities, potencies and efficacies of both DAMGO and
290 morphine, as well as the binding affinity of the peptide antagonist, CTAP.[29] Similarly, for the delta-opioid
291 receptor (δOR), the binding affinities of peptide and non-peptide agonists are reduced, while antagonists
292 bind equally well to both mutated and wild-type receptors.[30] Additional mutagenesis studies of this residue
293 have shown classical δOR antagonists transformed into potent beta-arrestin-biased agonists – suggesting an
294 important role in mediating the R-R* equilibriums.[31] Similar experiments have been performed in a variety
295 of Class A GPCRs and lead to a variety of different pharmacological effects, including reduced downstream
296 signaling and receptor internalization, increases in agonist affinity with no change in antagonist affinity, no
297 change in agonist affinity, and changes in antagonist affinity while abolishing G protein signaling.[32,33,34,35]
298 However, mutating this residue at the β_1AR does not lead to significant differences in agonist binding
299 affinity - even in the presence of Na^+ - suggesting the activity of this receptor is not modulated by Na^+.[26]
300 The β_1AR is the only known Class A GPCR to not show modulation by Na^+ ions. Further functional, kinetic,
301 and structural studies will need to be performed on additional GPCRs to determine whether superagonist
302 insensitivity to Na^+ and guanine nucleotide is an opioid receptor phenomenon only (cite MOR/DOR
303 insensitivity here) and whether specific ligand-receptor interactions mediate this phenomenon.[36]

304 The structure of the allosteric Na^+ site has been finely tuned to delicately balance the transition between R
305 and R* through stabilizing GPCRs in their ligand-free state. Our results seem to indicate that superagonists
306 of μOR with high intrinsic efficacy may negate this physiological role of Na^+ through additionally binding
307 to a Na^+-bound receptor state, and facilitating it's transition into an active receptor state that is able to

308 efficiently couple to heterotrimeric G proteins. The implication of our findings is that a decrease in affinity
309 due to the presence of Na^+ is not a reliable predictor of efficacy when dealing with very potent compounds
310 and these observations are in contrast with previously accepted ideas.[6,12] It will be intriguing if this is the
311 reason why it is more difficult for naloxone to reverse respiratory depression caused by a highly potent
312 agonist like carfentanil.[37] Additionally, understanding the mechanisms and specific receptor-ligand
313 interactions that mediate Na^+ insensitivity may allow for the design of more potent GPCR agonists *in vivo*,
314 where there is an abundance of Na^+ and guanine nucleotide. It is important to note that this phenomenon
315 does not occur with existing endogenous opioid peptides, but exclusively with high affinity and intrinsic
316 efficacy ligands as observed so far.

317 The influence of sodium on GPCR activation should not be viewed as a uniform or as an obligatory
318 inhibitory mechanism. Sodium's allosteric effects appear to be probe-dependent, varying with the chemical
319 nature and coupling behavior of the bound ligand. Different agonists, or in this case superagonists, may
320 stabilize unique Na^+-bound intermediate or active-like receptor states that remain competent for G protein
321 engagement. In this context, superagonists may exploit sodium binding rather than oppose it, therefore
322 promoting receptor activation through Na^+-bound pathways that conventional agonists cannot access. Thus,
323 sodium's role in μOR activation may encompass multiple parallel trajectories, some requiring ion
324 displacement and others proceeding through stabilized Na^+-bound intermediates that facilitate G protein
325 coupling, as seen with superagonists like AFN42.

326 This work has broad implications for GPCR pharmacology – challenging foundational models of class A
327 GPCR activation and providing framework for designing next-generation GPCR therapeutics that retain
328 high affinity and potency under physiological ionic conditions. There is a significant negative correlation
329 between sodium sensitivity and intrinsic efficacy. The prevailing theory is that high efficacy ligands, such
330 as fentanyl and morphine, are more sensitive to Na^+ and guanine nucleotide compared to low efficacy
331 ligands. Here, instead of a positive correlation and what was previously thought to be true, there is a negative

correlation in which the ligands with the highest intrinsic efficacy are the least sensitive to Na^+ and guanine nucleotide and thus show a smaller affinity shift.

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 ($n \geq 3$).

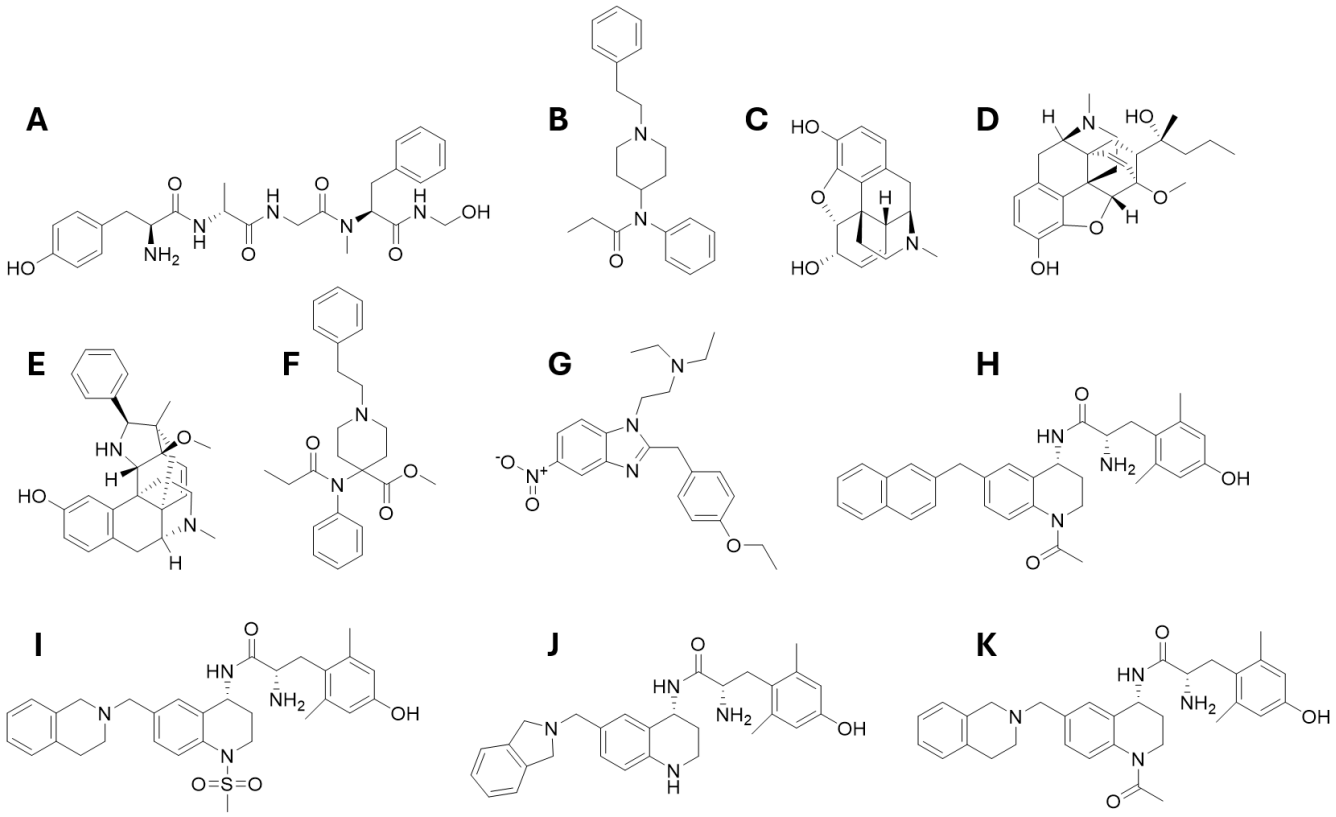

Figure 1: Chemical Structures of Opioid Agonists and Peptidomimetics. (A) DAMGO, (B) Fentanyl, (C) Morphine, (D) Etorphine, (E) BU72, (F) Carfentanil, (G) Etonitazene, (H) AAH8, (I) AFN42, (J) AMB46, and (K) AMB47

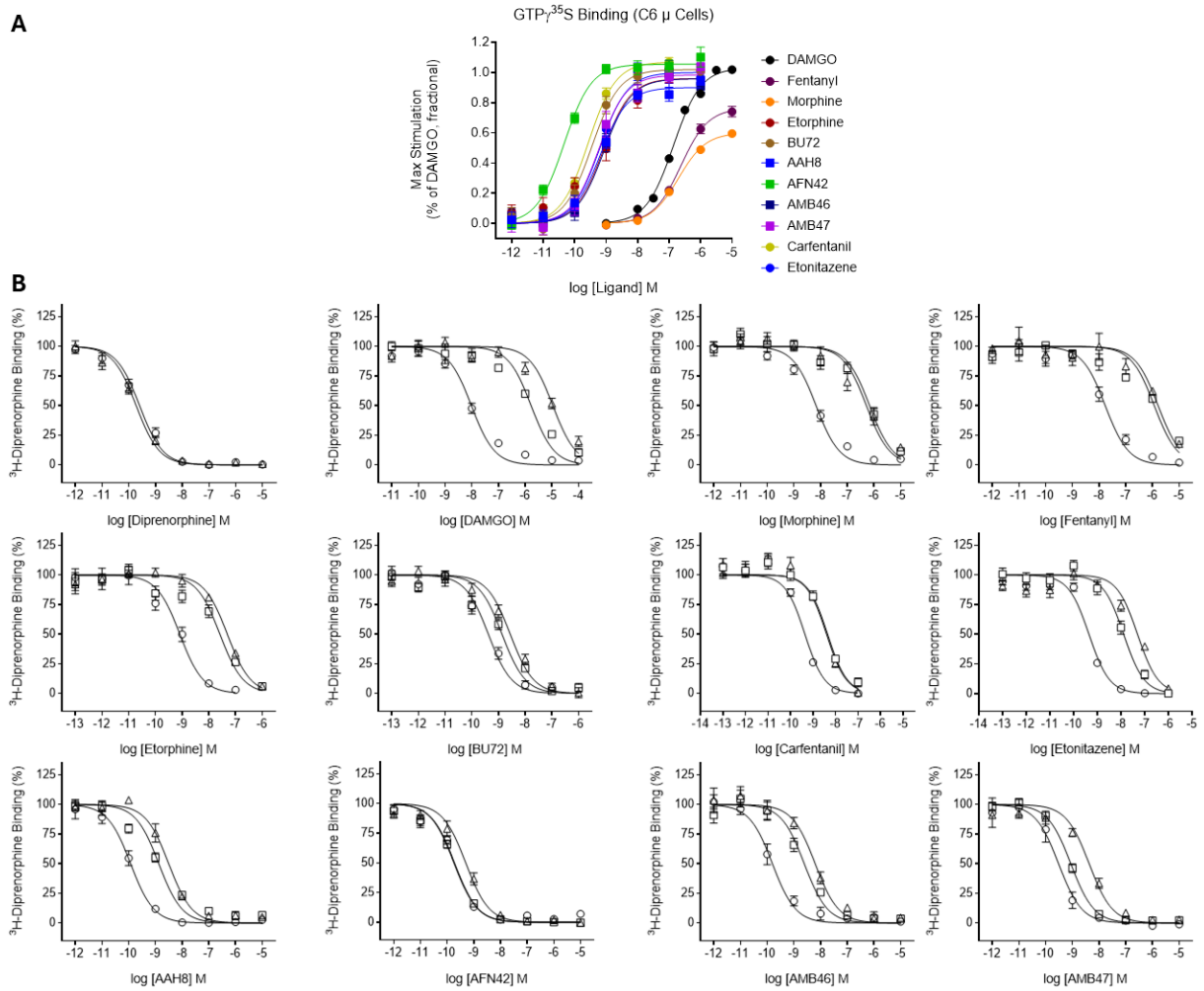

Figure 2: Stimulation of GTPγ^{35}S Binding and Inhibition of ^{3}H-Diprenorphine Binding Concentration-Response Curves in C6 μ Cells. (A) GTPγ^{35}S binding assays were performed using membranes from C6 cells with various concentrations of opioid agonists. Maximal stimulation of GTPγ^{35}S binding was 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 ($n \geq 3$).

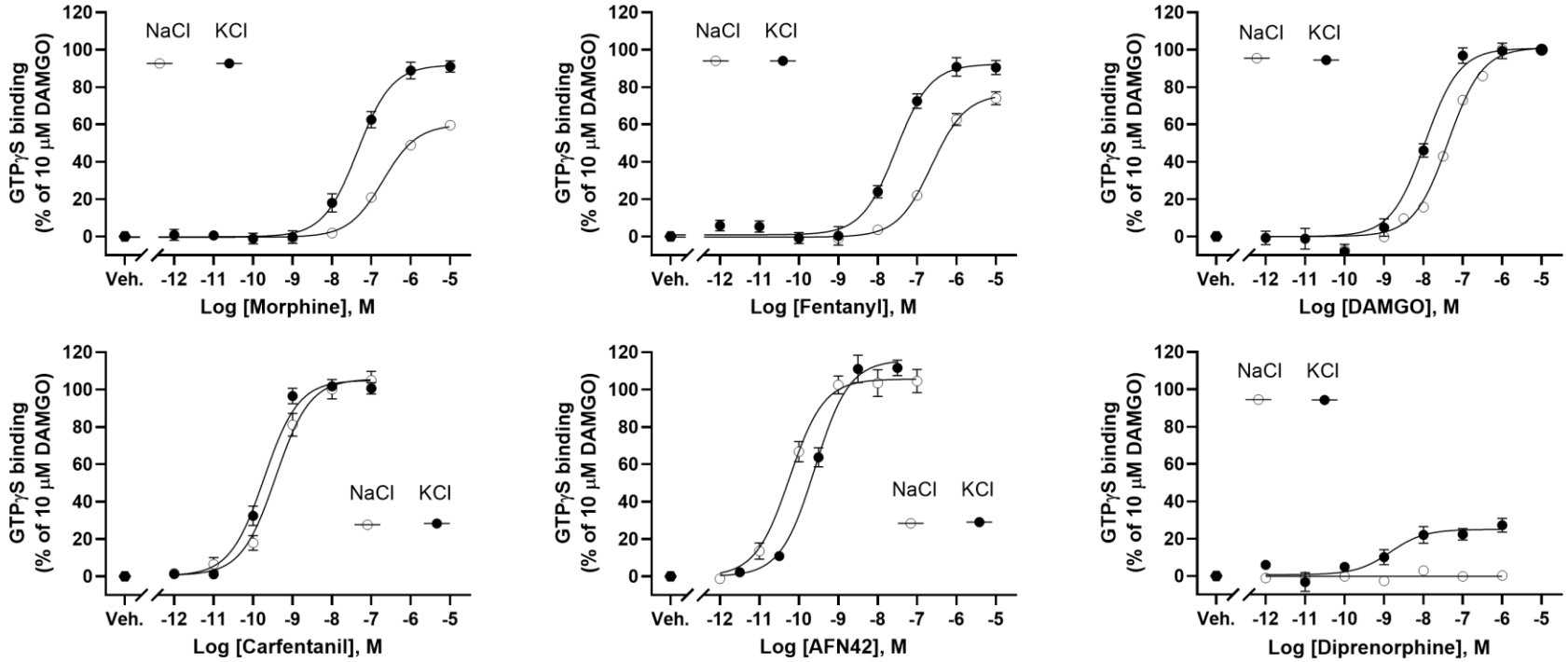

Figure 3: Stimulation of GTPγ^{35}S Binding in 100 mM NaCl and KCl buffer. Stimulation of GTPγ^{35}S binding at the μOR was performed using membranes from C6 cells with increasing concentrations of opioid agonists. Maximal stimulation of GTPγ^{35}S binding was determined by 10 μM of the reference agonist, 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)** 50 mM Tris	**K_i (nM)** + 100mM K^+ +10μM GTPγS	**Affinity Shift** $\frac{\text{Ki 100mM K +}}{\text{Ki 50mM Tris}}$
DAMGO	4.71 (3.63-6.13)	111 (66.8-187)	23.6
Fentanyl	8.33 (5.74-12.3)	218 (140-344)	26.2
Morphine	3.32 (2.47-4.42)	150 (93.9-246)	45.2
Diprenorphine	0.12 (0.094-0.16)	1.96 (1.29-2.94)	16.3
Carfentanil	0.17 (0.13-0.23)	1.19 (0.81-1.76)	7
AFN42	0.093 (0.072-0.12)	1.68 (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)** 50 mM Tris	**K_i (nM)** + 100mM Na^+ +10μM GTPγS	**Affinity Shift** $\frac{\text{Ki 100mM Na +}}{\text{Ki 50mM Tris}}$
DAMGO	0.65 (0.40-1.05)	529 (293-946)	814
BU72	0.27 (0.18-0.39)	0.73 (0.54-1.01)	2.7
Carfentanil	0.06 (0.035-0.10)	2.09 (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 ($n \geq 3$).

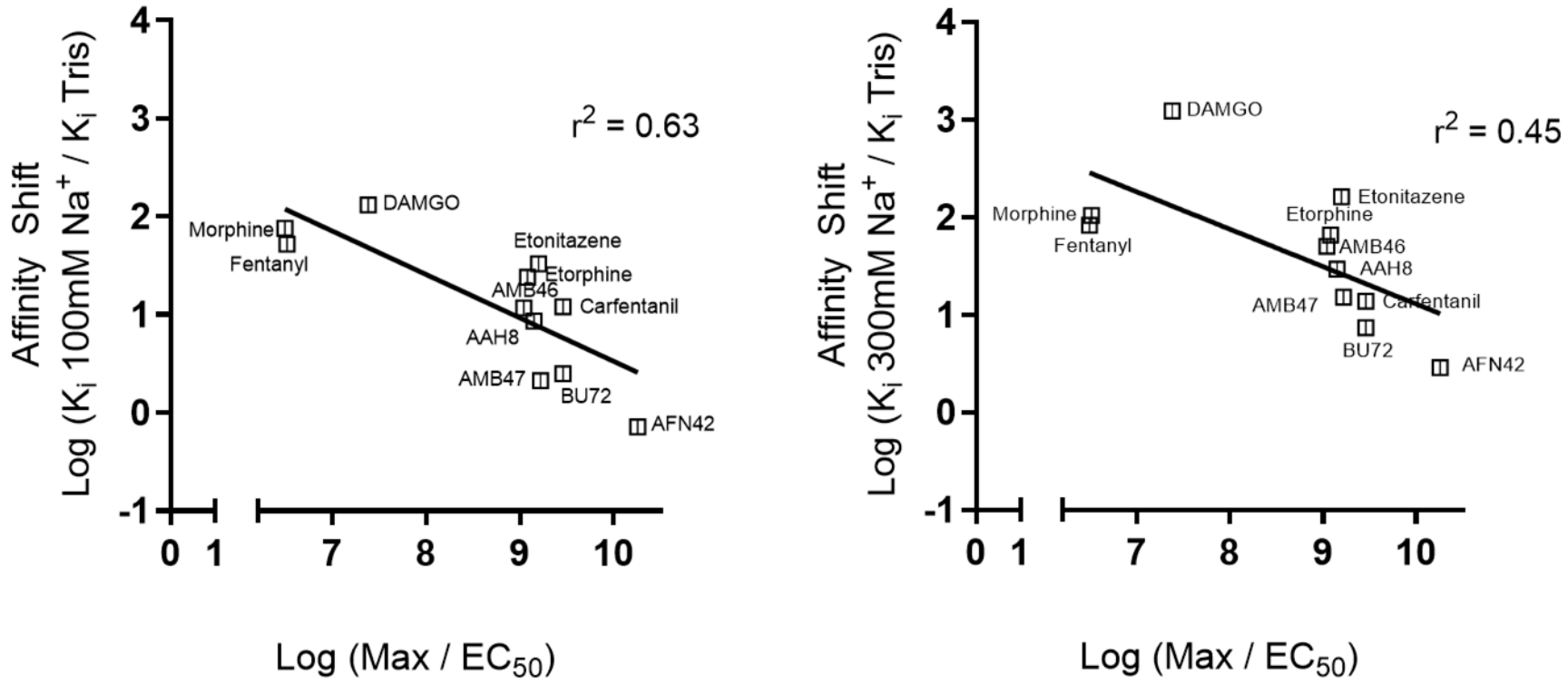

Figure 4: Intrinsic Efficacy Plots for C6 μ. Agonism (Log [Max/EC_{50}]) was determined from stimulation of GTPγ^{35}S binding assays and Affinity Shift (Log [Ki, 100 mM Na^+ / K_i 50 mM Tris] in **(A)** or Log [Ki, 300 mM Na^+ / K_i 50 mM Tris] in **(B)** was determined from ^{3}H-Diprenorphine competition 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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