Positive allosteric modulators of CB_1 signaling in autaptic hippocampal neurons.

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Abbreviations: DSE, depolarization-induced suppression of excitation; 2-AG, 2-arachidonoyl glycerol; NAM, negative allosteric modulator; PAM, positive allosteric modulator; EPSC, excitatory postsynaptic current;

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ABSTRACT (249 words)

The cannabinoid signaling system is found throughout the CNS and its involvement in several pathological processes makes it an attractive therapeutic target. Because orthosteric CB1 cannabinoid receptor ligands have undesirable adverse effects there has been great interest in the development of allosteric modulators -- both negative (NAMs) and positive (PAMs) -- of these receptors. NAMs of CB_1 appeared first on the scene, followed more recently by PAMs. Because allosteric modulation can vary depending on the orthosteric ligand it is important to study their function in a system that employs endogenous cannabinoids. We have recently surveyed first generation NAMs using cultured autaptic hippocampal neurons. These neurons exhibit CB_1 and 2-arachidonoyl glycerol (2-AG)-mediated depolarization induced suppression of excitation (DSE) and therefore are an excellent neuronal model of endogenous cannabinoid signaling in which to test CB_1 modulators.

In this study we find that while two related compounds, GAT211 and ZCZ011, each show PAM-like responses in autaptic hippocampal neurons, they also exhibit complex pharmacology. Notably we were able to separate the PAM- and agonist-like responses of GAT211 by examining the enantiomers of this racemic compound: GAT228 and GAT229. We find that GAT229 exhibits PAM-like behavior while GAT228 appears to directly activate the CB_1 receptor.

Both GAT229 and ZCZ011 represent the first PAMs that we have found to be effective in using this 2-AG utilizing neuronal model system. Because these compounds may exhibit both probe selectivity and biased signaling it will be important to test them with anandamide as well as other signaling pathways.

INTRODUCTION

Cannabinoid receptors are G protein-coupled receptors that mediate many of the salient effects of marijuana consumption (Varvel et al., 2005). These receptors were first identified in the 1980s (Matsuda et al., 1990, Munro et al., 1993) and have since been shown to be key components of an endogenous signaling system that is both phylogenetically highly conserved (Elphick and Egertova, 2001) and active throughout the body. Potent and efficacious orthosteric agonists (i.e. WIN55212-2 and CP55940) and antagonists (i.e. SR141716, AM251) have been extensively studied for more than 20 years. However there is a strong interest in the development of allosteric modulators at CB_1. This is partly due to the sometimes unfavorable therapeutic profiles of the orthosteric CB_1 ligands. Negative allosteric modulators (NAMs) inhibit binding and/or signaling, while positive allosteric modulators (PAMs) potentiate binding and/or signaling, by an orthosteric agonist (Christopoulos, 2014). NAMs/PAMs may act in a variety of ways but in its simplest form the concept is that a receptor may have one or more additional 'allosteric' sites that when engaged will modulate orthosteric signaling. This usually occurs by altering the binding kinetics of the orthosteric ligand and/or by potentiating/inhibiting the receptor's signaling via one or more signaling pathways. One desirable property of allosteric modulators is the ability to modulate only activated receptors. For the nearly ubiquitous cannabinoid signaling system and consequent risk of off-target action during a therapeutic intervention such selectivity is especially desirable.

We have recently examined the actions of first-generation CB_1 allosteric modulators on synaptic transmission using autaptic hippocampal neurons (Straiker et al., 2015), testing five negative and one positive allosteric modulator. Of these, three acted in a manner consistent with allosteric modulation. Autaptic neuronal cultures are a well-characterized model of endogenous cannabinoid signaling that expresses the machinery to produce and metabolize

endocannabinoids as well as presynaptic CB_1 receptors (Straiker and Mackie, 2005, Straiker et al., 2009, Jain et al., 2013). Depolarization of these neurons induces depolarization induced suppression of excitation (DSE) a form of retrograde inhibition involving endocannabinoids and CB_1 receptors found in many brain regions (Kano et al., 2009). The autaptic model is well-suited to an examination of allosterism with endocannabinoids since we have established that DSE is mediated by the endocannabinoid 2-arachidonoylglycerol (Straiker and Mackie, 2005). ZCZ011 was recently reported to serve as a PAM albeit with a mixed pharmacological profile that included agonist properties (Ignatowska-Jankowska et al., 2015) (Fig. 1A). This compound is derived from 3-(2-nitro-1-phenylethyl)-2-phenyl-1H-indole (Noland and Lange, 1959); CAS Registry Number: 102704-40-5; F-0870-0064) referred to here as GAT211 (Fig. 1B). We now report our study of these candidate positive allosteric modulators in a neuronal model of endogenous CB_1 signaling.

METHODS

Hippocampal culture preparation. All procedures used in this study were approved by the Animal Care Committee of Indiana University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Mouse hippocampal neurons isolated from the CA1-CA3 region were cultured on microislands as described previously (Furshpan et al., 1976, Bekkers and Stevens, 1991). Neurons were obtained from animals (age postnatal day 0-2) and plated onto a feeder layer of hippocampal astrocytes that had been laid down previously (Levison and McCarthy, 1991). Cultures were grown in high-glucose (20 mM) DMEM containing 10% horse serum, without mitotic inhibitors and used for recordings after 8 days in culture and for no more than three hours after removal from culture medium.

Electrophysiology. When a single neuron is grown on a small island of permissive substrate, it forms synapses—or "autapses"—onto itself. All experiments were performed on isolated autaptic neurons. Whole cell voltage-clamp recordings from autaptic neurons were carried out at room temperature using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The extracellular solution contained (in mM) 119 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.5 $MgCl_2$, 30 glucose, and 20 HEPES. Continuous flow of solution through the bath chamber (~2 ml/min) ensured rapid drug application and clearance. Drugs were typically prepared as stocks, and then diluted into extracellular solution at their final concentration and used on the same day.

Recording pipettes of 1.8-3 MΩ were filled with (in mM) 121.5 KGluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 MgATP, and 0.5 LiGTP. Access resistance and holding current were monitored and only cells with both stable access resistance and holding current were included for data analysis. Conventional stimulus protocol: the membrane potential was held at –70 mV and excitatory postsynaptic currents (EPSCs) were evoked every 20 seconds by triggering an unclamped action current with a 1.0 ms depolarizing step. The resultant evoked waveform consisted of a brief stimulus artifact and a large downward spike representing inward sodium currents, followed by the slower EPSC. The size of the recorded EPSCs was calculated by integrating the evoked current to yield a charge value (in pC). Calculating the charge value in this manner yields an indirect measure of the amount of neurotransmitter released while minimizing the effects of cable distortion on currents generated far from the site of the recording electrode (the soma). Data were acquired at a sampling rate of 5 kHz.

DSE stimuli: After establishing a 10-20 second 0.5 Hz baseline, DSE was evoked by depolarizing to 0 mV for 50 msec, 100 msec, 300 msec, 500 msec, 1 sec, 3 sec and 10 sec, followed in each case by resumption of a 0.5 Hz stimulus protocol for 20-80+ seconds, allowing EPSCs to recover to baseline values. This

approach allowed us to determine the sensitivity of the synapses to DSE induction. To allow comparison, baseline values (prior to the DSE stimulus) are normalized to one. DSE inhibition values are presented as fractions of 1, i.e. a 50% inhibition from the baseline response is 0.50 ± standard error of the mean. The x-axis of DSE depolarization-response curves are log-scale seconds of the duration of the depolarization used to elicit DSE. Depolarization response curves are obtained to determine pharmacological properties of endogenous 2-AG signaling by depolarizing neurons for progressively longer durations (50 msec, 100 msec, 300 msec, 500 msec, 1 sec, 3 sec and 10 sec). The data are fitted with a nonlinear regression, allowing calculation of an ED50, the effective dose or duration of depolarization at which a 50% inhibition is achieved. Statistical significance in these curves is taken as non-overlapping 95% confidence intervals.

RESULTS

GAT229 has PAM-like response in autaptic hippocampal neurons.

Depolarization of excitatory autaptic hippocampal neurons elicits a form of retrograde inhibition termed depolarization induced suppression of excitation mediated by 2-AG and requiring CB_1 receptors (DSE; (Straiker and Mackie, 2005)). DSE can be quantified by subjecting the neuron to a series of successively longer depolarizations (50ms, 100ms, 300ms, 500ms, 1sec, 3 sec, 10 sec) resulting in progressively greater inhibition of neurotransmission (Straiker et al., 2011). This yields a “depolarization-response curve” that permits the characterization of some pharmacological properties of cannabinoid signaling, including the calculation of an effective-dose 50 (ED50), corresponding in this case to the duration of depolarization that results in 50% of the maximal response. Because we do not see evidence for tonic activity via CB_1 in autaptic neurons (Straiker and Mackie, 2005), a positive allosteric modulator is not expected to directly inhibit synaptic transmission but should shift the depolarization response curve down and/or to the left by enhancing the action of

the 2-AG released by depolarization at CB_1 receptors. We found that GAT211 directly inhibited excitatory postsynaptic currents (EPSCs) in autaptic neurons cultured from wild type (WT) but not $CB_1^{-/-}$ mice (Fig. 2, Relative EPSC charge after GAT211 (1μM) in WT: 0.62 ± 0.08, n=14; in $CB_1^{-/-}$: 1.00 ± 0.02, n=5; $p<0.005$, unpaired t test vs. $CB_1^{-/-}$**)**. This substantial inhibition of EPSCs in a CB1-dependent fashion by GAT211 is inconsistent with pure positive allosteric modulation.

Because GAT211 is a racemic mixture of two enantiomers, we tested each enantiomer separately. One of these, GAT228, affected EPSCs bi-modally, inhibiting only a subset of CB1-expressing neurons (Fig. 3A,B, Relative EPSC charge after GAT228 (1μM) in WT: 0.81 ± 0.08, n=10; in $CB_1^{-/-}$: 0.99 ± 0.03, n=5). No inhibition by GAT228 was seen in any $CB_1^{-/-}$ neurons tested, indicating that when inhibition was present it likely occurred via CB_1 activation. In cells that were not directly inhibited by GAT228, there was no net effect of GAT228 on the depolarization response curve (Fig. 3C: ED50 before GAT228: 1.34 (1.19-1.50) sec; with GAT228: 1.00 (0.77-1.29) sec, n=5; 95% CI overlapping). However the DSE recovery time courses were slowed (Fig. 3D: DSE $t_{1/2}$ baseline: 6.0 (4.4-9.1) sec; with GAT228: 17.7 (13.6-25.4) sec, n=10; 95% CI non-overlapping). The response profile for GAT228 is therefore inconsistent with positive allosteric modulation of CB1 signaling, and consistent with modest agonism.

In contrast, the other enantiomer, GAT229 did not inhibit EPSCs when applied on its own (Fig. 4AB: relative EPSC charge GAT229 (1μM) in WT: 1.0 ± 0.05, n=10; in $CB_1^{-/-}$: 0.97 ± 0.04, n=6; $p>0.05$, unpaired t test). It did however enhance DSE. Figure 4C shows a DSE depolarization series before and after 1μM GAT229; DSE was more pronounced after treatment, particularly for longer depolarizations. These data are summarized in Figure 4D. In contrast to GAT228, GAT229 did not significantly slow recovery from DSE (Fig. 4: $t_{1/2}$ baseline: 9.8 (7.0-16.5) sec; with GAT229: 17.1 (11.2-36.6) sec; n=10, overlapping 95%CI).

ZCZ011 has both agonist and PAM-like properties in autaptic hippocampal neurons.

We also tested the structurally related ZCZ011 compound for PAM-like properties. We found that as with GAT211, ZCZ011 directly inhibited EPSCs in most wild type (Fig. 5A,C; 0.65 ± 0.08, n=9; $p<0.005$ unpaired t-test vs. $CB_1^{-/-}$) but not $CB_1^{-/-}$ knockout neurons (Fig 5C; 1.06 ± 0.02, n=4). But in contrast to GAT211, some neurons did not see a direct inhibition of EPSCs in response to ZCZ011 (Fig. 5B,D). Direct inhibition of currents is consistent with this compound possessing some orthosteric activity and makes PAM-like properties difficult to discern, but as shown in the example in Figure 5E, in some neurons that did not see a direct inhibition, ZCZ011 did however potentiate their DSE responses in a manner similar to that seen for GAT229. Furthermore, the DSE recovery time courses in the five neurons with the least agonist effect were slowed with ZCZ011 (Fig.5E and 5F: DSE $t_{1/2}$ baseline: 24.5 (21.9-27.2) sec; with ZCZ011: 59.5 (50.2-72.8) sec; 95% CI non-overlapping).

DISCUSSION

The potential of allosteric modulators of cannabinoid receptor signaling has generated considerable interest. We have recently surveyed the function of 'first generation' NAMs in a neuronal model that uses 2-AG as the endocannabinoid in an endogenous retrograde signaling system (Straiker et al., 2015) and now report the function of two structurally related candidate PAMs, using an autaptic model of endogenous cannabinoid signaling. Our chief findings are that these compounds can exhibit PAM-like responses in this system but that their pharmacology is complex, even in the architecturally simple autaptic model. Most intriguingly however we found that enantiomers of the racemic GAT211 were pharmacologically distinct and effectively separated out the 'undesired' direct (orthosteric) actions of GAT211 from the 'desired' PAM-like properties. Of

the compounds we tested, therefore, GAT229 appears to be a ‘pure’ PAM in this model. We have additionally found that the related ZCZ011, recently described as a PAM with agonist properties (Ignatowska-Jankowska et al., 2015) behaves in a similar manner (i.e. as a PAM with agonist properties) in autaptic neurons.

As we have recently noted (Straiker et al., 2015), our experiments can ‘rule out’ an allosteric candidate in terms of 2-AG modulation of synaptic transmission, but cannot rule one in: the definitive identification of a compound as an allosteric agent generally requires binding studies that characterize the impact of that compound on the binding kinetics of an orthosteric ligand. We do not therefore claim that the ZCZ011 and GAT compounds are or are not allosteric modulators. However we have shown whether they do/do not act in a manner consistent with allosteric modulation in their CB_1-dependent inhibition of glutamatergic transmission.

Our synaptic physiology studies suggest that GAT228 directly inhibits EPSCs via CB_1 in a manner that is inconsistent with “pure” positive allosteric modulation. As noted above, we have previously shown that these neurons do not exhibit detectable CB_1-dependent tonic activity and do not therefore expect that sufficient 2-AG is present under baseline conditions to serve as a substrate for allosteric modulation. But since GAT228 directly inhibits EPSCs via CB_1, one would expect this to shift the depolarization response curve to the right. Instead we see no shift at all. This may be explained as resulting from a dual action since GAT228 also slows the DSE recovery time course. This slowing may result from slowing the dissociation of 2-AG from CB_1, perhaps allosterically. If so GAT228 may be an ago-PAM in this neuronal setting though this does not explain the differential responses in some neurons versus others. Because GAT211 consists of both GAT228 and GAT229, the issues associated with GAT228 extend to GAT211. In contrast, GAT229 behaved in a manner consistent with positive allosteric modulation in this assay.

Our findings for the structurally similar ZCZ011 are similar to those for the GAT211/228/229 compounds but are also distinct in important ways. Like GAT229, ZCZ011 exhibited a PAM-like response in some neurons, but like GAT228 it also directly inhibited neurotransmission in many autaptic neurons. And unlike GAT211, ZCZ011 did not directly inhibit EPSCs in all neurons. Indeed this points up a puzzling attribute of GAT211 in that the racemic mixture appears to behave differently than one would predict based on the properties of the enantiomers when taken alone. That profile would look more like ZCZ011 – PAM properties in some neurons, agonist properties in others – but instead GAT211 appears to routinely exhibit agonist properties. The reasons for this are unclear but may involve a synergistic action of the enantiomers.

As noted above ZCZ011 was developed as a derivative of 3-(2-nitro-1-phenylethyl)-2-phenyl-1H-indole, referred to here as GAT211 but also known as F087 (Noland and Lange, 1959). The PAM-like properties of this compound were first described in a conference abstract in 2007 ("AZ-4" (Adam et al., 2007)). The structure of ZCZ011 suggests that it is also racemic. It would therefore be of interest to test whether the enantiomers also effectively separate the PAM and agonist properties we have seen for ZCZ011. These enantiomers are not however currently available. Furthermore there may be a limit to the value of studying ZCZ011 enantiomers: although these compounds represent an exciting development in terms of CB_1 allosteric pharmacology, they all suffer from an important shortcoming that greatly limits their application as therapeutic agents. The structures of GAT211/228/229 and ZCZ011 all include an aliphatic nitrogen moiety that is generally avoided in the design of pharmacotherapeutics. The challenge now will be to develop PAMs that offer similar pharmacological properties without the structural properties that limit their therapeutic relevance.

In summary, we have found that GAT211 behaves as an agonist at cannabinoid CB1 receptors in autaptic hippocampal neurons. Importantly the racemic GAT211 exhibits enantiomer-specific signaling; it is possible to dissect out the

PAM-like responses in one enantiomer while the other enantiomer exhibits the agonist properties. In the related compound, ZCZ011, also likely to be racemic, we similarly see agonist and PAM-like properties. These are the first compounds that we have observed to behave as PAMs in this model system. Our results indicate that PAMs can effectively enhance endogenous cannabinoid signaling via CB_1 and the characterization of this class of PAMs may pave the way for further development of clinically significant allosteric modulators and therefore expand upon the considerable clinical potential of cannabinoid-bases therapies.

Literature Cited

Adam L, Salois D, Rihakova L, Lapointe S, St-Onge S, Labrecque J, Payza K (2007) Positive Allosteric Modulators of CB1 Receptors. In: International Cannabinoid Research Society, p 86.

Bekkers JM, Stevens CF (1991) Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. Proc Natl Acad Sci U S A 88:7834-7838.

Christopoulos A (2014) Advances in G protein-coupled receptor allostery: from function to structure. Mol Pharmacol 86:463-478.

Elphick MR, Egertova M (2001) The neurobiology and evolution of cannabinoid signalling. Philos Trans R Soc Lond B Biol Sci 356:381-408.

Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD (1976) Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc Natl Acad Sci U S A 73:4225-4229.

Ignatowska-Jankowska BM, Baillie GL, Kinsey S, Crowe M, Ghosh S, Owens RA, Damaj IM, Poklis J, Wiley JL, Zanda M, Zanato C, Greig IR, Lichtman AH, Ross RA (2015) A Cannabinoid CB1 Receptor-Positive Allosteric Modulator Reduces Neuropathic Pain in the Mouse with No Psychoactive Effects. Neuropsychopharmacology 40:2948-2959.

Jain T, Wager-Miller J, Mackie K, Straiker A (2013) Diacylglycerol lipasealpha (DAGLalpha) and DAGLbeta cooperatively regulate the production of 2-arachidonoyl glycerol in autaptic hippocampal neurons. Mol Pharmacol 84:296-302.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89:309-380.

Levison SW, McCarthy KD (1991) Characterization and partial purification of AIM: a plasma protein that induces rat cerebral type 2 astroglia from bipotential glial progenitors. J Neurochem 57:782-794.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561-564.

Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61-65.

Noland WE, Lange RF (1959) The Nitroethylation of Indoles. III. A Synthetic Route to Substituted Tryptamines. J Am Chem Soc 81:6.

Straiker A, Hu SS, Long JZ, Arnold A, Wager-Miller J, Cravatt BF, Mackie K (2009) Monoacylglycerol lipase limits the duration of endocannabinoid-mediated depolarization-induced suppression of excitation in autaptic hippocampal neurons. Mol Pharmacol 76:1220-1227.

Straiker A, Mackie K (2005) Depolarization-induced suppression of excitation in murine autaptic hippocampal neurones. J Physiol 569:501-517.

Straiker A, Mitjavila J, Yin D, Gibson A, Mackie K (2015) Aiming for allosterism: Evaluation of allosteric modulators of CB1 in a neuronal model. Pharmacol Res 99:370-376.

Straiker A, Wager-Miller J, Hutchens J, Mackie K (2011) Differential signaling in human cannabinoid CB(1) receptors and their splice variants in autaptic hippocampal neurons. Br J Pharmacol.

Varvel SA, Bridgen DT, Tao Q, Thomas BF, Martin BR, Lichtman AH (2005) Delta9-tetrahydrocannbinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. J Pharmacol Exp Ther 314:329-337.

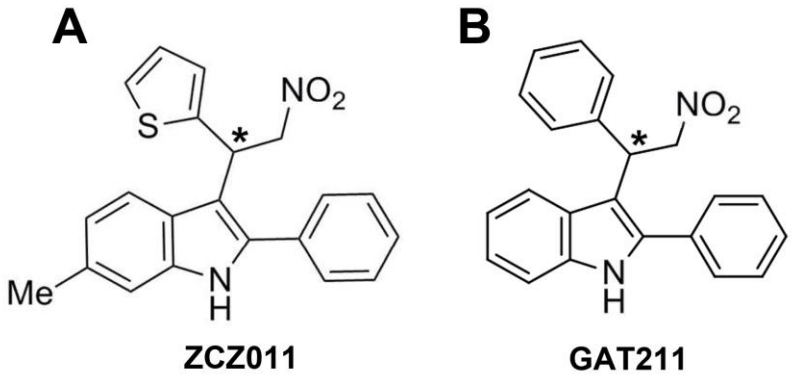

Figure. 1 Structures of ZCZ011 and GAT211

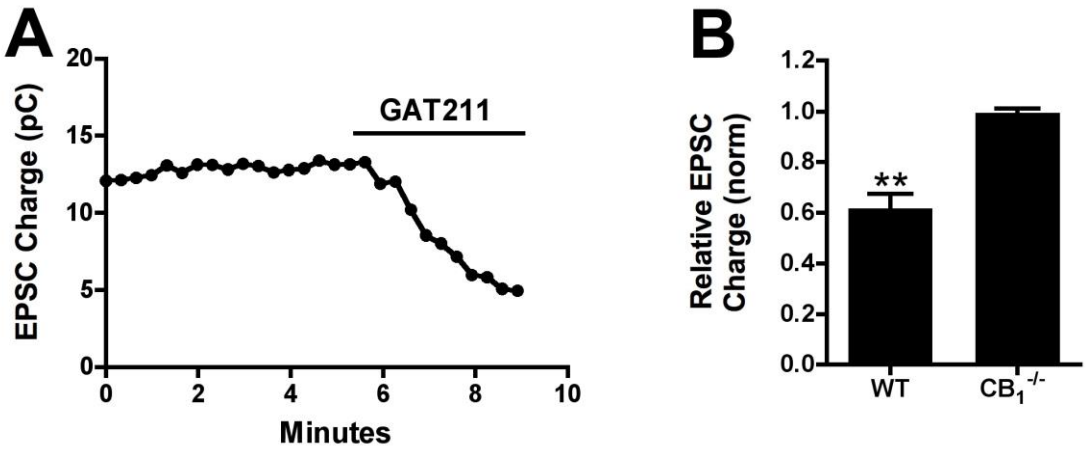

Figure 2. GAT211 inhibits EPSCs via CB_1 A) Sample time course shows GAT211 (1μM) inhibits integrated EPSC current (charge, pC). B) Summarized data shows that GAT211 inhibits EPSCs in WT (n=14) but not $CB_1^{-/-}$ neurons (n=6). **, $p < 0.005$, unpaired t-test vs. $CB_1^{-/-}$.

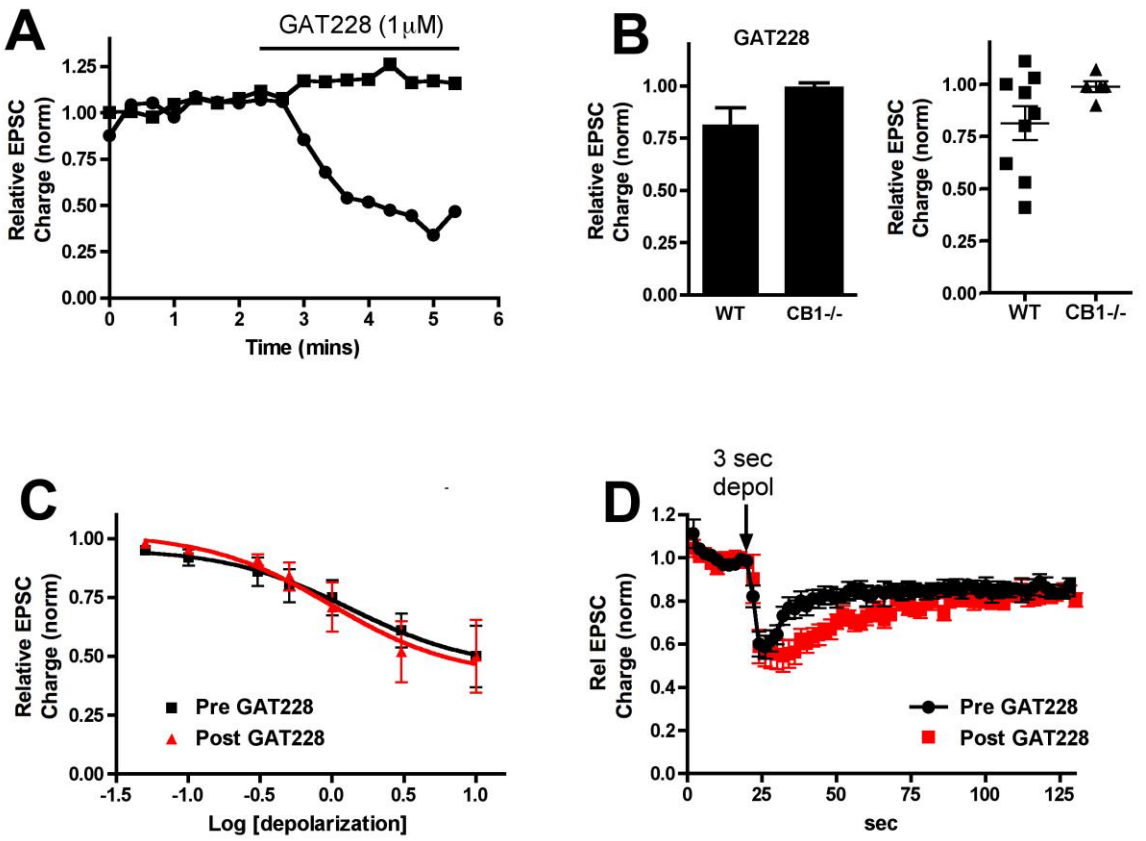

Figure 3. GAT228 inhibits EPSCs in some neurons. A) Sample time course shows GAT228 (1μM) inhibits EPSCs in some neurons. B) Summarized data as bar graph (left) and scatter plot (right) shows a bimodal inhibition of EPSCs in WT (n=10) but not $CB_1^{-/-}$ (n=5) neurons by GAT228. C) The DSE depolarization response profile in cells not directly inhibited by GAT228 treatment is unaltered (n=5). D) A summarized DSE (3 sec depolarization) time course before and after treatment shows that GAT228 does not change the peak inhibition but does slow recovery (n=10).

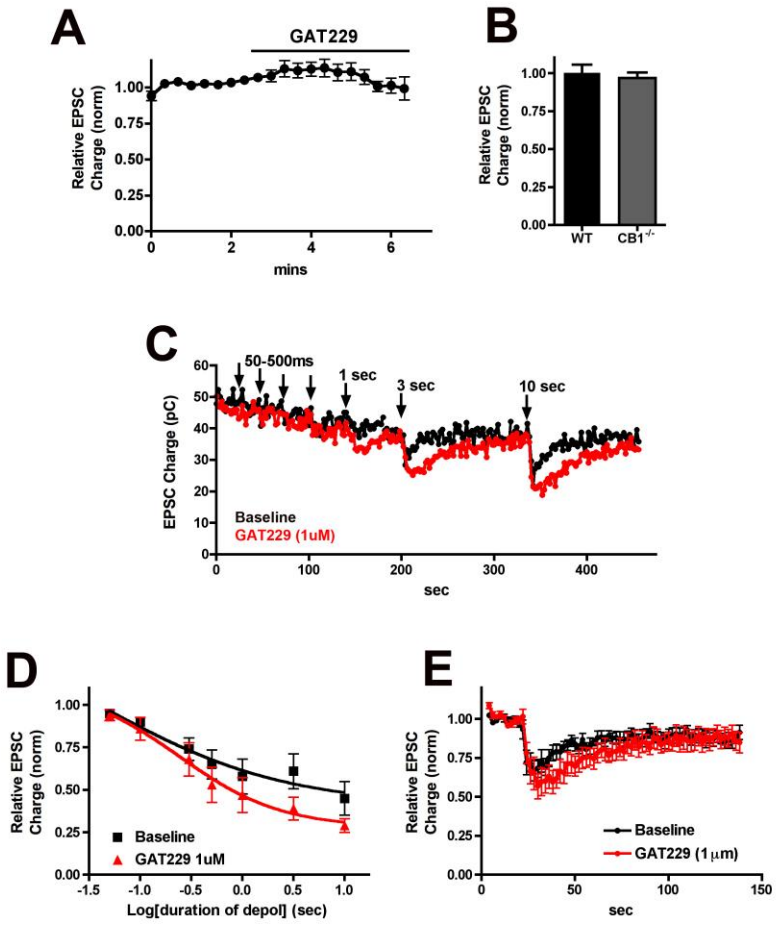

Figure 4. GAT229 acts in a manner consistent with positive allosteric modulation of CB1 in autaptic neurons. A) Average time course shows GAT229 (1μM) does not inhibit EPSCs. B) Summarized data from A) n=10, 6. C) Series of progressively longer depolarizations shows that treatment with GAT229 results in greater EPSC inhibition. D) DSE depolarization response curve is shifted down and to the left after GAT229 treatment (n=10). E) Summary of the 3 sec depolarization DSE time course before and after treatment with GAT229 (n=5).

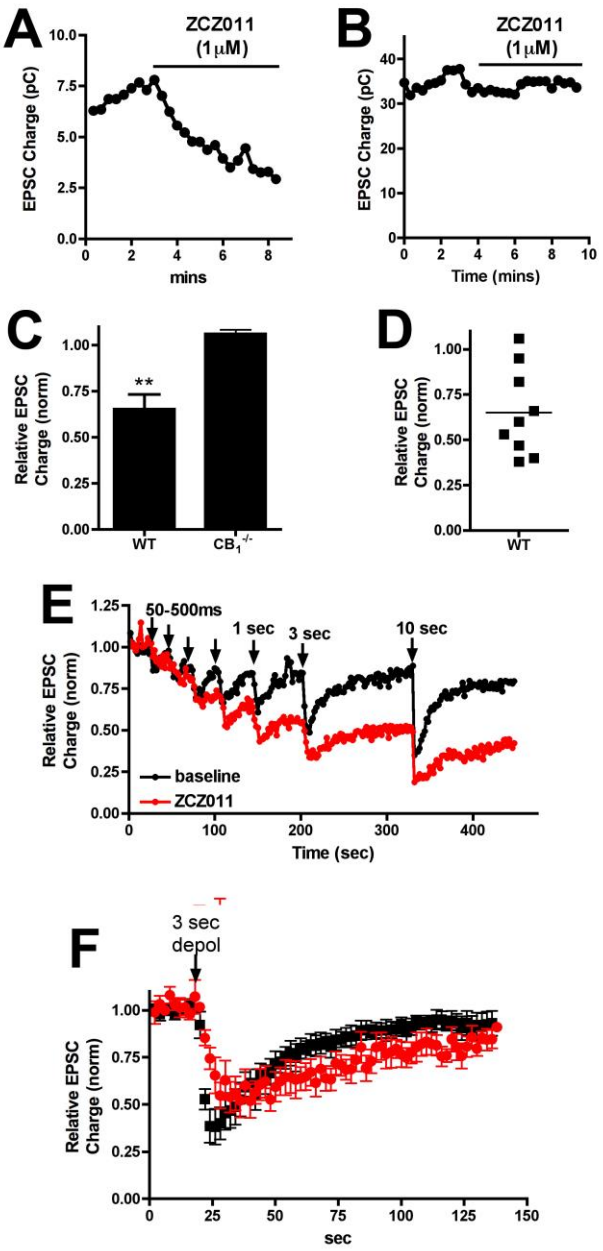

Figure 5. ZCZ011 exhibits both agonist and PAM-like properties in autaptic neurons. A-B) sample time courses where ZCZ011 (1μM) inhibits (A) or does not inhibit (B) EPSCs. C) Summary data for direct inhibition of neurotransmission in wild type (WT) and $CB_1^{-/-}$ neurons. D) Scatterplot of wild type data in C shows distribution of responses. E) Sample DSE series time course (50ms-1sec) in neuron that did not exhibit a direct inhibition in response to ZCZ011. F) Sample DSE time courses in neurons that saw limited or no inhibition in response to ZCZ011. **, $p<.005$, unpaired t-test vs. $CB_1^{-/-}$