# Assessment of high-efficacy agonism in synthetic cannabinoid receptor agonists containing l-*tert*-leucinate

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## Abstract

Synthetic cannabinoid receptor agonists (SCRAs) represent a class of new psychoactive substances that pose great health risks attributed to their wide-ranging and severe adverse effects. Recent evidence has shown that SCRAs with key moieties can confer superagonism, yet this phenomenon is still not well understood. In this study, we developed a structure-activity relationship (SAR) for SCRA superagonism by comparing eight compounds differing by their head moiety (l-valinate vs. l-*tert*-leucinate), core moiety (indole vs. indazole), and tail moiety (5-fluoropentyl vs. 4-fluorobenzyl) through different modes of bioluminescence resonance energy transfer (BRET). We found that l-*tert*-leucinate head moiety and indazole core moiety conferred superagonism across multiple $G\alpha_{i/o}$ proteins and β-arrestin 2. After generating CB1R mutant constructs, we found that transmembrane 2 (TM2) interactions to the head moiety of tested SCRAs at F170, F177, and H178 are key to eliciting activity. Finally, we found that l-*tert*-leucinate SCRAs confer a high-efficacy response in *ex vivo* slice electrophysiology.

## Introduction

The severe adverse effects observed with SCRA use are distinct from those of delta-9-tetrahydrocannabinol (THC), the active compound found in *Cannabis sativa* [1-4]. Due to their chemical structures, specifically aminoalkyl-heterocycle SCRAs, countless drugs can be illicitly made through iterative design, resulting in greatly increased efficacy and potency. Use of one of these SCRAs, MMB-FUBINACA, was responsible for a well-publicized case of SCRA intoxication that occurred in 2016 when thirty-three people exhibiting “zombie-like” cataleptic behavior [5]. In a separate clinical case, patients

experienced extreme agitation, aggressiveness, and seizures after using another SCRA ADB-PINACA [3]. Finally, SCRA overdose can be lethal, which is not reported with cannabis use [6, 7].

SCRAs are designed to target the cannabinoid type 1 receptor (CB1R), a highly expressed G-protein coupled receptor (GPCR) in the brain and CNS [1, 8]. CB1R plays a crucial role in downregulating neurotransmitter release, primarily through $G\alpha_{i/o}$ signaling pathways initiated by retrograde signaling at synaptic terminals [9, 10]. Upon G-protein activation, voltage-gated $Ca^{2+}$ channels are inhibited and G-protein-gated inward rectifying $K^+$ channels are activated, resulting in pre-synaptic hyperpolarization [9, 11]. Peripherally, the receptor also plays an important role in cardiovascular function as well as energy metabolism [12-14]. CB1R expression and function in multiple systems are complemented by the differential expression of the $G\alpha_{i/o}$ protein subfamily, consisting of ubiquitously ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$) and neuronally ($G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_{z}$) expressed inhibitory G-proteins [15-18]. Pharmacological studies have characterized a number of SCRAs based on CB1R-mediated cAMP inhibition as well as β-arrestin internalization [19-22]. SCRA-induced G-protein signaling within the subfamily of $G\alpha_{i/o}$ proteins, however, has only been investigated very recently [21, 23, 24]. Though these studies found SCRAs to show little functional selectivity or bias, a thorough comparison of moieties among structural analogs has not been attempted by using rigorous proximity assay approaches.

Understanding SCRA pharmacology has been a challenge mainly addressed through functional assays to assess potency and efficacy. Indeed, structure-activity relationships of these chemical structures, composed of three key moieties (head, core, and tail), have led to a characterization of SCRAs as “high efficacy” agonists [5, 7, 19, 20]. However, signal amplification in assays that measure activity downstream of receptor-transducer interactions can changes in efficacy, yet report increased potency [25]. Recently, we investigated the SAR between two compounds, 5F-MMB-PICA (M-PC) and 5F-MDMB-PICA (D-PC), that differed in a single methyl group in the “head” moiety [26]. D-PC, with an l-*tert*-leucinate head moiety, was shown to act as a “superagonist”, an agonist that has greater efficacy than that of the endogenous ligand, while M-PC acted as a full agonist[25, 26]. Notably, molecular dynamics simulations between these drugs revealed different levels of interaction with key residues in the extracellular TM2 domain, a region recently reported to be critical in activation of CB1R [26-29]. Although we found a SCRA that elicits superagonism, an understanding of what drives CB1R superagonism is still limited.

In the current study, we further explore differences in SCRA moieties to uncover CB1R superagonism based on differences in “head,” “core,” and “tail” moieties. With a panel of eight compounds we use different modes of bioluminescence resonance energy transfer (BRET) to assess the efficacy, bias, and functional selectivity of these compounds. Finally, we developed CB1R mutants in key TM2 residues to reveal key interactions with the head moiety l-*tert*-leucinate.

## Materials and Methods

**Mammalian Cell Culture** All *in vitro* assays are performed in human embryonic kidney 293 T (HEK-293T) cells cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine and incubated at 37°C and 5% $CO_2$. HEK-293T cells are cultured in 10-cm plates at a high cell density of 375,000 cells/ml (3 x $10^6$ cells/8 ml) twenty-four hours prior to transfection. Cells are maintained using aseptic technique and are used in pharmacological assays within 5-30 passages.

**Animals** C57Bl/6 mice are maintained in a vivarium provided by the Department of Laboratory Animal Medicine of Northeastern University. All experimental procedures performed on mice are in accordance with protocols approved by the Institutional Animal Care and Use Committee at Northeastern University.

**Compounds & Plasmid Constructs** All CB1R ligands used in this study are purchased or acquired from Cayman Chemical (Ann Arbor, Michigan) or NIDA Drug Supply Program (Rockville, Maryland). The ligands are dissolved in DMSO to a stock concentration of 10 mM. All DNA constructs are generated in pcDNA3 plasmid vectors. Alanine substitutions of *CNR1*-containing plasmid constructs were generated using the Quickchange Site-Directed Mutagenesis Kit (Agilent) and the following primers:
F170A forward primer 5' – TGGGGAGTGTCATTGCTGTCTACAGCTTCAT – 3',
reverse primer 5' – ATGAAGCTGTAGACAGCAATGACACTCCCCA – 3';
S173A forward primer 5' – TCATTTTTGTCTACGCCTTCATTGACTTCCA – 3',
reverse primer 5' – TGGAAGTCAATGAAGGCGTAGACAAAAATGA 3';
F177A forward primer 5' – TTTTTGTCTACAGCGCCATTGACTTCCACG – 3',
reverse primer 5' – CGTGGAAGTCAATGGCGCTGTAGACAAAAA – 3';
H178A forward primer 5' – GCTTCATTGACTTCGCCGTGTTCCACCGCA – 3',
reverse primer 5' – TGCGGTGGAACACGGCGAAGTCAATGAAGC – 3'.

**Bioluminescence Resonance Energy Transfer (BRET) receptor function assays** BRET assays described below contain variations in DNA constructs transfected and in method of experimentation. Consistent with all experiments is a pre-incubation of 5-15 µg DNA (per 10-cm plate, DNA amounts vary by experiment) with 30 µg/cell plate linear polyethyleneimine (PEI) in serum-free DMEM twenty minutes before addition to cells. After overnight treatment, media is fully replaced with fresh, supplemented DMEM. After 48 hours of transfection, cells are washed with phosphate-buffered saline (PBS), harvested, and resuspended in PBS containing 0.1% glucose, 200 µM sodium bisulfite and 1 mg/mL fatty acid-free bovine serum albumin (BSA), which serves as a drug stabilizer. Cells are evenly distributed amongst wells in white flat-bottom 96 well plates.

On the day of experiment, serially diluted drugs are transferred to cells three minutes after 5 μM coelenterazine H incubation. Luminescence and fluorescence values are recorded 10 minutes after drug treatment in a PheraStar FSX plate reader (bioluminescence at 480 nm, fluorescence at 530 nm). BRET ratio is calculated based on the measurement of fluorescence divided by that of luminescence. BRET ratios are then normalized to the basal BRET ratio calculated by the non-linear regression generated by GraphPad Prism 10.

*G-protein/β-arrestin engagement* – Plasmid DNA in engagement BRET assays have been reported [26] as follows: 0.5 μg CB1R tagged with *Renilla* luciferase 8 (CB1R-RLuc), 5 μg $G\alpha_{i1}$ tagged with Venus ($G\alpha_{i1}V$), and 4 μg of both $G\beta_1$ and $G\gamma_2$. For β-arrestin engagement, 6 μg of β-arrestin-2 tagged with Venus (βArr2V) and 5 μg of G-protein coupled receptor kinase 2 (GRK2) are used. They have been reported previously.

*G-protein activation* – Plasmid DNA in activation BRET assays have been reported [30, 31] as follows: 3.5 μg CB1R untagged, 0.5 μg Gα tagged with *Renilla* luciferase 8 ($G\alpha_{i1}$-RLuc, $G\alpha_{i2}$-RLuc, $G\alpha_{i3}$-RLuc, $G\alpha_{oA}$-RLuc, $G\alpha_{oB}$-RLuc, or $G\alpha_z$-RLuc), 4 μg of $G\beta_1$ and 5 μg of $G\gamma_2$ tagged with Venus ($G\gamma_2V$).

**Ex vivo slice electrophysiology** Mice anesthetized with isoflurane were intracardially perfused with slicing artificial cerebral spinal fluid (slicing ACSF) containing (in mM): 92 NMDG, 20 HEPES, 25 glucose, 30 NaHCO3, 1.2 NaH2PO4, 2.5 KCl, 5 sodium ascorbate, 3 sodium pyruvate, 2 thiourea, 10 MgSO4, 0.5 CaCl2, 300–310 mOsm, at pH 7.3–7.4. After decapitation, the mouse brain is fixed to a stage, submerged in cold slicing ACSF saturated with 95% O2 and 5% CO2 (carbogen), and sectioned in a para-sagittal manner at 300 μm using a vibratome. Brain slices are transferred to 37°C slicing ACSF for seven minutes, then transferred to room temperature holding ACSF saturated with carbogen containing (in mM): 92 NaCl, 20 HEPES, 25 glucose, 30 NaHCO3, 1.2 NaH2PO4, 2.5 KCl, 5 sodium ascorbate, 3 sodium pyruvate, 2 thiourea, 1 MgSO4, 2 CaCl2, 300–310 mOsm, at pH 7.3–7.4. Slices are maintained in holding ACSF for 1.5 to 2 hours prior to experimentation. Brain slices are transferred into a recording chamber filled with recording ACSF that contains (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgCl2, 26 NaHCO3, 11 glucose, 2.4 CaCl2, 0.05 caffeine, 300–310 mOsm, at pH 7.3–7.4. The recording ACSF is saturated with carbogen, maintained at 31-32°C, and perfused at 2 ml/min using a peristaltic pump. Caffeine, an adenosine A1 receptor antagonist, is used to reduce the effects of endogenous adenosine on observing CB1R function. Brain slices rest in perfused recording ACSF for 20 minutes before recording. A borosilicate recording electrode filled with recording ACSF is placed in the CA1 region of the hippocampus within the stratum radiatum, while a bipolar stimulating wire is placed in the CA3a region of the hippocampus. CA3 stimulation occurred at a frequency of 0.033 Hz, and were recorded using a MultiClamp 700b amplifier (10 kHz low-pass Bessel filter), digitized with a Digidata 1550b (20 kHz digitization), and visualized on pClamp software.

Current amplitude is set at approximately 25-30% of max output, determined by generating an input/output curve. Baseline is recorded for 10 minutes before a 60 minute drug perfusion.

**Data Analysis**. All data was processed and analyzed in GraphPad Prism 10 (San Diego, California). Data points were transformed to individual BRET ratio values and further normalized to the minimal and maximal responses by CP55,940 at 10 min as 0% and 100% respectively within each respective transducer. $E_{max}$ and $pEC_{50}$ parameters were obtained from the non-linear fit of normalized transformed data and multi-comparison data analyses at the 10 min time point were conducted. Based on the extrapolated curves, $E_{max}$ and $pEC_{50}$ are determined. $E_{max}$ on a non-plateaued curve and $pEC_{50}$ less than 5 are still reported in the tables. In those cases, $E_{max}$ is reported as the efficacy at the highest concentration observed (10 μM). To evaluate whether SCRAs exhibited G protein subunit signaling bias, bias factors were calculated as reported previously [32]. This method yields bias factors similar to the operational model. Briefly, $\Delta\log(E_{max}/EC_{50})$ value for each transducer was calculated by subtracting the $\log(E_{max}/EC_{50})$ value of the agonist by that of the reference compound. $\Delta\Delta\log(E_{max}/EC_{50})$ is determined by subtraction of $\Delta\log(E_{max}/EC_{50})$ between two transducers. The comparison between the two transducers shows the bias direction of a certain ligand towards one of the two transducers. Throughout the experiments, a triplicate of at least four independent experiments was performed per each condition. Statistical grouped analyses on $E_{max}$ and $pEC_{50}$ were conducted using one-way ANOVA with a post-hoc Dunnett test for multiple comparisons. For extracellular recordings, the fEPSP slope was determined in Clampfit from a 1.5-2 ms range in the rise phase post-stimulation. Recordings are averaged in 2-minute bins. Inhibition determined as the fEPSP slope percentage as compared to the baseline fEPSP slope. Max inhibition is determined by averaging the fEPSP slope of the last six minutes of the drug prefusion. Tau was obtained from the non-linear regression of the time course. Statistical significance of max inhibition and tau was determined using one-way ANOVA with a post-hoc Dunnett test for multiple comparisons

## Results and Discussion

### L-*tert*-leucinate head moiety leads to high efficacy in SCRA derivatives.

After recently investigating the drastic efficacy differences between l-valinate and l-*tert*-leucinate (*i.e.*, MMB- and MDMB-) head moieties of 5-fluoropenylindoles, we expanded our investigation on core and tail moieties in combination to the head moiety [26]. Clinical studies have reported on the severe impact of indazole-based SCRA abuse, including death from overdose [3, 5, 33, 34]. Furthermore, original derivatives patented by Pfizer, as well as the drug responsible for the 2016 Brooklyn outbreak, carried a 4-fluorobenzyl tail moiety [5]. Therefore, we selected 5F-MMB-PICA (M-PC), 5F-MDMB-PICA (D-PC),

5F-MMB-PINACA (M-PN), 5F-MDMB-PINACA (D-PN), MMB-FUBICA (M-FC), MDMB-FUBICA (D-FC), MMB-FUBINACA (M-FN), MDMB-FUBINACA (D-FN) for our study panel to develop a SAR of these moieties for CB1R superagonism (Fig. 1).

We began our study with measuring the engagement of $G\alpha_{i1}$ and β-arrestin-2 to CB1R using an "engagement" mode of BRET where the receptor and Gα-protein or β-arrestin are tagged with luciferase and fluorescent proteins, respectively (Fig. 2A, 1D). Both M-PC and D-PC displayed superagonism, although M-PC is ~20x less potent than D-PC in $G\alpha_{i1}$ engagement (Fig. 2B). We see a separation in both efficacy and potency for the 4-fluorobenzyl analogues M-FC and D-FC (Fig. 2C). Interestingly, the difference in efficacy between MMB- and MDMB- compounds is negligible amongst indazole SCRAs (*i.e.*, M-PN, D-PN, M-FN, D-FN), as all confer superagonism, although there is still a noticeable potency shift (~6-fold) for the MDMB- series (Fig. 2B-C, Table 1). Within β-arrestin-2, seven of the eight SCRAs are more efficacious than the reference compound CP55940, with only M-FC acting as a full agonist (2E-F, Table 1). Although an insignificant difference for $G\alpha_{i1}$ engagement, the 5-fluoropentyl SCRAs were all approximately 20-75% greater in efficacy than their 4-fluorobenzyl analogues (2E-F, Table 1).

Previous studies assessed a number of SCRAs used here as "high efficacy" agonists [35, 36]. By using the BRET "engagement" mode, we better address issues associated with signal amplification and receptor reserves, which can result in falsely high efficacy and potency activity in downstream functional assays [25, 37], as the engagement mode detect direct coupling between the receptor and transducer. Contrary to our previous results, M-PC displays superagonism for both BRET engagement modes [26]. This likely resulted from adding 1 mg/mL BSA to the assay bugger as a carrier protein to these highly lipophilic compounds for their solubility and stability, as used in previous studies [21,22]. Indazoles, regardless of head or tail moiety within the structure, drastically increases SCRA efficacy and potency, the latter of which has been well reported for indazole SCRA derivatives [20, 21]. The superagonist activity of D-PC, D-PN, and D-FN, in both $G\alpha_{i/o}$ and β-arrestin engagement, may be a determining factor in the severity of the adverse effects, as cannabis use, thus moderate efficacy of THC on CB1R, rarely causes the severe adverse effects and toxicities [7]. The increased efficacy in β-arrestin-2 engagement may be associated with increased tolerance found in SCRA use as β-arrestins mediate CB1R internalization [38, 39]. Indeed, SCRAs have been reported to induce rapid CB1R internalization through β-arrestin [21, 24]. These studies as well as our findings indicate that high efficacy in β-arrestin likely plays a role in adverse effects reported in human use.

**SCRA superagonism is observed across activation of most $G\alpha_{i/o}$ subtypes.**

Numerous studies have reported on SCRA-mediated CB1R activities without identifying the involvement of specific $G\alpha_{i/o}$ subtypes. Concurrently, there are limited findings on

SCRA agonism amongst the six main inhibitory G proteins ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_z$). Therefore, we aim to study the extent of SCRA superagonism across the $G\alpha_{i/o}$ subtypes, as well as the contribution of key moieties in the functional selectivity of these SCRAs.

To investigate this, we use an "activation" mode of BRET to measure the G-protein heterotrimer dissociation via untagged CB1R (Fig. 3A) [31, 40, 41]. In general, MDMB-compounds are more potent than MMB- compounds. Amongst indole SCRAs, there was a noticeable increase in both efficacy and potency for MDMB series compared to MMB series (Fig. 3B-G), but only an efficacy difference (>20%) for M-FC and D-FC in line with the $G\alpha_{i1}$ "engagement" assay results (Fig. 2A-C). M-FC showed the lowest efficacy and potency for $G\alpha_i$ subtypes; in the latter case, sharing the least potency with M-PC. For indazoles,MDMB- compounds are more potent than MMB- compounds, but there is no clear difference in efficacy in general. In $G\alpha_{oA}$, we found that D-PN was the single instance of an MDMB- indazole displaying noticeably greater efficacy (~50%) than its MMB-analogue (Fig. 3E). Interestingly, a general high efficacy trend among all SCRAs tested (except for D-FC) was observed for $G\alpha_{i3}$ (>150%), $G\alpha_{oA}$ (>125%), and $G\alpha_z$ (>124%).

We further analyzed $G\alpha_{i/o}$ subtype activation by estimating each compound's functional selectivity by calculating bias factors. Here, we determined the $\Delta\log(E_{max}/EC_{50})$ of each SCRA to compare two different transducers then taking the difference between the Δ values of a particular SCRA and the reference agonist CP55940, obtaining $\Delta\Delta\log(E_{max}/EC_{50})$ (Table 3). Here we found that none of the compounds had a ΔΔlog value outside the range of -1 to 1, thus suggesting the tested SCRAs are not biased towards any specific pathway.

Through BRET activation, we determined that the panel of tested SCRAs displays superagonism across $G\alpha_{i/o}$ subtypes. This finding is crucial as most previous reports have not focused on SCRA characterization of all $G\alpha_{i/o}$ subtypes and the diverse $G\alpha_{i/o}$ subtype expression in different organs and cell-types may lay background for biased pharmacology. Indeed, the $G\alpha_i$ are ubiquitously expressed throughout the body and are the dominant $G\alpha_{i/o}$ subtypes in the periphery, as the $G\alpha_o$ proteins and $G\alpha_z$ are the dominant proteins in the CNS. SCRA-induced superagonism across these subtypes may suggest a connection to the wide-range of adverse effects from SCRA use, even though these compounds were found to be relatively balanced. Further processing of ΔΔlog values to yield ΔΔΔlog and cross-comparison within the pairs of a differing core, head or tail moiety did not identify any significant bias beyond the -1 or 1 threshold (data not shown). Clinically reported SCRAs have also been found relatively balanced using similar *in vitro* assays [21-23]. Since there are a multitude of combinatorial diversity among different head, core and tail moieties for SCRAs, characterization of signaling bias remains to be explored and incomplete at present. Here though, we have determined that

across $G\alpha_{i/o}$ subtypes, the panel of paired SCRA analogs at head, core and tail moieties display CB1R superagonism.

**F170, F177, and H178 are key residues in SCRA activation, not exclusive to l-*tert*-leucinate.**

After having established the consistent increase in efficacy and potency for l-*tert*-leucinate (*i.e.*, MDMB-) containing SCRAs, we investigated residues within the binding pocket essential for conferring superagonism. Recent research has found that extracellular transmembrane domain 2 (TM2) residues are implicated in CB1R activation as they rotate inwards in the presence of a full agonist [27, 28]. Indeed, we previously reported on the conformational stability of TM2 being associated with 5F-MDMB-PICA superagonism [26]. Here, we expanded our investigation by generating alanine substitutions along the TM2 residues in order to isolate the essential residues implicated in CB1R superagonism.

We conducted $G\alpha_{oA}$ "activation" BRET with single-point alanine-substituted CB1R constructs at F170, S173, F177, and H178, intracellular to extracellular on TM2, using CP55940 and two representative SCRAs M-PC, and D-PC (Fig. 4A). By establishing the $E_{max}$ of CP55940 for the wildtype CB1R as 100% efficacy, mutational effects were studied on the SCRAs. The F170A substitution ablated CP55 efficacy, and reduced M-PC and D-PC efficacy and potency (Fig. 4C). The S173A substitution reduced the efficacy of the agonists to a partial level (Fig. 4D). The F177A mutation nearly ablated the efficacy of CP55 and M-PC, but only partially for D-PC (Fig. 4E). Similarly to F177A, the H178A mutation reduced the efficacy of CP55 and M-PC to partial agonism, whereas D-PC conferred high agonism (Fig. 4F).

In this mutational study, we investigated the TM2 residues implicated in SCRA-mediated CB1R superagonism. Recent reports have indicated that H178 is implicated in the activation of the CB1R [27, 29]. We confirmed that to be the case as the alanine substitution drastically reduced the effect of both CP55 and MPC. This phenomenon implicates the ring structure and/or positive charge of histidine as essential in CB1R engagement to an agonist in general, beyond head moiety interaction in the aminoalkylindole agonists, warranting further investigation. Interestingly, CP55 efficacy was ablated in both F170A and F177A, implicating these residues as essential in activation by CP55. F177A also drastically reduced the efficacy of M-PC, indicating F177 as a residue important in stabilizing the MMB- head moiety, whereas D-PC with the MDMB- head moiety maintains high efficacy (>100%).. Similarly, we expected the head moiety of M-PC and D-PC to fully interact with S173 and the S173A mutation to lower their efficacy [26]. We found this phenomenon in our investigation, but we also see that M-PC and D-PC are still more efficacious than CP55, implicating S173 as important for CB1R activation regardless of cannabinoid structure. Further investigation on these and

other interacting residues beyond TM2 will provide more insight into SCRA superagonism and key interactions with SCRA core and tail moieties as well.

**L-*tert*-leucinate SCRAs confer high efficacy agonism in glutamate release inhibition in the hippocampus.**

To investigate the pharmacological profiles of SCRAs at a neurophysiological level, we tested the effects of l-*tert*-leucinate SCRAs through *ex vivo* brain slice electrophysiology. As mentioned above, CB1R is the most abundant GPCR in the brain and responsible for attenuation of anterograde neurotransmitter release through its inhibition of voltage-gated calcium channels and activation of G-protein-gated inward rectifying K+ channels. We have previously shown that M-PC and D-PC have a drastic difference in the degree of efficacy in inhibiting glutamate release in the hippocampus. Here, we test the efficacy and kinetics of other l-*tert*-leucinate SCRAs on glutamate release.

We performed para-sagittal slicing of young adult mice brains to reveal the hippocampus and then perfused 1 μM of SCRAs over the course of 60 minutes while recording field extracellular postsynaptic potentials (fEPSP) in the hippocampus. We used M-PC to serve as a reference SCRA, resulting in approximately 25% inhibition of the fEPSP slope. All four MDMB- compounds resulted in 40-55% inhibition, significantly greater than that of M-PC. We also compared the kinetics of efficacy increase via CB1R over time with tau values of these compounds. Interestingly, there is a separation based on the tail moiety of the compound, whereby D-FC and D-FN both had tau greater than 30 minutes, whereas M-PC, D-PC, and D-PN were under 10 minutes.

We confirmed here that l-*tert*-leucinate SCRAs confer a high degree of efficacy at a neurophysological level. Interestingly, M-PC confers a distinctly lower level of efficacy to D-PC in electrophysiology that we do not see in BRET studies. Although all MDMB-compounds had much greater levels of inhibition, the tail moiety seemed to play a key role in the kinetics of these drugs, given the large difference in tau between 4-fluorobenzyl and 5-fluoropentyl compounds. This difference may be due to the higher lipophilicity of the benzyl ring in the tail moiety (e.g., cLogP values: D-FC – 4.7, D-FN – 4.6, compared to M-PC – 3.9, D-PC – 4.3, and D-PN – 4.2), potentially leading to poor tissue penetration. Indeed, kinetic differences were not observed among MDMB- compounds in *in vitro* BRET assays as there is no tissue penetration issue. As such, our electrophysiological recordings can be a viable method to distinguish ligand accessibility to native CB1Rs.

## Conclusion

The iterative design of SCRAs in their modular components has led to an explosive number of substances, many manifested toxicities. In this study, we attempted to understand the involvement of TM2 in a SAR for SCRA-induced CB1R superagonism.

Using structural pairwise comparison within head, core, and tail moieties, we detailed superagonism conferred by the l-*tert*-leucinate head moiety. We found that SCRAs, depending on their key moieties, can elicit superagonism through multiple $G\alpha_{i/o}$ subtypes and β-arrestin, and that F170, F177, and H178 in TM2 are implicated in conferring such drug action. This pairwise comparison approach within ligand structure in conjunction with extensive transducer analysis may be useful for revealing specific activation profiles in the future SAR studies for novel SCRAs.

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**Author Contributions** C.L., H.Y. designed the experiments. C.L., C.P., J.D. performed experiments. C.L., C.P., J.D., H.Y. analyzed the data. C.L., H.Y. wrote the manuscript.

## References


1. Huffman JW, Dai D., Martin B.R., Compton D.R.: **Design, synthesis and pharmacology of cannabimimetic indoles**. *Bioorganic & Medicinal Chemistry Letters* 1994, **4**(4):563-566.
2. Wiley JL, Compton DR, Dai D, Lainton JA, Phillips M, Huffman JW, Martin BR: **Structure-activity relationships of indole- and pyrrole-derived cannabinoids**. *J Pharmacol Exp Ther* 1998, **285**(3):995-1004.
3. Schwartz MD, Trecki J, Edison LA, Steck AR, Arnold JK, Gerona RR: **A Common Source Outbreak of Severe Delirium Associated with Exposure to the Novel Synthetic Cannabinoid ADB-PINACA**. *J Emerg Med* 2015, **48**(5):573-580.
4. Zapata F, Matey JM, Montalvo G, García-Ruiz C: **Chemical classification of new psychoactive substances (NPS)**. *Microchemical Journal* 2021, **163**:105877.
5. Adams AJ, Banister SD, Irizarry L, Trecki J, Schwartz M, Gerona R: **"Zombie" Outbreak Caused by the Synthetic Cannabinoid AMB-FUBINACA in New York**. *N Engl J Med* 2017, **376**(3):235-242.
6. Zaurova M, Hoffman RS, Vlahov D, Manini AF: **Clinical Effects of Synthetic Cannabinoid Receptor Agonists Compared with Marijuana in Emergency Department Patients with Acute Drug Overdose**. *J Med Toxicol* 2016, **12**(4):335-340.
7. Ford BM, Tai S, Fantegrossi WE, Prather PL: **Synthetic Pot: Not Your Grandfather's Marijuana**. *Trends Pharmacol Sci* 2017, **38**(3):257-276.
8. Marsicano G, Lutz B: **Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain**. *Eur J Neurosci* 1999, **11**(12):4213-4225.
9. Kano M, Ohno-Shosaku T, Maejima T: **Retrograde signaling at central synapses via endogenous cannabinoids**. *Mol Psychiatry* 2002, **7**(3):234-235.
10. Piomelli D: **The molecular logic of endocannabinoid signalling**. *Nat Rev Neurosci* 2003, **4**(11):873-884.
11. Ohno-Shosaku T, Tsubokawa H, Mizushima I, Yoneda N, Zimmer A, Kano M: **Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses**. *J Neurosci* 2002, **22**(10):3864-3872.
12. Ravinet Trillou C, Delgorge C, Menet C, Arnone M, Soubrié P: **CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity**. *Int J Obes Relat Metab Disord* 2004, **28**(4):640-648.
13. Mendizábal VE, Adler-Graschinsky E: **Cannabinoids as therapeutic agents in cardiovascular disease: a tale of passions and illusions**. *Br J Pharmacol* 2007, **151**(4):427-440.
14. Malinowska B, Baranowska-Kuczko M, Schlicker E: **Triphasic blood pressure responses to cannabinoids: do we understand the mechanism?** *Br J Pharmacol* 2012, **165**(7):2073-2088.

15. Jiang M, Bajpayee NS: **Molecular mechanisms of go signaling**. *Neurosignals* 2009, **17**(1):23-41.
16. Foerster K, Groner F, Matthes J, Koch WJ, Birnbaumer L, Herzig S: **Cardioprotection specific for the G protein Gi2 in chronic adrenergic signaling through beta 2-adrenoceptors**. *Proc Natl Acad Sci U S A* 2003, **100**(24):14475-14480.
17. Cha HL, Choi JM, Oh HH, Bashyal N, Kim SS, Birnbaumer L, Suh-Kim H: **Deletion of the α subunit of the heterotrimeric Go protein impairs cerebellar cortical development in mice**. *Mol Brain* 2019, **12**(1):57.
18. Hultman R, Kumari U, Michel N, Casey PJ: **Gαz regulates BDNF-induction of axon growth in cortical neurons**. *Mol Cell Neurosci* 2014, **58**:53-61.
19. Banister SD, Longworth M, Kevin R, Sachdev S, Santiago M, Stuart J, Mack JB, Glass M, McGregor IS, Connor M *et al*: **Pharmacology of Valinate and tert-Leucinate Synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and Their Analogues**. *ACS Chem Neurosci* 2016, **7**(9):1241-1254.
20. Banister SD, Moir M, Stuart J, Kevin RC, Wood KE, Longworth M, Wilkinson SM, Beinat C, Buchanan AS, Glass M *et al*: **Pharmacology of Indole and Indazole Synthetic Cannabinoid Designer Drugs AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA, and 5F-ADBICA**. *ACS Chem Neurosci* 2015, **6**(9):1546-1559.
21. Finlay DB, Manning JJ, Ibsen MS, Macdonald CE, Patel M, Javitch JA, Banister SD, Glass M: **Do Toxic Synthetic Cannabinoid Receptor Agonists Have Signature in Vitro Activity Profiles? A Case Study of AMB-FUBINACA**. *ACS Chem Neurosci* 2019, **10**(10):4350-4360.
22. Patel M, Manning JJ, Finlay DB, Javitch JA, Banister SD, Grimsey NL, Glass M: **Signalling profiles of a structurally diverse panel of synthetic cannabinoid receptor agonists**. *Biochem Pharmacol* 2020, **175**:113871.
23. Ryalls B, Patel M, Sparkes E, Banister SD, Finlay DB, Glass M: **Investigating selectivity and bias for G protein subtypes and β-arrestins by synthetic cannabinoid receptor agonists at the cannabinoid CB1 receptor**. *Biochemical Pharmacology* 2024, **222**:116052.
24. Zagzoog A, Brandt AL, Black T, Kim ED, Burkart R, Patel M, Jin Z, Nikolaeva M, Laprairie RB: **Assessment of select synthetic cannabinoid receptor agonist bias and selectivity between the type 1 and type 2 cannabinoid receptor**. *Sci Rep* 2021, **11**(1):10611.
25. Schrage R, De Min A, Hochheiser K, Kostenis E, Mohr K: **Superagonism at G protein-coupled receptors and beyond**. *Br J Pharmacol* 2016, **173**(20):3018-3027.
26. Yano H, Chitsazi R, Lucaj C, Tran P, Hoffman AF, Baumann MH, Lupica CR, Shi L: **Subtle Structural Modification of a Synthetic Cannabinoid Receptor Agonist Drastically Increases its Efficacy at the CB1 Receptor**. *ACS Chemical Neuroscience* 2023, **14**(21):3928-3940.
27. Krishna Kumar K, Robertson MJ, Thadhani E, Wang H, Suomivuori CM, Powers AS, Ji L, Nikas SP, Dror RO, Inoue A *et al*: **Structural basis for activation of CB1 by an endocannabinoid analog**. *Nat Commun* 2023, **14**(1):2672.

28. Krishna Kumar K, Shalev-Benami M, Robertson MJ, Hu H, Banister SD, Hollingsworth SA, Latorraca NR, Kato HE, Hilger D, Maeda S *et al*: **Structure of a Signaling Cannabinoid Receptor 1-G Protein Complex**. *Cell* 2019, **176**(3):448-458.e412.
29. Brust CA, Swanson MA, Tsoutsouvas CI, Dimova ST, Dang VQ, Stahl EL, Ho J-H, Nikas SP, Makriyannis A, Bohn LM: **Comparison of Agonist Activity between CB1 and CB2 Receptors with Orthosteric Site Mutations**. *Receptors* 2024, **3**(3):380-396.
30. Semeano A, Garland R, Bonifazi A, Lee KH, Famiglietti J, Zhang W, Jo YJ, Battiti FO, Shi L, Newman AH *et al*: **Linkers in Bitopic Agonists Shape Bias Profile among Transducers for the Dopamine D2 and D3 Receptors**. *ACS Pharmacology & Translational Science* 2024, **7**(8):2333-2349.
31. Sánchez-Soto M, Bonifazi A, Cai NS, Ellenberger MP, Newman AH, Ferré S, Yano H: **Evidence for Noncanonical Neurotransmitter Activation: Norepinephrine as a Dopamine D<sub>2</sub>-Like Receptor Agonist**. *Molecular Pharmacology* 2016, **89**(4):457-466.
32. Bonifazi A, Yano H, Guerrero AM, Kumar V, Hoffman AF, Lupica CR, Shi L, Newman AH: **Novel and Potent Dopamine D(2) Receptor Go-Protein Biased Agonists**. *ACS Pharmacol Transl Sci* 2019, **2**(1):52-65.
33. Usui K, Fujita Y, Kamijo Y, Kokaji T, Funayama M: **Identification of 5-fluoro ADB in human whole blood in four death cases**. *Journal of analytical toxicology* 2018, **42**(2):e21-e25.
34. Boland DM, Reidy LJ, Seither JM, Radtke JM, Lew EO: **Forty‐three fatalities involving the synthetic cannabinoid, 5‐fluoro‐ADB: Forensic pathology and toxicology implications**. *Journal of forensic sciences* 2020, **65**(1):170-182.
35. Cabanlong CV, Russell LN, Fantegrossi WE, Prather PL: **Metabolites of Synthetic Cannabinoid 5F-MDMB-PINACA Retain Affinity, Act as High Efficacy Agonists and Exhibit Atypical Pharmacodynamic Properties at CB1 Receptors**. *Toxicol Sci* 2022, **187**(1):175-185.
36. Banister SD, Connor M: **The Chemistry and Pharmacology of Synthetic Cannabinoid Receptor Agonists as New Psychoactive Substances: Origins**. *Handb Exp Pharmacol* 2018, **252**:165-190.
37. Schrage R, Seemann WK, Klöckner J, Dallanoce C, Racké K, Kostenis E, De Amici M, Holzgrabe U, Mohr K: **Agonists with supraphysiological efficacy at the muscarinic M2 ACh receptor**. *Br J Pharmacol* 2013, **169**(2):357-370.
38. Daigle TL, Kwok ML, Mackie K: **Regulation of CB1 cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism**. *J Neurochem* 2008, **106**(1):70-82.
39. Jin W, Brown S, Roche JP, Hsieh C, Celver JP, Kovoor A, Chavkin C, Mackie K: **Distinct domains of the CB1 cannabinoid receptor mediate desensitization and internalization**. *J Neurosci* 1999, **19**(10):3773-3780.
40. Galés C, Van Durm JJJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M: **Probing the activation-promoted structural rearrangements in**

**preassembled receptor–G protein complexes**. *Nature Structural & Molecular Biology* 2006, **13**(9):778-786.

41. Yano H, Sánchez-Soto M, Ferré S: **Bioluminescence Resonance Energy Transfer Assay to Characterize Gi-Like G Protein Subtype-Dependent Functional Selectivity**. *Curr Protoc Neurosci* 2017, **81**:5.33.31-35.33.13.

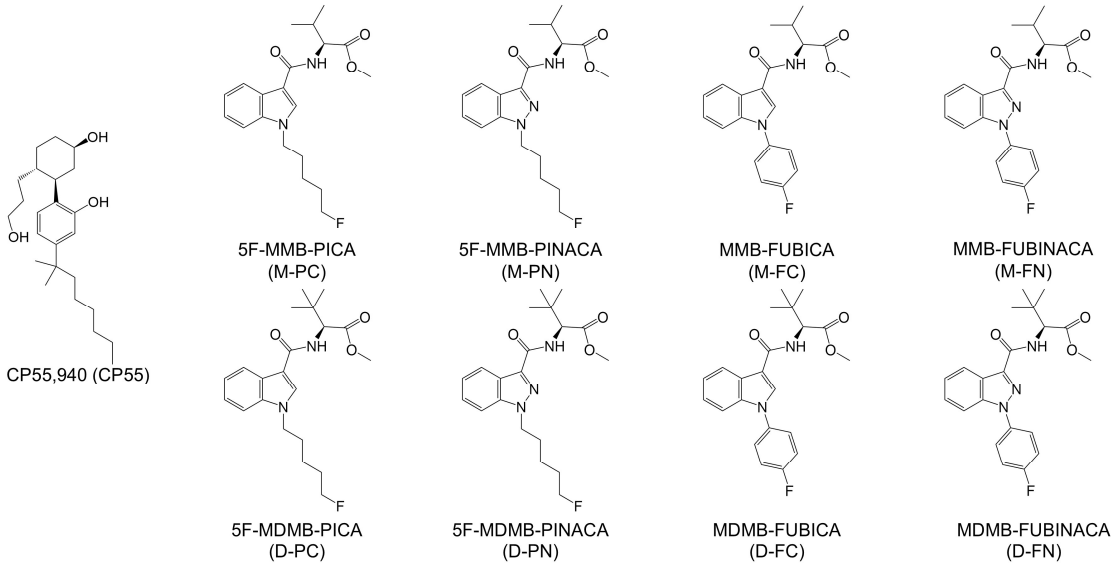


**Figure 1: Structures of SCRAs used in this study.** CP55,940 serves as the reference ligand for all experiments.

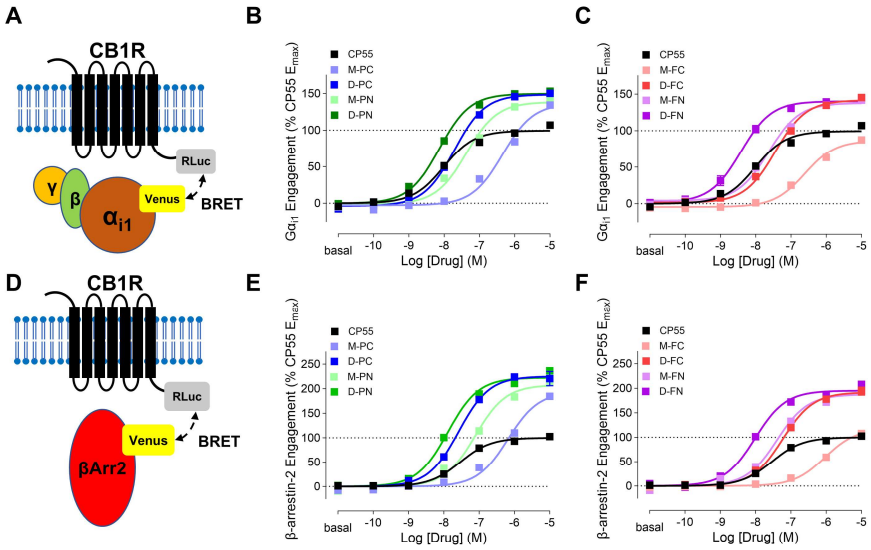


**Figure 2: SCRA-induced $G\alpha_{i1}$ and β-Arr2 engagement to CB1R.** Drug-induced BRET between CB1R-RLuc and $G\alpha_{i1}$-Venus (A-C) or β-Arr2-Venus (D-F) are shown. Concentration-response curves of SCRA-induced BRET are plotted as a percentage of CP55 $E_{max}$ (B-C, $G\alpha_{i1}$; E-F, β-Arr2) and organized by tail moiety (B,E 5-fluoropentyl; C,F 4-fluorobenzyl). Data are presented as means ± SEM of n=4 experiments.

| Table 1: Pharmacological comparison of SCRAs in CB1R-transducer engagement BRET | | | | | | |
|---|---|---|---|---|---|---|
| | $G\alpha_{i1}$ | | βArr2 | | Bias Factor | |
| | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | ΔΔlog | $G\alpha_{i1}$ - βArr2 |
| **CP55** | 100.0 ± 1.75 | 8.01 ± 0.05 | 100.0 ± 1.59 | 7.54 ± 0.10 | | |
| **M-PC** | 136.5 ± 3.97* | 6.34 ± 0.06* | 193.7 ± 5.71* | 6.19 ± 0.05* | **M-PC - CP55** | -0.47 |
| **D-PC** | 148.6 ± 1.71* | 7.71 ± 0.03* | 225.1 ± 4.17* | 7.58 ± 0.06 | **D-PC - CP55** | -0.52 |
| **M-PN** | 138.5 ± 2.79* | 7.37 ± 0.06* | 207.6 ± 3.92* | 7.11 ± 0.06* | **M-PN - CP55** | -0.39 |
| **D-PN** | 149.6 ± 2.07* | 8.14 ± 0.04 | 222.5 ± 2.74* | 7.89 ± 0.09* | **D-PN - CP55** | -0.40 |
| **M-FC** | 86.5 ± 3.42* | 6.61 ± 0.09* | 117.3 ± 4.60* | 6.04 ± 0.13* | **M-FC - CP55** | -0.03 |
| **D-FC** | 131.0 ± 2.40* | 7.44 ± 0.06* | 191.9 ± 3.31* | 7.23 ± 0.05* | **D-FC - CP55** | -0.40 |
| **M-FN** | 137.8 ± 2.45* | 7.63 ± 0.05* | 187.5 ± 2.98* | 7.40 ± 0.08 | **M-FN - CP55** | -0.37 |
| **D-FN** | 140.2 ± 2.50* | 8.42 ± 0.06* | 195.2 ± 3.29* | 8.02 ± 0.08* | **D-FN - CP55** | -0.21 |

**Data analyzed by one-way ANOVA followed by post-hoc Dunnett test. Data presented as means ± SEM. Significance of * represents p>0.05 compared to CP55 $E_{max}$ and $pEC_{50}$ values for respective transducer.**

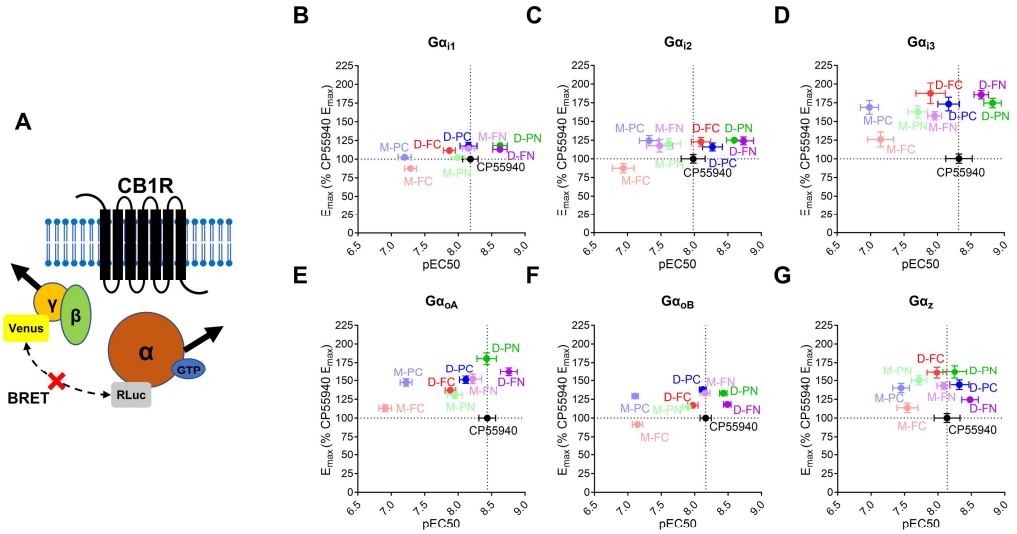


**Figure 3: SCRA-activated $G\alpha_{i/o}$ subtype protein dissociation BRET assay.** Cartoon illustrating BRET interaction between CB1R and Gα-RLuc and $G\gamma_2$-Venus with untagged CB1R (A). SCRA-induced BRET efficacy, as a percentage of CP55 $E_{max}$, and pEC50 were plotted for $G\alpha_{i/o}$ subtype transducers: $G\alpha_{i1}$ (B), $G\alpha_{i2}$ (C), $G\alpha_{i3}$ (D), $G\alpha_{oA}$ (E), $G\alpha_{oB}$ (F), and $G\alpha_z$ (G). Data presented as means ± SEM of n=4 experiments.

**Table 2: Pharmacological comparison of SCRAs in G protein dissociation BRET**

| | $G\alpha_{i1}$ | | $G\alpha_{i2}$ | | $G\alpha_{i3}$ | | $G\alpha_{oA}$ | | $G\alpha_{oB}$ | | $G\alpha_{z}$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $E_{max}$ (% CP55) | $pEC_{50}$ | $E_{max}$ (% CP55) | $pEC_{50}$ | $E_{max}$ (% CP55) | $pEC_{50}$ | $E_{max}$ (% CP55) | $pEC_{50}$ | $E_{max}$ (% CP55) | $pEC_{50}$ | $E_{max}$ (% CP55) | $pEC_{50}$ |
| **CP55** | 100.0± 3.66 | 8.18± 0.12 | 100.0± 5.87 | 7.98± 0.18 | 100.3± 6.19 | 8.32± 0.20 | 100.0± 3.63 | 8.44± 0.12 | 100.0± 2.68 | 8.17± 0.084 | 100.0± 5.89 | 8.14± 0.19 |
| **M-PC** | 102.6± 3.97 | 7.20± 0.10* | 124.4± 6.77 | 7.32± 0.15* | 168.9± 9.32* | 6.98± 0.13* | 147.4± 5.06* | 7.22± 0.09* | 129.2± 2.51* | 7.11± 0.05* | 140.0± 6.17* | 7.45± 0.12* |
| **D-PC** | 117.6± 4.83* | 8.15± 0.13 | 115.9± 5.36 | 8.27± 0.15 | 173.3± 9.10* | 8.17± 0.16 | 151.0± 4.89* | 8.12± 0.09 | 137.9± 2.33* | 8.12± 0.052 | 144.4± 6.37* | 8.33± 0.14 |
| **M-PN** | 102.1± 3.68 | 7.99± 0.11 | 120.1± 7.12 | 7.62± 0.17 | 162.8± 8.30* | 7.70± 0.15* | 130.9± 4.88* | 7.95± 0.11* | 114.6± 3.36* | 7.91± 0.086 | 150.3± 5.98* | 7.72± 0.12 |
| **D-PN** | 118.3± 3.63* | 8.63± 0.11* | 125.1± 4.20* | 8.60± 0.12 | 174.7± 6.40* | 8.82± 0.13 | 180.4± 7.84* | 8.43± 0.14 | 133.1± 2.20* | 8.43± 0.056 | 162.0± 8.83* | 8.26± 0.18 |
| **M-FC** | 87.5± 2.90 | 7.29± 0.09* | 87.5± 6.17 | 6.94± 0.16* | 126.0± 9.93 | 7.15± 0.19* | 113.0± 4.64 | 6.91± 0.10* | 91.6± 2.81 | 7.15± 0.08* | 113.7± 6.38 | 7.55± 0.16* |
| **D-FC** | 111.6± 3.45 | 7.87± 0.09 | 122.8± 5.91 | 8.10± 0.14 | 187.7± 13.79* | 7.90± 0.22* | 136.9± 3.73* | 7.89± 0.08* | 116.9± 3.12* | 7.98± 0.078 | 161.1± 7.86* | 7.99± 0.15 |
| **M-FN** | 114.4± 3.33 | 8.15± 0.09 | 117.5± 8.48 | 7.48± 0.20 | 157.5± 5.57* | 7.96± 0.10* | 152.1± 6.38* | 8.22± 0.13 | 133.2± 3.13* | 8.16± 0.073 | 142.9± 4.71* | 8.09± 0.10 |
| **D-FN** | 113.1± 3.47 | 8.62± 0.11* | 124.0± 5.29 | 8.73± 0.15 | 185.8± 5.68* | 8.66± 0.11 | 162.2± 5.79* | 8.76± 0.13 | 118.2± 1.95* | 8.49± 0.056 | 124.4± 4.30* | 8.48± 0.12 |

**Data analyzed by one-way ANOVA followed by post-hoc Dunnett test. Data presented as means ± SEM. Significance of * represents p>0.05.**

**Table 3: SCRA-mediated activation bias of $G\alpha_{i/o}$ subtypes**

| ΔΔlog | $G\alpha_{i1}$ - $G\alpha_{i2}$ | $G\alpha_{i1}$ - $G\alpha_{i3}$ | $G\alpha_{i1}$ - $G\alpha_{oA}$ | $G\alpha_{i1}$ - $G\alpha_{oB}$ | $G\alpha_{i1}$ - $G\alpha_{z}$ | $G\alpha_{i2}$ - $G\alpha_{i3}$ | $G\alpha_{i2}$ - $G\alpha_{oA}$ | $G\alpha_{i2}$ - $G\alpha_{oB}$ | $G\alpha_{i2}$ - $G\alpha_{z}$ | $G\alpha_{i3}$ - $G\alpha_{oA}$ | $G\alpha_{i3}$ - $G\alpha_{oB}$ | $G\alpha_{i3}$ - $G\alpha_{z}$ | $G\alpha_{oA}$ - $G\alpha_{oB}$ | $G\alpha_{oA}$ - $G\alpha_{z}$ | $G\alpha_{oB}$ - $G\alpha_{z}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M-PC - CP55** | -0.41 | 0.14 | 0.07 | -0.03 | -0.43 | 0.55 | 0.48 | 0.38 | -0.02 | -0.07 | -0.17 | -0.57 | -0.10 | -0.50 | -0.40 |
| **D-PC - CP55** | -0.31 | -0.04 | 0.18 | -0.05 | -0.30 | 0.27 | 0.49 | 0.26 | 0.01 | 0.22 | -0.01 | -0.26 | -0.24 | -0.49 | -0.25 |
| **M-PN - CP55** | 0.10 | 0.23 | 0.19 | 0.02 | 0.06 | 0.13 | 0.09 | -0.08 | -0.04 | -0.04 | -0.20 | -0.17 | -0.17 | -0.13 | 0.04 |
| **D-PN - CP55** | -0.19 | -0.23 | 0.27 | 0.13 | 0.19 | -0.03 | 0.46 | 0.32 | 0.39 | 0.50 | 0.35 | 0.42 | -0.14 | -0.08 | 0.06 |
| **M-FC - CP55** | 0.15 | 0.12 | 0.52 | 0.11 | -0.42 | -0.03 | 0.37 | -0.04 | -0.57 | 0.40 | -0.01 | -0.54 | -0.41 | -0.94 | -0.52 |
| **D-FC - CP55** | -0.47 | -0.11 | 0.15 | -0.14 | -0.32 | 0.36 | 0.62 | 0.33 | 0.15 | 0.26 | -0.03 | -0.21 | -0.29 | -0.47 | -0.18 |
| **M-FN - CP55** | 0.46 | 0.20 | 0.06 | -0.09 | -0.08 | -0.26 | -0.40 | -0.55 | -0.54 | -0.13 | -0.28 | -0.27 | -0.15 | -0.14 | 0.01 |
| **D-FN - CP55** | -0.35 | -0.11 | -0.04 | 0.10 | 0.06 | 0.24 | 0.31 | 0.45 | 0.41 | 0.07 | 0.20 | 0.16 | 0.14 | 0.10 | -0.04 |

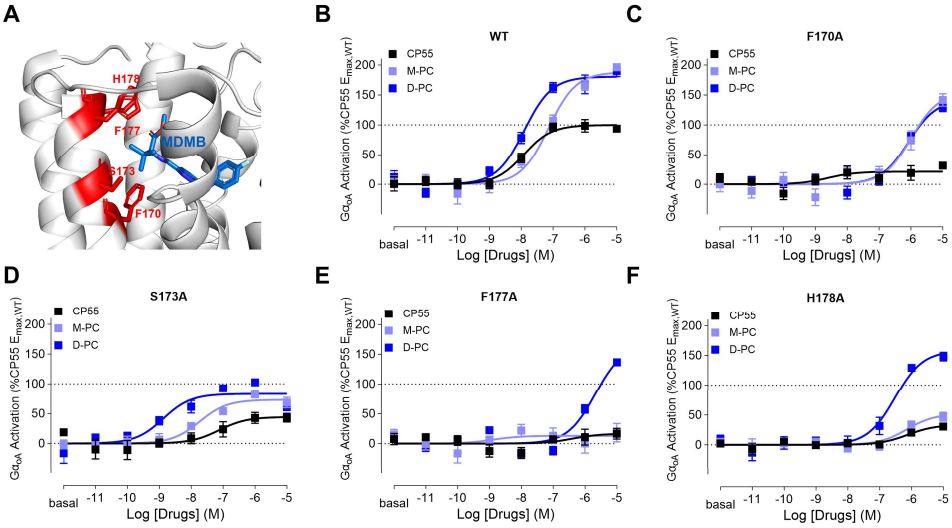


**Figure 4: CB1R-TM2 mutational analysis of SCRA head moiety interaction.** A close-up view of TM2-MDMB-FUBINACA (PDB:6N4B) with residues (red) within 4 Å of the MDMB-head moiety (blue) using PyMOL 3.0 (Schrödinger) for visualization (A). Through BRET $G\alpha_{oA}$ Activation mode, 5F-MMB-PICA and 5F-MDMB-PICA are compared to CP55 in the presence of CB1R with the following TM2 mutations: Wildtype (B), F170A (C), S173A (D), F177A (E), H178A (F). Concentration-response curves of SCRA-induced BRET plotted as a percentage of CP55 $E_{max,WT}$. Data presented as means ± SEM of n≥3 experiments.

**Table 4: CB1R-TM2 Mutational Analysis with SCRA Head Moiety**

| | WT | | F170A | | S173A | | F177A | | H178A | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | $E_{max}$ (% CP55) | $pEC_{50}$ (M) | $E_{max}$ (% CP55) | $pEC_{50}$ (M) |
| **CP55** | 100.0 ± 5.49 | 7.92 ± 0.16 | 21.2 ± 5.52 | 8.61 ± 0.88 | 44.2 ± 8.79 | 7.13 ± 0.49 | 16.6 ± 11.34 | N.D. | 33.4 ± 7.10 | 6.12 ± 0.38 |
| **M-PC** | 188.1 ± 8.28 | 7.15 ± 0.11 | 155.1 ± 16.91 | 5.99 ± 0.19 | 73.1 ± 5.06 | 7.73 ± 0.20 | 12.9 ± 6.80 | N.D. | 51.8 ± 8.80 | 6.14 ± 0.31 |
| **D-PC** | 180.5 ± 6.43 | 7.91 ± 0.11 | 142.4 ± 9.95 | 6.08 ± 0.12 | 83.0 ± 5.14 | 8.88 ± 0.22 | 166.1 ± 14.33 | 5.67 ± 0.13 | 157.8 ± 10.35 | 6.51 ± 0.15 |

**Data analyzed by one-way ANOVA followed by post-hoc Dunnett test. Data presented as means ± SEM. Significance of * represents p>0.05 compared to CP55 $E_{max}$ of the wildtype construct. For concentration-response curves with an $E_{max}$ less than 20% or with a poor-fit pEC50 were not determined (N.D.)**

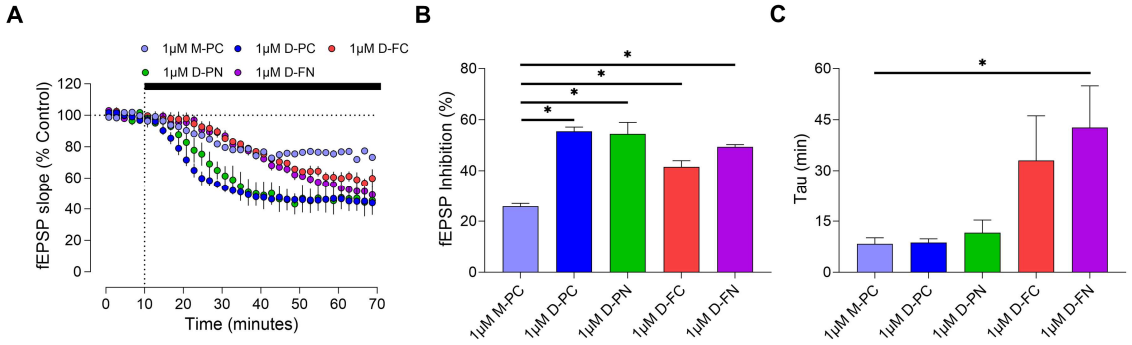


**Figure 5: Extracellular field recordings in the CA1 hippocampus after perfusion of L-*tert*-leucinate SCRAs.** Field extracellular postsynaptic potentials (fEPSPs) were recorded every 30 seconds in the CA1 hippocampus during a perfusion of SCRAs. After 10 minutes of baseline, SCRAs were perfused for 60 minutes (A). Maximum inhibition was calculated from the average of the last five minutes of SCRA perfusion (B). The time decay constant tau was calculated from the fitted curve of the timecourse recording (C). (N=3 mice; n≥3 recordings; means ± SEM).