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Aiming for allosterism: Evaluation of allosteric modulators of CB_1 in a neuronal model

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Abstract

Cannabinoid pharmacology has proven nettlesome with issues of promiscuity a common theme among both agonists and antagonists. One recourse is to develop allosteric ligands to modulate cannabinoid receptor signaling. Cannabinoids have come late to the allosteric table. The 'first-generation' negative and positive allosteric modulators (NAMs and PAMs) represent an important first effort. However, most studies have relied on synthetic agonists, often tested in over-expression systems rather than a defined neuronal model system that utilizes endogenously synthesized and released cannabinoids. We have systematically examined first-generation NAMs and a PAM on endocannabinoid modulation of synaptic transmission in cultured autaptic hippocampal neurons. These neurons exhibit CB_1 and 2-arachidonoyl glycerol (2-AG)-mediated depolarization induced suppression of excitation (DSE) and therefore serve as a model to test CB_1 modulators in a neuronal model of endogenous cannabinoid signaling.

We find ORG27569, PSNCBAM-1, and PEPCAN12 attenuate DSE and do not directly inhibit CB_1 receptors. Of these PSNCBAM-1 is the most efficacious while PEPCAN12 has the distinction of being an endogenous NAM. The reported NAMs pregnenolone and hemopressin as well as the reported PAM lipoxin A4 are without effect in this model of endocannabinoid signaling.

In summary, three of the allosteric modulators evaluated function in a manner consistent with allosterism in a neuronal 2-AG-based model of endogenous cannabinoid signaling.

Graphical Abstract

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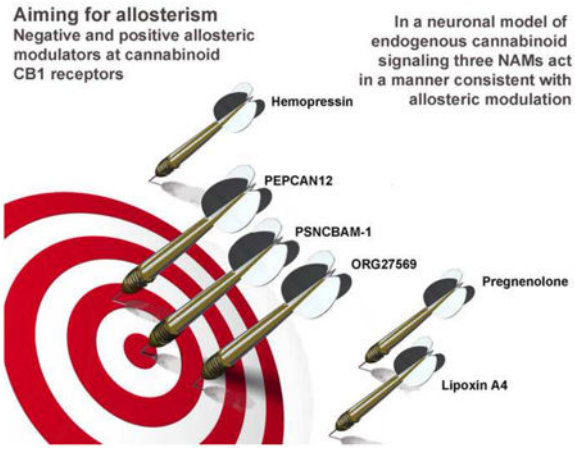
CONFLICT OF INTEREST

The authors declare that they do not have a conflict of interest relating to this work.



Keywords

allosterism; allosteric; orthosteric; cannabinoid; depolarization-induced suppression of excitation; tetrahydrocannabinol; excitatory postsynaptic current

INTRODUCTION

Cannabinoid receptors were first identified in the early 1990s [1,2]. These G protein-coupled receptors mediate most of the salient effects of marijuana consumption and also are also key components of an endogenous signaling system that is both phylogenetically highly conserved [3] and active throughout the body. The cannabinoid research field benefitted from the early identification of potent and efficacious orthosteric agonists (i.e. WIN55212-2 and CP55940) and antagonists (i.e. SR141716, AM251), all still in experimental use. However, due to the sometimes unfavorable therapeutic profiles of the orthosteric CB_1 ligands, there has been a strong interest in the development of allosteric modulators at CB_1. Negative allosteric modulators (NAMs) inhibit binding and/or signaling while positive allosteric modulators (PAMs) potentiate binding and/or signaling by an orthosteric agonist [4]. 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. A major advantage of allosteric modulators is the potential to modulate only activated receptors. This selectivity is especially relevant to the nearly ubiquitous cannabinoid signaling system with its high level of endogenous activity and consequent risk of off-target action during a therapeutic intervention.

Allosteric modulation is not a new idea. For instance several important classes of drugs are allosteric modulators at $GABA_A$ receptors (e.g. benzodiazepines [5]). However the first two CB_1 NAMs, ORG27569 and PSNCBAM-1 were characterized less than ten years ago [6–9], while the first CB_1 PAM has been described more recently [10]. These first generation allosteric modulators generated excitement but additional studies have found that they possess complex pharmacological profiles. For instance, ORG27569 lowers cAMP

inhibition consistent with NAM activity [6]. But ORG27569 also increases binding of CP55940 [9], stimulates CB_1 internalization [6], and potentiates Gα-mediated pERK production [11]. Similarly PSNCBAM-1 has been demonstrated to enhance binding affinity [8]. Adding to the complexity, a recent study that examined ORG27569 and PSNCBAM-1 found that even the reported NAM-like reduced CB_1 internalization [11], may in fact be a secondary consequence of enhanced orthosteric binding and consequent desensitization [12]. Therefore these compounds do not behave as 'pure' NAMs under the conditions that they have been tested thus far.

PEPCAN12 is a member of a novel class of endogenous compounds, since the cannabinoid field is dominated by lipid ligands [13]. Discovered in 2012, PEPCAN12 may be an endogenous CB_1-modulating peptide acting as a NAM. More recently levels of the important steroid hormone precursor, pregnenolone, were reported to be upregulated *in vivo* by Δ^9THC; pregnenolone acted as a NAM for Δ^9THC at CB_1 receptors [14]. While this is a highly interesting finding, the interaction of pregnenolone with CB_1 receptors activated by endogenous cannabinoids was not evaluated. And lastly lipoxin A4 was reported as one of the first CB_1 PAMs [10]. The identification of a PAM is of particular interest chiefly because many of the therapeutic applications for cannabinoids involve CB_1 activation. PAMs offer the possibility of targeting a subset of CB1 receptors (i.e. those currently active). Additionally, a pathway-specific PAM may further limit off-target action and may avoid undesired psychoactive effects, particularly when CB_1 is activated by endogenous ligands.

The pharmacology of allosterism can become quite complex, particularly because allosteric modulation can be probe-dependent, i.e. it may depend greatly on the orthosteric ligand being used [4]. Generally speaking, the therapeutic use of an allosteric modulator will involve the endogenous ligand, yet the characterizations of most CB1 allosteric modulators have used synthetic agonists such as CP55940 or WIN55212-2 or the phytocannabinoid, Δ^9THC. We have examined the effect of a range of first-generation CB_1 allosteric modulators on synaptic transmission using autaptic hippocampal neurons. These 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 [15–17]. 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 [18]. The autaptic model is well-suited to an examination of allosterism with the endocannabinoids since we have established that DSE is mediated by the endocannabinoid 2-arachidonoylglycerol [15]. We now report our study of first-generation allosteric modulators in a neuronal model of endogenous CB1 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 (CD1 strain) hippocampal neurons isolated from the CA1-CA3 region were cultured on microislands as described previously [19,20]. 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 [21]. 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 95% confidence interval.

Drugs

Concentrations of the drugs were tested at 1μM considered to be a concentration that was high enough to expect to see an effect but not so high as to introduce an artifact or off-target effect. The exception was PEPCAN12 which has been found to be effective at very low concentrations. Thus we tested this compound at the lower lower concentrations of 200nM. PEPCAN12 was the generous gift of Dr. Juerg Gertsch (Universität Bern). ORG27569 was the generous gift of Dr. Ruth Ross (University of Toronto). Hemopressin was the generous gift of Dr. Lakshmi Devi (Mt. Sinai University). PSNCBAM-1, lipoxin A4 and pregnenolone were purchased from Tocris Bioscience (Bristol, UK), Cayman Chemicals (Ann Arbor, MI), and Sigma Aldrich (St. Louis, MO), respectively.

RESULTS

We examined the signaling properties of candidate CB_1 allosteric modulators in autaptic hippocampal neurons, a model of endogenous cannabinoid signaling. CB_1 activation inhibits neurotransmitter release in these neurons via βγ subunit-dependent inhibition of calcium channels, resulting in a smaller excitatory postsynaptic current (EPSC) [15,22,23]. A negative allosteric modulator (NAM) at CB_1 should not inhibit EPSCs on its own but it should reduce the inhibition induced by CB_1 activation. Because 2-AG mediated depolarization induced suppression of excitation (DSE) can be elicited in these neurons, we can test the effect of a given candidate NAM on 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). A NAM should shift the DSE response curve up and/or to the right, while a PAM should shift the curve down and/or to the left.

ORG27569 and PSNCBAM-1 act in a manner consistent with negative allosteric modulation

We first tested the two original negative allosteric modulators: ORG27569 [6,7,9] and PSNCBAM-1 [8]. ORG27569 did not directly inhibit EPSCs directly at 1μM (Fig 1C, relative EPSC charge (1μM ORG): 0.99 ± 0.01, n=6). We also found that at 1μM ORG27569 did not shift the ED50 of the DSE depolarization response curve (Fig 1A, ED50(baseline): 1.25 (1.15–1.36) ED50(ORG27569): 1.30 sec (1.12–1.51); n=5, overlapping 95% CIs). However, there was a statistically significant difference in inhibition for longer depolarizations (Figure 1A, relative EPSC charge (baseline, 10 sec depol): 0.38 ± 0.04; (ORG): 0.59 ± 0.03, n=6; $p<0.01$, 2 way ANOVA with Bonferroni posthoc test at 3 and 10 secs). The DSE time course (three-second depolarization) was unaltered by the drug treatment (Fig. 1B). 100 nM ORG27569 had no effect on the DSE response profile (data not shown). ORG27569 can therefore be said to act in a manner consistent with a negative allosteric modulator of the endocannabinoid 2-AG in this neuronal model.

Turning to PSNCBAM-1, the results for this candidate NAM were quite striking: at 1μM the compound substantially diminishes cannabinoid signaling (Fig 2A, ED50(pre): 2.1 sec (1.5–2.8); ED50(PSNCBAM-1): 3.8 sec (3.3–4.3); n=5, non-overlapping 95 CI). We found that PSNCBAM did not inhibit neurotransmission on its own during the five-minute incubation period (Fig 2C, Relative EPSC charge in WT: 1.00 ± 0.08; n=5, $p<0.05$ by unpaired t-test).

This indicates that PSNCBAM-1 may be an efficacious negative allosteric modulator of 2-AG at CB_1.

PEPCAN12 modestly inhibits cannabinoid signaling while hemopressin does not

PEPCAN12, also known as RVD-hemopressin, is a 'peptide cannabinoid' that was described in 2012 as a potential NAM [13]. PEPCANs are interesting both because they represent non-lipid modulators of cannabinoid signaling and also because they may be produced endogenously. We tested PEPCAN12 at 200 nM as these peptides were reported to act at relatively low concentrations [13]. We found that PEPCAN12 has no direct effect on EPSCs (Fig. 3C, Relative EPSC charge (PEPCAN12 (200nM): 0.97 ± 0.03, n=11) but that it does inhibit CB_1 responses, an effect that is consistent with NAM action (Fig 3, ED50(pre): 0.58 sec (0.52–0.65); ED50(PEPCAN12): 1.86 sec (1.60–2.15); n=10, non-overlapping 95% CIs).

The structurally similar hemopressin (PEPCAN12: Arg - Val - Asp - Pro - Val - Asn - Phe - Lys - Leu - Leu - Ser–His; Hemopressin: Pro - Val – Asn – Phe - Lys – Phe – Leu – Leu – His) was reported to be an antagonist at CB_1 [24]. We include it here because of its relationship to PEPCAN12 and the possibility that its antagonist properties might be due negative allosterism. Bath-applied hemopressin (2μM) did not inhibit DSE (Fig 4A, relative EPSC charge after 3 sec DSE (baseline): 0.32 ± 0.04; (HP 2μM): 0.38 ± 0.05, n=5; $p>0.05$ unpaired t-test). Because of the conflict with published results, we hypothesized that the peptide might have trouble crossing the lipid bilayer and therefore included hemopressin in the recording pipette, but saw no effect even after 15 minutes of dialysis (Fig 4B, relative EPSC charge after 3 sec DSE (0 mins): 0.44 ± 0.04; (15 mins): 0.47 ± 0.06, n=6; $p>0.05$ 1 way ANOVA with Bonferroni posthoc test).

Pregnenolone does not attenuate 2-AG signaling in autaptic neurons

Lastly among the NAMs we tested the steroid hormone precursor, pregnenolone. In a recent report pregnenolone was found to act in a manner consistent with negative allosteric modulation of CB1 [14] a potentially significant finding for this important hormone precursor. However, we found that pregnenolone at 1μM was without effect on endocannabinoid signaling in autaptic neurons (Fig 5, ED50(pre): 2.7 sec (2.4–3.0); ED50(Preg 1μM): 2.2 sec (2.0–2.4); Relative EPSC charge after preg: 1.01 ± 0.01, n=5).

Lipoxin A4 antagonizes cannabinoid inhibition of neurotransmission

To finish on a positive note, we turned to the putative PAM lipoxin A4 [10]. In contrast to NAMs, we would expect lipoxin A4 to shift the DSE curve to the left. However we found that if anything 1μM lipoxin A4 shifted the curve to the right, suggesting *antagonism* of CB_1 signaling (Fig. 6, ED50(baseline): 2.2 sec (2.1–2.4); (drug): 3.3 sec (3.0–3.6); n=5; non-overlapping 95% CIs). Because lipoxin A4 did not inhibit EPSCs when applied on its own (Fig 6C, relative EPSC charge: 1.02 ± 0.02, n=5), it is possible that lipoxin A4 serves as a NAM rather than a PAM with 2-AG as the ligand.

DISCUSSION

Allosteric modulators of cannabinoid receptor signaling are relatively new arrivals on the pharmacotherapeutic stage. Since CB_1 receptors (and the associated therapeutic potential) are abundant throughout the CNS and since 2-AG is implicated in many of the known forms of CB_1-mediated plasticity, we have tested several of the first-generation negative and positive allosteric modulators in a neuronal model that uses 2-AG as the endocannabinoid in an endogenous retrograde signaling system. Our chief finding is that two of the six candidates act in a manner consistent with their predicted allosteric modulation while the remaining compounds have a diversity of actions, including agonist effects. While our experiments can 'rule out' an allosteric candidate (in regard to 2-AG modulation of synaptic transmission), they cannot rule one in: the definitive identification of a compound as an allosteric agent requires binding studies that characterize the impact of that compound on the binding kinetics of an orthosteric ligand. We do not therefore conclude that a given compound is or is not an allosteric modulator but have determined whether a compound does/does not act in a manner consistent with allosteric modulation in the specific pathway of 2-AG inhibition of glutamatergic transmission. And importantly all is not lost for compounds that fail to act as negative allosteric modulators in a neuronal model of inhibition of synaptic transmission. As has been shown for ORG27569, a given compound may serve as a negative allosteric modulator for one signaling pathway but not another. Indeed this may be a preferred outcome. The CB_1 antagonist SR141716 (aka Rimonabant/Accomplia) ran afoul of antihedonic properties during clinical use following EMA approval [25,26] that led to its failure to be approved in the US and eventual withdrawal from the market in Europe. A NAM that avoids inhibition of the receptors/pathways responsible for this antihedonic action may produce the desired therapeutic/cosmetic outcome free of this side-effect. Conversely one of the allures of positive allosteric modulators would be the ability to induce a therapeutic effect without the undesired psychoactive responses, perhaps via signaling pathway-specific actions. Our results add a notch in favor of ORG27569 as a NAM. As noted above ORG27569 has a mixed profile that includes an enhancement of CP55940 binding as well as an unexpected enhancement of pERK signaling [11]. Most studies of ORG27569 action have examined its interaction with the synthetic CB_1 agonist CP55940 or the endocannabinoid anandamide [11]. Though relatively well-characterized and easier to handle than the readily-metabolized 2-AG, it is likely that the NAM profile differs depending on the orthosteric agonists active in a given circuit. The interaction with endogenous cannabinoids such as 2-AG or anandamide is presumably more therapeutically relevant but one could envision a 'one-two' synthetic agonist/allosteric modulator drug combination tailored to a specific pathway to achieve a particular end. As noted above the peptide nature of PEPCAN12 offers interesting possibilities. However the NAM effect of PEPCAN12 was only significant over part of the range of DSE depolarizations even at the relatively (for PEPCANs) high concentration of 200nM. Another notable aspect of PEPCAN12 is that in contrast to the synthetic compounds tested, PEPCAN12 is found endogenously as has recently been shown in CNS and adrenal medulla [27].

Our negative result for pregnenolone suggests that the allosteric inhibition observed by Vallee et al may be probe-dependent (i.e., restricted to Δ^9-THC and perhaps WIN55212-2).

Since the role of pregnenolone was presented as protective against Δ^9-THC toxicity, a lack of interaction with the endogenous cannabinoid signaling may be particularly advantageous —disruption of Δ^9-THC actions, while maintaining those mediated by 2-AG.

Lastly our results for lipoxin A4, the only putative PAM tested for this study, are perhaps the most surprising since the profile we observed better fits a NAM rather than a PAM. Pamplona et al. did however report that lipoxin A4 exhibits a probe-dependence favoring anandamide over 2-AG. It is possible therefore that lipoxin A4 will exhibit a PAM-like effect at synapses and in cells that utilize anandamide-based signaling. Unfortunately, there is not currently a culture neuronal model of anandamide-based cannabinoid signaling to test the potential probe-dependence of lipoxin A4.

In summary we see mixed results for first-generation allosteric modulators in a neuronal model of endogenous cannabinoid inhibition of neurotransmission by 2-AG, with three compounds PSNCBAM-1, ORG27569 and PEPCAN12 acting in a manner consistent with their predicted allosteric modulation. Of these PSNCBAM-1 is relatively efficacious while PEPCAN12 has the distinction of being an endogenous NAM. The greatest challenge in developing allosteric modulators is identifying first-in-class compounds. Barring considerable good fortune, these compounds are unlikely to be optimal, but can help to identify structural motifs and the allosteric site (or sites) [14,28]. It is therefore not greatly surprising that these first-generation allosteric modulators are mostly, for lack of a better term, namby-pamby. It is likely that the next generation of allosteric modulators will come closer to fulfilling their considerable promise as an addendum to the therapeutic canon of cannabinoids.

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Abbreviations

DSE	depolarization-induced suppression of excitation
2-AG	2-arachidonoly glycerol
NAM	negative allosteric modulator
PAM	positive allosteric modulator
Δ^9THC	tetrahydrocannabinol
EPSC	excitatory postsynaptic current
pERK	phospho extracellular signaling related kinase

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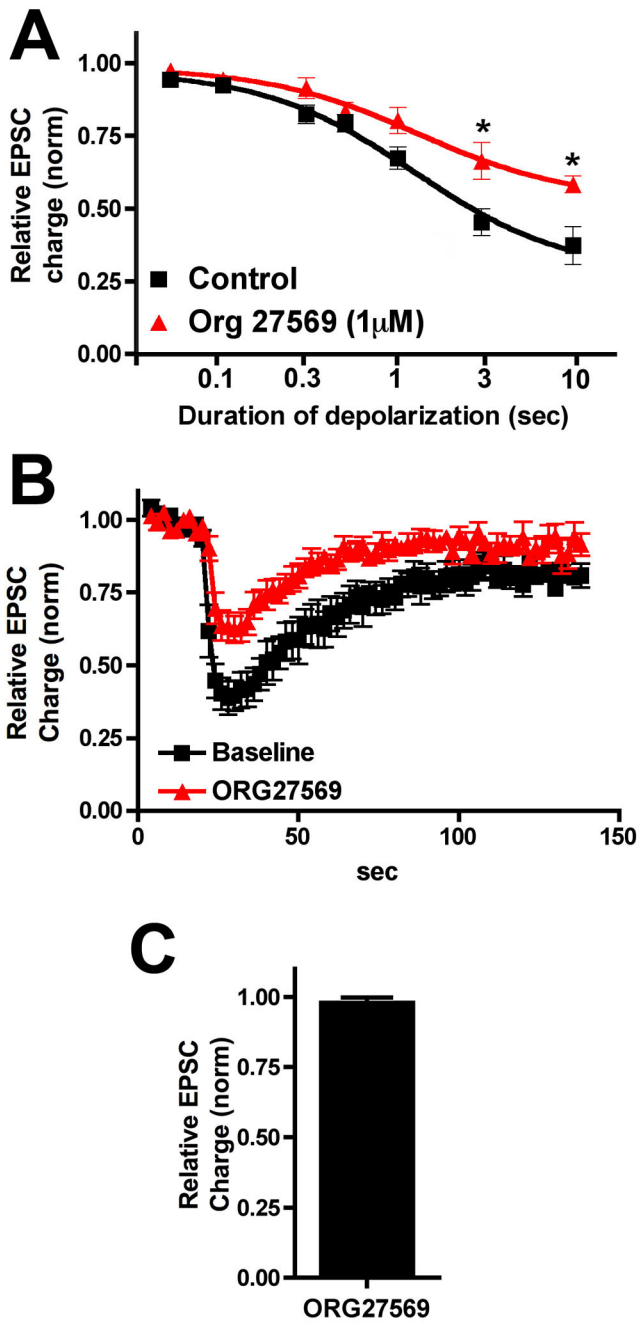

Figure 1. ORG27569 inhibits DSE

A) Depolarization response curve before (black squares) and after/during 1μM ORG27569 treatment (red triangles). B) Averaged DSE (3 sec depolarization) time courses before and after drug. C) Effect of 1μM ORG27569 alone on EPSCs.

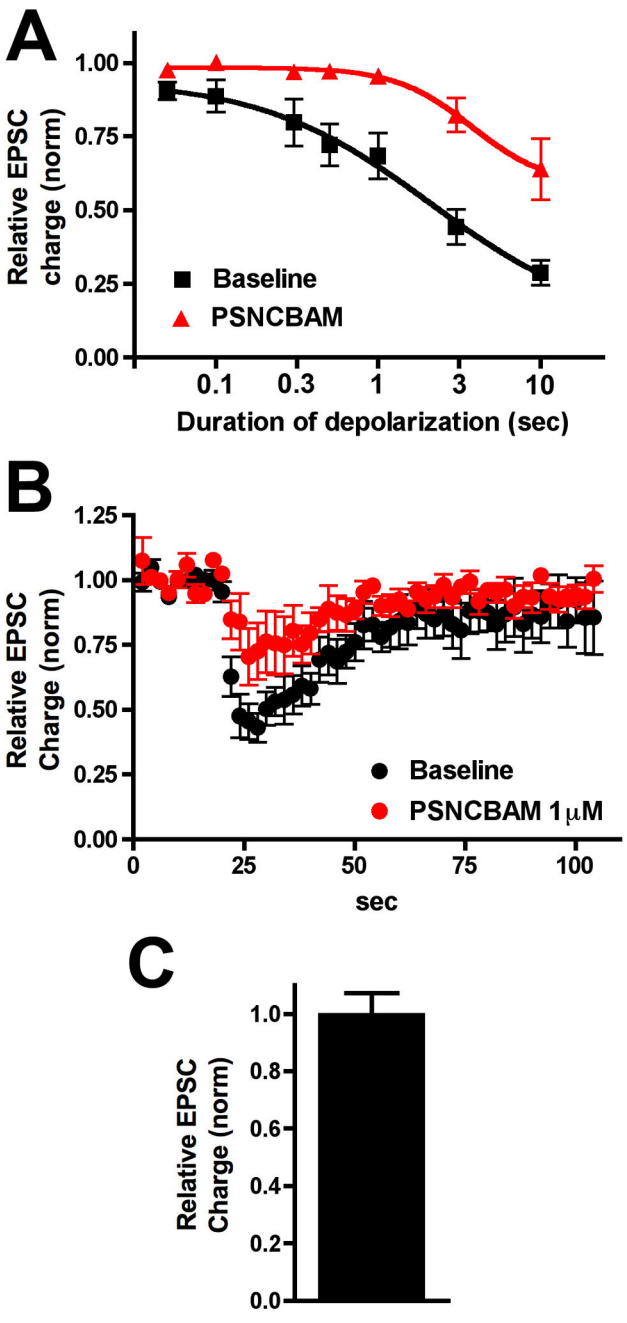

Figure 2. PSNCBAM-1 substantially inhibits DSE

A) Depolarization response curve before (black squares) and after/during 1μM PSNCBAM-1 treatment (red triangles). B) Averaged DSE (3 sec depolarization) time courses before and after drug. C) Effect of 1μM PSNCBAM-1 alone on EPSCs in WT neurons.

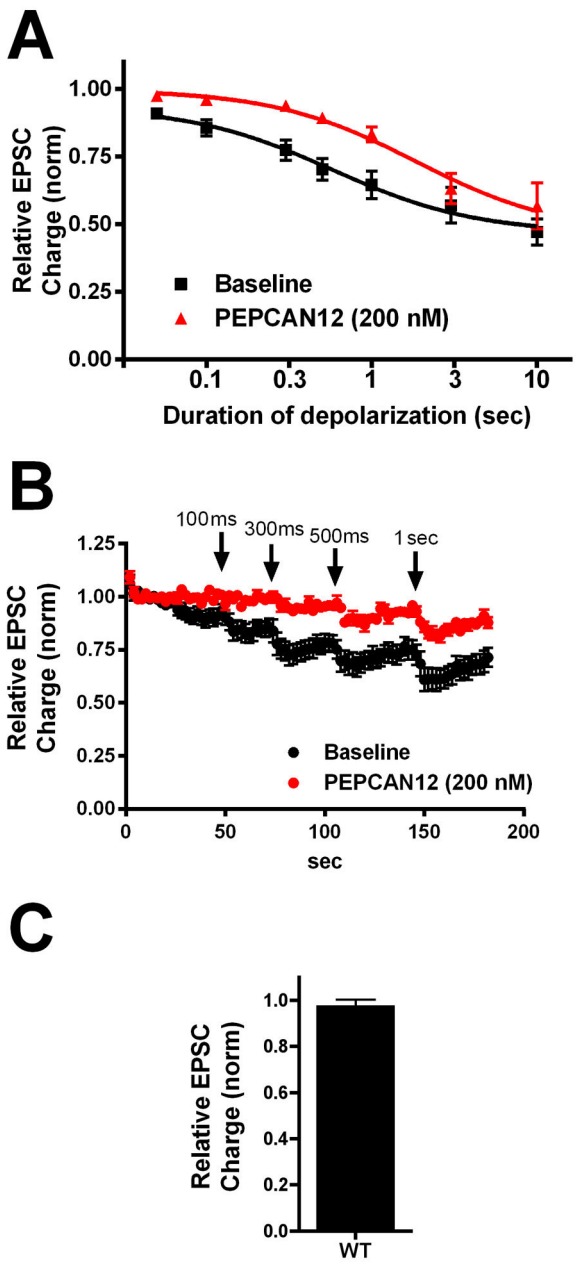

Figure 3. PEPCAN12 inhibits DSE

A) Depolarization response curve before (black squares) and after/during 200nM PEPCAN12 treatment (red triangles). B) Averaged DSE (series of depolarizations 100msec to 1 sec) time courses before and after drug. C) Effect of 200nM PEPCAN12 alone on EPSCs.

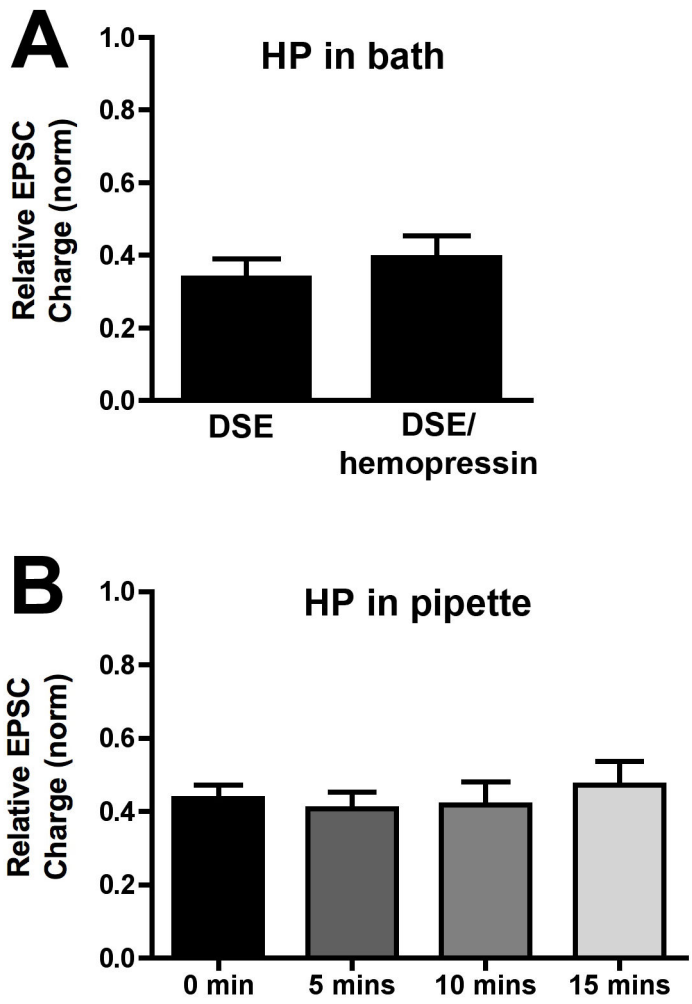

Figure 4. Hemopressin does not inhibit neurotransmission via CB_1

A) DSE response before and after 1μM hemopressin. B) Effect of 2μM hemopressin (pipette) on DSE at indicated time alone on EPSCs in WT neurons.

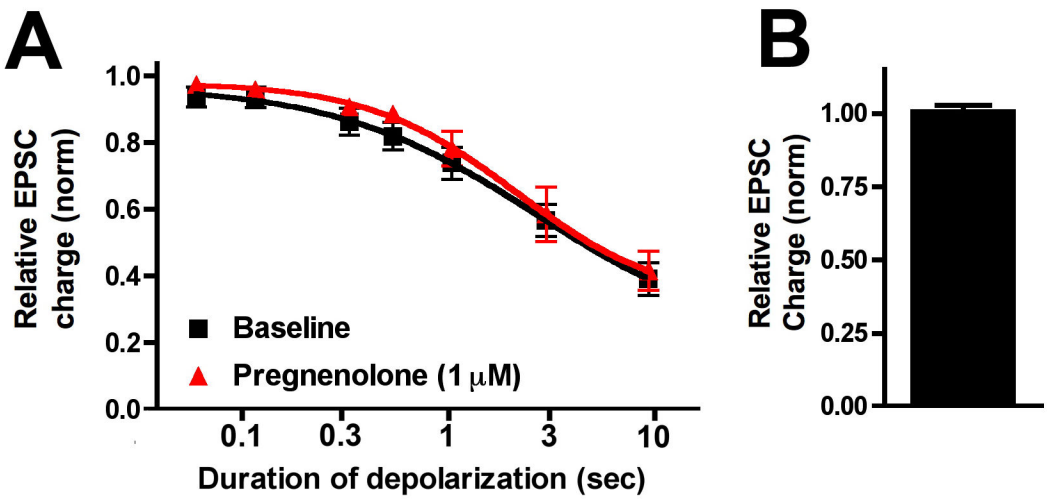

Figure 5. Pregnenolone does not inhibit neurotransmission via CB_1

A) Depolarization response curve before (black squares) and after/during 1μM pregnenolone treatment (red triangles). B) Effect of 1μM pregnenolone alone on EPSCs in WT neurons.

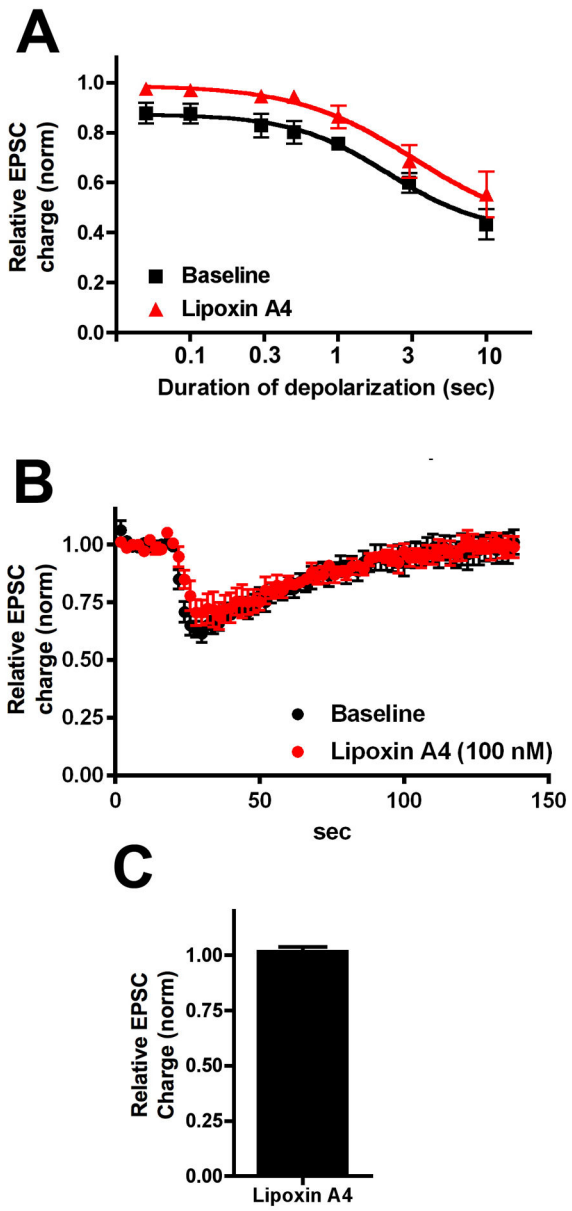

Figure 6. The PAM Lipoxin A4 (LPA4) inhibits DSE in autaptic neurons

A) Depolarization response curve before (black squares) and after/during 1μM LPA4 treatment (red triangles). B) Averaged DSE (3 sec depolarization) time courses before and after drug. C) Effect of 1μM LPA4 alone on EPSCs in WT neurons.