

Modulation of Recombinant Human T-type calcium Channels by Δ^9 -tetrahydrocannabinolic acid *in vitro*

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Abstract

Introduction: Low voltage-activated T-type calcium channels (T-type I_{Ca}), Cav3.1, Cav3.2, and Cav3.3 are opened by small depolarizations from the resting membrane potential in many cells and have been associated with neurological disorders including absence epilepsy and pain. Δ^9 -tetrahydrocannabinol (THC) is the principal psychoactive compound in *Cannabis* and also directly modulates T-type I_{Ca} , however, there is no information about functional activity of most phytocannabinoids on T-type calcium channels, including Δ^9 -tetrahydrocannabinol acid (THCA), the natural non-psychoactive precursor of THC. The aim of this work was to characterize THCA effects on T-type calcium channels.

Materials and Methods: We used HEK293 Flp-In-TREx cells stably expressing Cav3.1, 3.2 or 3.3. Whole-cell patch clamp recordings were made to investigate cannabinoid modulation of I_{Ca} .

Results: THCA and THC inhibited the peak current amplitude Cav3.1 with a pEC_{50} s of 6.0 ± 0.7 and 5.6 ± 0.4 , respectively. $1 \mu\text{M}$ THCA or THC produced a significant negative shift in half activation and inactivation of Cav3.1 and both drugs prolonged Cav3.1 deactivation kinetics. THCA ($10 \mu\text{M}$) inhibited Cav3.2 by $53\% \pm 4$ and both THCA and THC produced a substantial negative shift in the voltage for half inactivation and modest negative shift in half activation of Cav3.2. THC prolonged the deactivation time of Cav3.2 while THCA did not. THCA inhibited the peak current of Cav3.3 by $43\% \pm 2$ ($10 \mu\text{M}$) but did not notably affect Cav3.3 channel activation or inactivation, however, THC caused significant hyperpolarizing shift in Cav3.3 steady state inactivation.

Discussion:

THCA modulated T-type I_{Ca} currents *in vitro*, with significant modulation of kinetics and voltage dependence at low μM concentrations. This study suggests that THCA may have

potential for therapeutic use in pain and epilepsy via T-type channel modulation without the unwanted psychoactive effects associated with THC.

Keywords: T-type Calcium Channels, Phytocannabinoids; Δ^9 - tetrahydrocannabinol; Δ^9 - tetrahydrocannabinol acid; Pain, Epilepsy; Electrophysiology

ABBREVIATIONS

I_{Ca}	Voltage gated calcium channel current
THC	Δ^9 -tetrahydrocannabinol
THCA	Δ^9 - tetrahydrocannabinolic acid
CBD	Cannabidiol
TRP	Transient Receptor Potential

Introduction

Cannabis sativa has been used for thousands of years as a medicinal plant for the relief of pain and seizures¹⁻³. There is a growing body of evidence suggesting cannabinoids are beneficial for a range of clinical conditions including pain⁴ inflammation⁵ epilepsy⁶⁻⁸, sleep disorders⁹, symptoms of multiple sclerosis¹⁰, and other conditions^{11,12}. Phytocannabinoids, derived from diterpenes in *Cannabis*, have a range of distinct pharmacological actions¹³. The best characterised phytocannabinoid is Δ^9 -tetrahydrocannabinol (THC), well known for its psychoactive effects¹⁴, mediated by its activation of the cannabinoid receptor CB₁¹⁵. The next most abundant phytocannabinoid is cannabidiol (CBD), which is non-psychoactive and proposed to have potential therapeutic effects in a broad range of neurological disorders¹⁶⁻¹⁸ and which has been shown to inhibit signalling via at both CB₁ and CB₂ receptors^{16,19,20}. Cannabinoids can also interact with a wide variety of ion channels including Transient Receptor Potential (TRP) channels, ligand gated channels and voltage dependent channels²¹. THC was identified as a prototypic agonist of TRPA1 and subsequently it and other phytocannabinoids have been reported to activate or inhibit many other TRP channels²². THC and CBD inhibit evoked currents through recombinant 5-HT₃ receptors independently of cannabinoid receptors²³; and THC caused significant inhibition of native receptor in mammalian neurons²⁴. THC and CBD also potentiate glycine receptor function through an allosteric mechanism²⁵.

Voltage gated ion channels also modulated by phytocannabinoids. CBD and cannabigerol (CBG) are able to inhibit voltage-gated Na (Nav) channels *in vitro*^{26,27} which has been suggested to contribute to anti-epileptic effects. A wide range of cannabinoids have been shown to modulate T type *I*_{Ca} channels, including endogenous cannabinoids anandamide and N-arachidonoyl dopamine²⁸, endogenous lipoamino acids such as N-arachidonoyl 5-HT and N-arachidonoyl glycine, as well as the phytocannabinoids THC and CBD²⁹⁻³¹. These effects are

thought to be mediated by direct interaction of the ligands with channels, as the experiments were done in cells do not express cannabinoid receptors.

Voltage-dependent Ca^{2+} channels are categorized into three families: L-type channels ($\text{Cav}1$), the neuronal N-, P/Q- and R-type channels ($\text{Cav}2$) and the T-type channels ($\text{Cav}3$)³². T-type Ca^{2+} channels ($\text{Cav}3$), can activate upon small depolarizations of the plasma membrane and are present in many excitable cells³³ where they are critical for neuronal firing and neurotransmitter release and physiological processes such as slow wave sleep³⁴⁻³⁶. Cells expressing T-type calcium channels are involved in epilepsy, pain and other diseases and there is substantial evidence supporting the idea that modulating T type calcium channels is a potential therapeutic option in these conditions³⁷⁻³⁹. T-type calcium are encoded by three $\text{Cav}3$ subunits ($\text{Cav}3.1$, $\text{Cav}3.2$, and $\text{Cav}3.3$). Much smaller membrane depolarizations are required for opening, and at typical neuronal resting membrane potentials a significant number of T-type channels are inactivated. They markedly differ in some of their electrophysiological properties^{40,41}. The most notable of these are that $\text{Cav}3.1$ and $\text{Cav}3.2$ have much faster activation and inactivation kinetics, than $\text{Cav}3.3$ ^{42,43}.

Δ^9 -tetrahydrocannabinolic acid (THCA) is the precursor of THC in *Cannabis*. THCA is acutely decarboxylated to form THC by heating⁴⁴. Importantly, THCA has low affinity at CB_1 receptor⁴⁵ but interestingly, THCA has been reported to have neuroprotective, anti-inflammatory, and immunomodulatory effects⁴⁴, raising the possibility of therapeutic activity without unwanted psychotropic effects.

Previous work from our lab have shown that THC and CBD modulate T-type calcium channels⁴⁶, however, there is no information surrounding the effects of other phytocannabinoids including THCA on these channels. The aim of this work was to characterize THCA modulatory effects on the T-type calcium channels and compare its effects with THC. If THCA could also modulate $\text{Cav}3$ channels, this may provide potential therapeutic activity in pain and

other disorders involving the peripheral nervous system without having psychoactive properties.

Methods

Transfection and Cell culture

Flp-In T-REx 293 HEK cells (ThermoFisher) were stably transfected with pcDNA5/FRT/TO vector encoding human Cav3.1 (NM 018896.4), Cav3.2 (NM 021098.2), or Cav3.3 (NM 021096.3) (GenScript). The integration of this vector to the Flp-In site was mediated by pOG44, Flp-recombinase expression vector pOG44, which was co-transfected as per manufacturer's recommendation (ratio 9:1). Transfections were done using Fugene HD transfection agent (Promega) at ratio 1:4 (w/v) total DNA: Fugene HD. Selection of stably expressing cells were performed using 150µg/mL Hygromycin B Gold (InvivoGen) as per kill curve (data not shown). Flp-In T-Rex 293 HEK cells (expressing Cav3.1, Cav3.2, or Cav3.3) do not express CB1 or CB2 receptors⁴⁷. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and 1% penicillin-streptomycin. HEK-Cav3.1, Cav3.2, and Cav3.3 were passaged in media with 15µg/ml Blasticidin (InvivoGen) and 100µg/ml Hygromycin. Cells were maintained in 5% CO₂ at 37°C in a humidified atmosphere. Channel expression was induced by adding 2µg/mL tetracycline.

Electrophysiology

Currents in Flp-In T-REx 293 HEK cells expressing Cav3.1, Cav3.2, or Cav3.3 channels were recorded in the whole-cell configuration of the patch clamp method at room temperature. Dishes were constantly perfused with external recording solution containing (in mM) (1MgCl₂, HEPES, 10 Glucose, 114 CsCl, 5 BaCl₂) (pH to 7.4 with CsOH, osmolarity =330). 2-4 MΩ recording electrodes were filled with internal solutions containing (in mM) :126.5 CsMeSO₄, 11 EGTA, 10 HEPES adjusted to pH 7.3 with CsOH. Immediately before use, internal solution was added to a concentrated aliquot of GTP and ATP to yield final concentrations of 0.6 mM

and 2mM, respectively. All recordings were measured using an Axopatch 200B amplifier in combination with Clampex 9.2 software (Molecular Devices, Sunnyvale, CA). All data were sampled at 5-10 kHz and filtered at 1 kHz. All currents were leak subtracted using P/N4 protocol.

THC and THCA were prepared daily from concentrated DMSO stocks and diluted in external solution to appropriate concentrations and applied locally to cells via a custom-built gravity driven micro perfusion system. Before running drugs in test of activation and inactivation of Cav3 channels, external control solution was applied about 5 minutes in each experiment to observe in the absence of drugs, vehicle controls itself have no effects on Cav3 channel kinetics. All solutions did not exceed 0.1% DMSO and this concentration of vehicle had no effect on current amplitude or on half activation and half-inactivation potentials (Table1).

This voltage step was repeated at 12 second intervals (1 sweep) for at least 3 mins to achieve a stable peak I_{Ca} . Perfusion was then switched to 10 μ M drugs until maximum inhibition was attained (determined when no more I_{Ca} inhibition was observed after 3 successive sweeps). Finally, drug was “washed out” by switching perfusion back to control solution consisting of external buffer with vehicle control.

In order to test whether THCA used contained an appreciable amount of THC, we examined the activity of THCA in a fluorescent assay of CB1-dependent activation of inwardly rectifying K channels (described in detail in ⁴⁸). In these experiments, THC (1 μ M) produced a change in fluorescence of 12.8 ± 1.2 %. In parallel experiments, THCA (1 μ M) did not significantly alter the fluorescence ($1.0 \pm 0.6\%$). pEC_{50} for THC in this assay is about 300nM ⁴⁸ and 100nM THC produces a robust change in fluorescence ⁴⁹, the lack of effect of THCA at 1 μ M suggests that there was no significant contamination of THCA with THC.

Drugs and reagents

The THC and THCA used in this study were a kind gift from University of Sydney's Lambert Institute for Cannabinoid Therapeutics. Drugs (30 mM) were aliquoted and stored as concentrated stocks in DMSO and stored at -30 C. Daily dilutions were made fresh before each use in external recording solution to give a final vehicle concentration of 0.1%.

Statistics

Data are reported as the mean and standard error of at least 6 independent experiments. Concentration response curves, steady state inactivation and activation were generated by fitting data to a Boltzmann sigmoidal equation in Graph Pad Prism 8. Statistical significance for comparing the $V_{0.5}$ values of activation and inactivation were determined using one-way ANOVA comparing values of $V_{0.5}$ calculated for individual experiments. In order to compare the changes in the time to peak and decay time of deactivation, unpaired t-test was used. All values are reported as mean $\pm$ standard errors and were fitted with a modified Boltzmann equation: $I = [G_{max} \cdot (V_m - E_{rev})] / [1 + \exp((V_{0.5} \text{ act} - V_m) / k_a)]$, where V_m is the test potential, $V_{0.5} \text{ act}$ is the half-activation potential, E_{rev} is the reversal potential and G_{max} is the maximum slope conductance. Steady-state inactivation curves were fitted using Boltzmann equation: $I = 1 / (1 + \exp((V_m - V_h) / k))$, where V_h is the half-inactivation potential and k is the slope factor.

Results

Superfusion of THCA and THC on Cav3 inhibited the peak of the I_{Ca} evoked by a step from -100mV to -30 mV (Fig 1). At a concentration of $10\text{ }\mu\text{M}$, THC or THCA blocked the current amplitude of Cav3.1 almost completely, and inhibited Cav3.2 by $56 \pm 2\%$ and $53 \pm 4\%$ respectively ($n=6$). $10\mu\text{M}$ THC did not affect Cav3.3 I_{Ca} while $10\mu\text{M}$ THCA inhibited Cav3.3 by $43\% \pm 2$ (Fig 1A). Cav3.1 was inhibited by THC and THCA with pEC_{50} 6 ± 0.7 and 5.6 ± 0.4 respectively (Fig1B). The effects of THCA and THC on Cav3.1, 3.2 and 3.3 currents are illustrated in Fig 2 (THCA) and Fig 3 (THC), the drug effects did not readily reverse on washout.

THC and THCA effects on activation and inactivation kinetics

We examined the voltage-dependence of activation Cav3 channels by repetitively stepping cells from -75mV to 50mV from a holding potential of -100mV . After a control I/V relationship was generated, it was repeated after 5 min perfusion of THCA (Fig 4A). The voltage-dependence of activation for Cav3.1 was affected by THCA, notably it increased current amplitudes for depolarisations between -75mV to -45mV and inhibited current amplitude for depolarisations between -35 and 50mV (Fig 4B). THCA produced a significant hyperpolarizing shift in the half activation potential of Cav3.1; these shifts were not seen with time-matched vehicle controls (Table 1). Steady-state inactivation, where cells were voltage clamped at potentials between $(-110\text{ mV}$ and $-20\text{ mV})$ for 2s before current were evoked by stepping them to test potentials of -30mV , showed that THCA also caused large shifts in steady-state inactivation of Cav3.1 (Fig 4C). Activation and inactivation changes for cells exposed to vehicle alone for 5 min were less than -1mV (Table1). Using the same protocols, it was found that THCA also shifted Cav3.2 half activation to negative potentials and caused a larger shift in half inactivation of Cav3.2 (Fig 4D). THCA caused small positive shift and significant negative shift in half activation and inactivation of Cav3.3 (Fig 4E).

1 μ M THC also affected steady state inactivation and activation of Cav3.1. THC shifted half activation and inactivation of Cav3.1 to more negative voltages (Fig 5C). THC shifted half activation of Cav3.2 to negative potentials and caused significant negative shift in inactivation of Cav3.2 (Fig 5D). THC at 10 μ M had no effect on the half activation of Cav3.3 however THC negatively shifted the half inactivation of Cav3.3 significantly (Fig 5E).

Effects of THC and THCA on time to peak and kinetics of current deactivation of Cav3 channels

THC and THCA caused no significant changes on time to peak on any of the T-type channels at any voltage (Fig 6A-F). The effects of THC and THCA on deactivation of currents elicited during the standard I/V protocol, were measured by fitting a monophasic exponential to the inward “tail” currents that resulted immediately following the voltage step. 1 μ M THCA slowed deactivation of Cav3.1 (Fig. 7A, C), however, the deactivation of both Cav3.2 (Fig 7E) and Cav3.3 (not shown) were unaffected by THCA at 10 μ M. THC slowed deactivation of Cav3.1 (1 μ M, Figure 7B, D) and Cav3.2 (10 μ M, Figure 7F) but THC did not change deactivation of Cav3.3 (not shown).

Discussion

The major finding of this study is that THCA inhibited T-type calcium channels with most potent effects on Cav3.1. THC also most potently affected Cav3.1, and Cav3.2 was moderately inhibited by both drugs at 10 μ M with less inhibition of Cav3.3. THCA shifted the half activation and inactivation voltages of Cav3.1 and Cav3.2 to more negative potentials, THC behaved in a similar fashion. THCA and THC also slowed the time constant deactivation of Cav3.1 however at 10 μ M only THC slowed the deactivation of Cav3.2. Both THCA and THC produced modest shifts in Cav3.3 inactivation without any effects on the deactivation kinetics. The presence of the carboxylic acid moiety in THCA does not result in substantial differences in modulation of T type calcium channel compared with THC.

THC has higher affinity to cannabinoid receptors CB₁ and CB₂¹⁵ and causes a distinctive intoxication via activation of the CB₁⁵⁰ receptors, however, studies of affinity of THCA for the CB₁ receptor have produced different results, but studies where THCA was tested for THC produced by THCA degradation, there was little activity attributable to THCA^{51,52}. Verhoeckx *et al* examined THC and THCA affinity using radioligand binding assay and determined that THC had greater affinity compared to THCA at CB₁⁴⁴. However, Ahmed *et al* reported no affinity of THCA on CB₁⁵³ while Husni *et al.*, found some activity on CB₁⁵⁴, while the one study that reported THC and THCA had similar affinity for CB₁, did not examine the potential contamination of THCA with THC⁵¹. We tested the activity of our THCA in a membrane potential assay in AtT20 cells expressing CB₁ receptors. THC (1 μ M) produced a significant hyperpolarization of the cells, as reported many times previously, while THCA did not produce changes in fluorescence, suggesting that in our experiments, THC contamination of the THCA was insignificant.

In current study, THCA like THC shifted steady state inactivation of the Cav3.1 and Cav3.2 channels to more negative potentials, reducing the number of channels that can open when the cell is depolarised, preventing their transition to an inactivated state. THCA had the same effect as THC on Cav3.1 steady state activation, causing a hyperpolarising shift so that when the cells are depolarised, more channels are available for activation. THCA effects on Cav3.2 kinetics were less pronounced than THC, causing a more negative shift in both activation and inactivation of Cav3.2. The effects of THCA and THC on in half activation of Cav3.3 was not significant. Conversely, THCA and THC caused a significant shift in steady state inactivation of Cav3.3. Interestingly, THCA and THC potentiated Cav3.1 current evoked by modest depolarization and then inhibited current amplitudes following stronger depolarisation. These data suggest that THCA and THC may increase the initial depolarizing drive produced by Cav3.1 in some circumstances, despite the overall inhibitory effects on the channels.

The results with THC are in good agreement with previous studies from our lab. In general, THC showed modestly higher potency to inhibit Cav3.2 and Cav3.3 in the study of Ross *et al*, this can be attributed to the subtle different recording conditions where potency was determined in cells voltage clamped at slightly more depolarized potentials (-100mV vs -86mV)²⁸.

Both THC and THCA have been reported to activate TRPA1 and TRPV2 channels and showed the similar antagonist activity on TRPV1 and TRPM8^{21,22,55}. Together with the results of our study, these data show that THCA and THC generally behave in a similar manner for ion channel modulation, but they have very different activity on cannabinoid GPCR. The very limited permeability of THCA to cross the blood brain barrier suggests a potential role as a drug for treatment of pain and inflammation in the periphery, and THCA has been shown to

reduce inflammation in the gut⁵⁷. While the mechanism(s) underlying this are still unknown, inhibition of T-Type I_{Ca} is a possible contributor.^{58,59}

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Author Disclosure statement

No competing financial interests exist

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References

1. Bridgeman MB, Abazia DT. Medicinal cannabis: History, pharmacology, and implications for the acute care setting. *P T*. 2017;42:180-188.
2. Zlas J, Stark H, Seligman J, et al. Early medical use of cannabis. *Nature*. 1993;363:215.
3. Friedman D, Sirven JJ. Historical perspective on the medical use of cannabis for epilepsy: Ancient times to the 1980s. *Epilepsy Behav*. 2017;70:298-301.
4. Russo EB. Cannabinoids in the management of difficult to treat pain. *Ther Clin Risk Manag*. 2008;4:245-259.
5. Mechoulam R, Sumariwalla PF, Feldmann M, et al. Cannabinoids in models of chronic inflammatory conditions. *Phytochemistry Reviews*. 2005;4:11-18.
6. Rosenberg EC, Patra PH, Whalley BJ. Therapeutic effects of cannabinoids in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection. *Epilepsy Behav*. 2017;70:319-327.
7. Friedman D, Devinsky O. Cannabinoids in the Treatment of Epilepsy. *N Engl J Med*. 2015;373:1048-1058.
8. Devinsky O, Marsh E, Friedman D, et al. Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *Lancet Neurol*. 2016;15(3):270–8.
9. Gates PJ, Albertella L, Copeland J. The effects of cannabinoid administration on sleep: a systematic review of human studies. *Sleep Med Rev*. 2014;18:477-487.
10. Notcutt WG. Clinical Use of Cannabinoids for Symptom Control in Multiple Sclerosis. *Neurotherapeutics*. 2015;12:769-777.
11. Dariš B, Verboten MT, Knez Ž, et al. Cannabinoids in cancer treatment: Therapeutic potential and legislation. *Bosn J Basic Med Sci*. 2019;19:14–23.
12. Bielawiec P, Harasim-Symbor E, Chabowski A. Phytocannabinoids: Useful Drugs for the Treatment of Obesity? Special Focus on Cannabidiol. *Frontiers in Endocrinology*.

- 2020;11 :114.
13. Pisanti S, Malfitano AM, Ciaglia E, et al. Cannabidiol: State of the art and new challenges for therapeutic applications. *Pharmacol Ther.* 2017;175:133-150.
 14. Maccarrone M, Bab I, B  r   T, et al. Endocannabinoid signaling at the periphery: 50 years after THC. *Trends in Pharmacological Sciences.* 2015; 36:277–96.
 15. Pertwee RG. The diverse CB₁ and CB₂ receptor pharmacology of three plant cannabinoids: Δ^9 -tetrahydrocannabinol, cannabidiol and Δ^9 -tetrahydrocannabivarin. *Br J Pharmacol.* 2008;153:199-215.
 16. Elsaid S, Kloiber S, Le Foll B. Effects of cannabidiol (CBD) in neuropsychiatric disorders: A review of pre-clinical and clinical findings. *Prog Mol Biol Transl Sci* 2019;167:25-75.
 17. Devinsky O, Patel AD, Cross JH, et al. Effect of Cannabidiol on Drop Seizures in the Lennox–Gastaut Syndrome. *N Engl J Med.* 2018;378:1888-1897.
 18. Campos AC, Foga  a M V, Sonego AB, et al. Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacol Res.* 2016;112:119-127.
 19. Thomas A, Baillie GL, Phillips AM, et al. Cannabidiol displays unexpectedly high potency as an antagonist of CB₁ and CB₂ receptor agonists in vitro. *Br J Pharmacol.* 2007;150:613-623.
 20. Laprairie RB, Bagher AM, Kelly MEM, et al. Cannabidiol is a negative allosteric modulator of the cannabinoid CB₁ receptor. *Br J Pharmacol.* 2015;172:4790–805.
 21. Muller C, Morales P, Reggio PH. Cannabinoid ligands targeting TRP channels. *Front Mol Neurosci.* 2019;11.
 22. De Petrocellis L, Ligresti A, Moriello AS, et al. Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *Br J Pharmacol.* 2011;163:1479-1494.

23. Barann M, Molderings G, Brüss M, et al. Direct inhibition by cannabinoids of human 5-HT_{3A} receptors: Probable involvement of an allosteric modulatory site. *Br J Pharmacol.* 2002;137:589-596.
24. Yang KHS, Isaev D, Morales M, et al. The effect of δ 9-tetrahydrocannabinol on 5-HT₃ receptors depends on the current density. *Neuroscience.* 2010;171:40-49.
25. Hejazi N, Zhou C, Oz M, et al. Δ 9-Tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. *Mol Pharmacol.* 2006;69:991-997.
26. Mohammad X, Ghovanloo R, Shuart NG, et al. Inhibitory effects of cannabidiol on voltage-dependent sodium currents. *J Biol Chem.* 2018:16546-16558.
27. Hill AJ, Jones NA, Smith I, et al. Voltage-gated sodium (NaV) channel blockade by plant cannabinoids does not confer anticonvulsant effects per se. *Neurosci Lett.* 2014;566:269-274.
28. Ross HR, Gilmore AJ, Connor M. Inhibition of human recombinant T-type calcium channels by the endocannabinoid N-arachidonoyl dopamine. *Br J Pharmacol.* 2009;156:740-750.
29. Chemin J. Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO J.* 2001;20:7033-7040.
30. Gilmore AJ, Heblinski M, Reynolds A, et al. Inhibition of human recombinant T-type calcium channels by N-arachidonoyl 5-HT. *Br J Pharmacol.* 2012;167:1076-1088.
31. Barbara G, Alloui A, Nargeot J, et al. T-type calcium channel inhibition underlies the analgesic effects of the endogenous lipoamino acids. *J Neurosci.* 2009;29:13106-13114.
32. View of Voltage-gated calcium channels (version 2019.4) in the IUPHAR/BPS Guide to Pharmacology Database | IUPHAR/BPS Guide to Pharmacology CITE. Accessed August 1, 2020. <http://journals.ed.ac.uk/gtopdb-cite/article/view/3232/4318>

33. Cribbs LL, Lee J-H, Yang J, et al. Cloning and Characterization of $\alpha 1H$ From Human Heart, a Member of the T-Type Ca^{2+} Channel Gene Family. *Circ Res.*1998;83:103-109.
34. Huguenard JR. Low-Threshold Calcium Currents in Central Nervous System Neurons. *Annu Rev Physiol.*1996;58:329-348.
35. Crunelli V, David F, Leresche N, et al. Role for T-type Ca^{2+} channels in sleep waves
Role for T-type Ca^{2+} channels in sleep waves. *Pflügers Arch Eur J Physiol.* 2014;466:735-745.
36. Iftinca MC, Zamponi GW. Regulation of neuronal T-type calcium channels. *Trends Pharmacol Sci.* 2009;30:32-40.
37. Zamponi GW, Snutch TP. Recent advances in the development of T-type calcium channel blockers for pain intervention. *Br J Pharmacol.* 2018;175:2375-2383.
38. Monteil A, Chausson P, Boutourlinsky K, et al. Inhibition of Cav3.2 T-type calcium channels by its intracellular I-II loop. *J Biol Chem.* 2015;290:16168-16176.
39. Chen Y, Parker WD, Wang K. The role of T-type calcium channel genes in absence seizures. *Front Neurol.* 2014;5:45.
40. Cain SM, Snutch TP. Contributions of T-type calcium channel isoforms to neuronal firing. *Channels.* 2010;4:475-482.
41. Chemin J, Monteil A, Perez-Reyes E, et al . Specific contribution of human T-type calcium channel isoforms ($\alpha 1G$, $\alpha 1H$ and $\alpha 1I$) to neuronal excitability. *J Physiol.* 2002;540:3-14.
42. McRory JE, Santi CM, Hamming KSC, et al. Molecular and Functional Characterization of a Family of Rat Brain T-type Calcium Channels. *J Biol Chem.* 2001;276:3999-4011.
43. Klöckner U, Lee J-H, Cribbs LL, et al. Comparison of the Ca^{+2} currents induced by expression of three cloned $\alpha 1$ subunits, $\alpha 1G$, $\alpha 1H$ and $\alpha 1I$, of low-voltage-activated T-type Ca^{+2} channels. *Eur J Neurosci.* 1999;11:4171-4178.

44. Verhoeckx KCM, Korthout HAAJ, Van Meeteren-Kreikamp AP, et al. Unheated Cannabis sativa extracts and its major compound THC-acid have potential immunomodulating properties not mediated by CB₁ and CB₂ receptor coupled pathways. *Int Immunopharmacol*. 2006;6:656-665.
45. McPartland JM, MacDonald C, Young M, et al. Affinity and Efficacy Studies of Tetrahydrocannabinolic Acid A at Cannabinoid Receptor Types One and Two. *Cannabis Cannabinoid Res*. 2017;2:87-95.
46. Ross HR, Napier I, Connor M. Inhibition of recombinant human T-type calcium channels by Δ^9 -tetrahydrocannabinol and cannabidiol. *J Biol Chem*. 2008;283:16124-16134.
47. Atwood BK, Lopez J, Wager-Miller J, et al. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics*. 2011;12:14.
48. Sachdev S, Vemuri K, Banister SD, et al. In vitro determination of the efficacy of illicit synthetic cannabinoids at CB₁ receptors. *Br J Pharmacol*. 2019;176:4653-4665.
49. Santiago M, Sachdev S, Arnold JC, et al. Absence of Entourage: Terpenoids Commonly Found in Cannabis sativa Do Not Modulate the Functional Activity of Δ^9 -THC at Human CB₁ and CB₂ Receptors. *Cannabis Cannabinoid Res*. 2019;4:165-176.
50. Huestis MA, Gorelick DA, Heishman SJ, et al. Blockade of effects of smoked marijuana by the CB₁-selective cannabinoid receptor antagonist SR141716. *Arch Gen Psychiatry*. 2001;58:322-328.
51. Rosenthaler S, Pöhn B, Kolmanz C, et al. Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures. *Neurotoxicol Teratol*. 2014;46:49-56.
52. McPartland JM, MacDonald C, Young M, et al. Affinity and Efficacy Studies of

- Tetrahydrocannabinolic Acid A at Cannabinoid Receptor Types One and Two. *Cannabis cannabinoid Res.* 2017;2:87-95.
53. Ahmed SA, Ross SA, Slade D, et al. Cannabinoid ester constituents from high-potency *Cannabis sativa*. *J Nat Prod.* 2008;71:536-542.
 54. Husni AS, McCurdy CR, Radwan MM, et al. Evaluation of phytocannabinoids from high-potency *Cannabis sativa* using in vitro bioassays to determine structure-activity relationships for cannabinoid receptor 1 and cannabinoid receptor 2. *Med Chem Res.* 2014;23:4295-4300.
 55. Gaston TE, Friedman D. Pharmacology of cannabinoids in the treatment of epilepsy. *Epilepsy Behav.* 2017;70:313-318.
 56. Anderson LL, Low IK, Banister SD, et al. Pharmacokinetics of Phytocannabinoid Acids and Anticonvulsant Effect of Cannabidiolic Acid in a Mouse Model of Dravet Syndrome. *J Nat Prod.* 2018;82: 3047-3055.
 57. Nallathambi R, Mazuz M, Ion A, et al. Anti-Inflammatory Activity in Colon Models Is Derived from Δ^9 -Tetrahydrocannabinolic Acid That Interacts with Additional Compounds in Cannabis Extracts. *Cannabis Cannabinoid Res.* 2017;2:167-182.
 58. Picard E, Carvalho FA, Agosti F, et al. Inhibition of Cav3.2 calcium channels: A new target for colonic hypersensitivity associated with low-grade inflammation. *Br J Pharmacol.* 2019;176:950-963.
 59. Scanzi J, Accarie A, Muller E, et al. Colonic overexpression of the T-type calcium channel Cav3.2 in a mouse model of visceral hypersensitivity and in irritable bowel syndrome patients. *Neurogastroenterol Motil.* 2016;28:1632-1640.

Table 1. The effects of THCA and THC on the parameters of steady state activation and inactivation of Cav3 channels

Drug	Cav3	Change in $V_{0.5}$	
		Activation	Inactivation
THCA	3.1	$-7 \pm 2^{****}$	$-8 \pm 3^{****}$
THCA	3.2	$-4.8 \pm 2^{**}$	$-6 \pm 2^{***}$
THCA	3.3	3 ± 1	$-4 \pm 1^*$
THC	3.1	$-7 \pm 2^{****}$	$-8 \pm 1^{****}$
THC	3.2	$-5 \pm 1^{***}$	$-9 \pm 2^{****}$
THC	3.3	-1 ± 0.7	$-8 \pm 1^{****}$
No drug	3.1	0.7 ± 0.2	-1 ± 0.2
No drug	3.2	-1 ± 0.4	-0.6 ± 0.2
No drug	3.3	-0.5 ± 0.2	-1 ± 0.5

Cells expressing recombinant Cav3 channels were voltage-clamped at -100mV then stepped to potential above -75mV(activation) stepped every 5mV. The results of peak currents were fitted to a Boltzmann sigmoidal equation. Changes in the voltage for half activation/inactivation ($V_{0.5}$) of the curve are reported in Table 1. No drug represents time dependent changes under our recording conditions. One-way ANOVA **** indicates p value < 0.0001 , *** indicates p value < 0.001 , ** indicates p value < 0.01 and * indicates p value < 0.02 .

Figure Legends

Figure 1. Effects of 10 μ M THCA and THC on T-type calcium channel current and concentration response curve for THCA and THC effects on Cav3. (A) Peak I_{Ca} was elicited by a step from -100 mV to -30 mV; Cav3.1 current was almost completely inhibited by 10 μ M application of THC and THCA. 10 μ M THC and THCA blocked Cav3.2 calcium current about 52% $\pm$ 3. Cav3.3 current was not affected by 10 μ M THC but THCA decreased Cav3.3 calcium current by 43% $\pm$ 4. **(B)** Concentration response curves were created to determine the potency of these compounds at Cav3.1. Each point represents the mean $\pm$ SEM of 6 cells.

Figure 2. THCA effects on Cav3 current amplitude. Each trace represents the current elicited by a voltage step from -100 mV to -30 mV. **(A)** 1 μ M THCA inhibited calcium current of Cav3.1. **(B)** Time course of inhibition and degree of reversibility THCA inhibition of Cav3.1 is illustrated. **(C)** THCA 10 μ M inhibited calcium current of Cav3.2. **(D)** Time course of inhibition and degree of reversibility THCA inhibition of Cav3.2 is illustrated. **(E)** THCA at 10 μ M inhibited current amplitude of Cav3.3. **(F)** The inhibition of Cav3.3 by 10 μ M THCA was not washed out shown in time course inhibition of Cav3.3.

Figure 3. THC effects on Cav3 current amplitude. Recording of Cav3 channel was made as outlined under experimental procedures. Each trace represents the current elicited by a voltage step from -100 mV to -30 mV. **(A)** 1 μ M THC inhibited Cav3.1 calcium current. **(B)** inhibitory effects of THC on Cav3.1 was not washed out by using external solution. **(C)** THC inhibited Cav3.2 calcium current at 10 μ M. **(D)** A reversal of THC (10 μ M) inhibition of Cav3.2 was not seen by washing. **(E)** THC at 10 μ M had little effect on calcium current of Cav3.3. **(F)** Inhibition by THC at 10 μ M was not reversible.

Figure 4. THCA effects on the activation and inactivation of Cav3 channels. (A) Current-Voltage (I-V) relationship showing the activation of Cav3.1 from a holding membrane potential of -100mV in the absence and presence of 1 μ M THCA. The peak current amplitude is plotted, (B) example traces of this experiment illustrating the effects of 1 μ M THCA at testing membrane potential of -51mV and -22mV: current is enhanced at lower test potentials then inhibited at more depolarized potentials. (C) 1 μ M THCA affected half activation and inactivation of Cav3.1 expressed in HEK293 to negative potentials. (D) Steady state activation and inactivation of Cav3.2 expressed in HEK293 in the presence and absence of THCA showed a significant shift in inactivation of Cav3.2 however 10 μ M THCA created slight shift in activation of Cav3.2. (E) THCA caused a small positive shift in activation kinetics of Cav3.3 and a small negative shift in inactivation of Cav3.3. Each data points represent the mean $\pm$ SEM of 6 cells.

Figure 5. Effects of THC on the voltage-dependence of Cav3 activation and Inactivation.

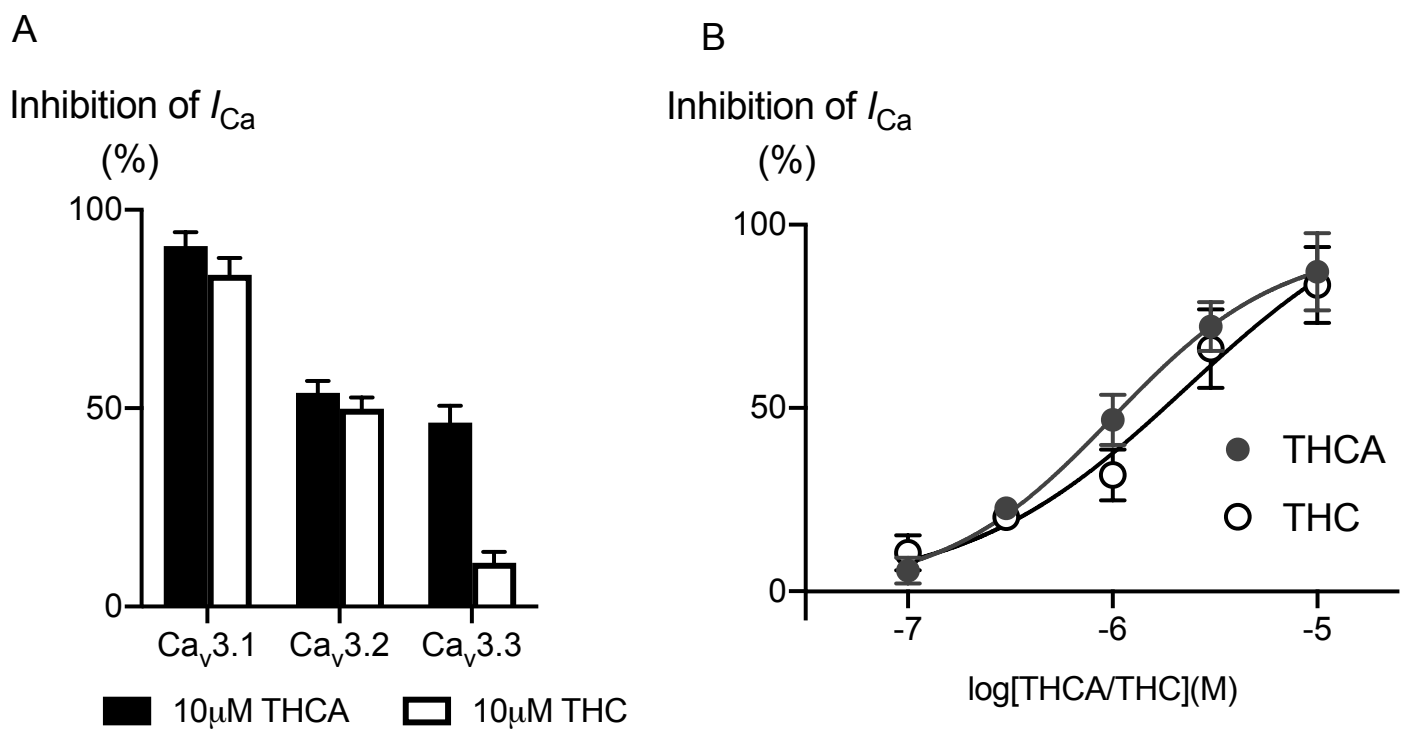
(A) Current- Voltage (I-V) relationship showing the activation of Cav3.1 from a holding membrane potential of -100mV in the absence and presence of 1 μ M THC.(B) The peak current amplitude is plotted at testing membrane potential of -51mV and -22mV. Example traces of this experiment illustrating the effects of 1 μ M THC: current is enhanced at lower test potentials then inhibited at more depolarized potentials.(C) THC effect on Cav3 channels kinetics when HEK293 cells were voltage clamped at -100mV, depolarized to 50mV from -75mV showed that 1 μ M THC shifted activation and inactivation of Cav3.1 to negative potentials significantly.(D) 10 μ M THC effects on activation and inactivation kinetics of Cav3.2 indicated steady state inactivation was shifted to negative potentials significantly however THC caused -5mV shift in activation kinetics of Cav3.2.(E) 10 μ M THC effects on activation and inactivation kinetics of Cav3.3; THC had no effects on steady state activation however THC

caused significant shift in inactivation kinetics of Cav3.3. Each data point represent the mean $\pm$ SEM of six cells.

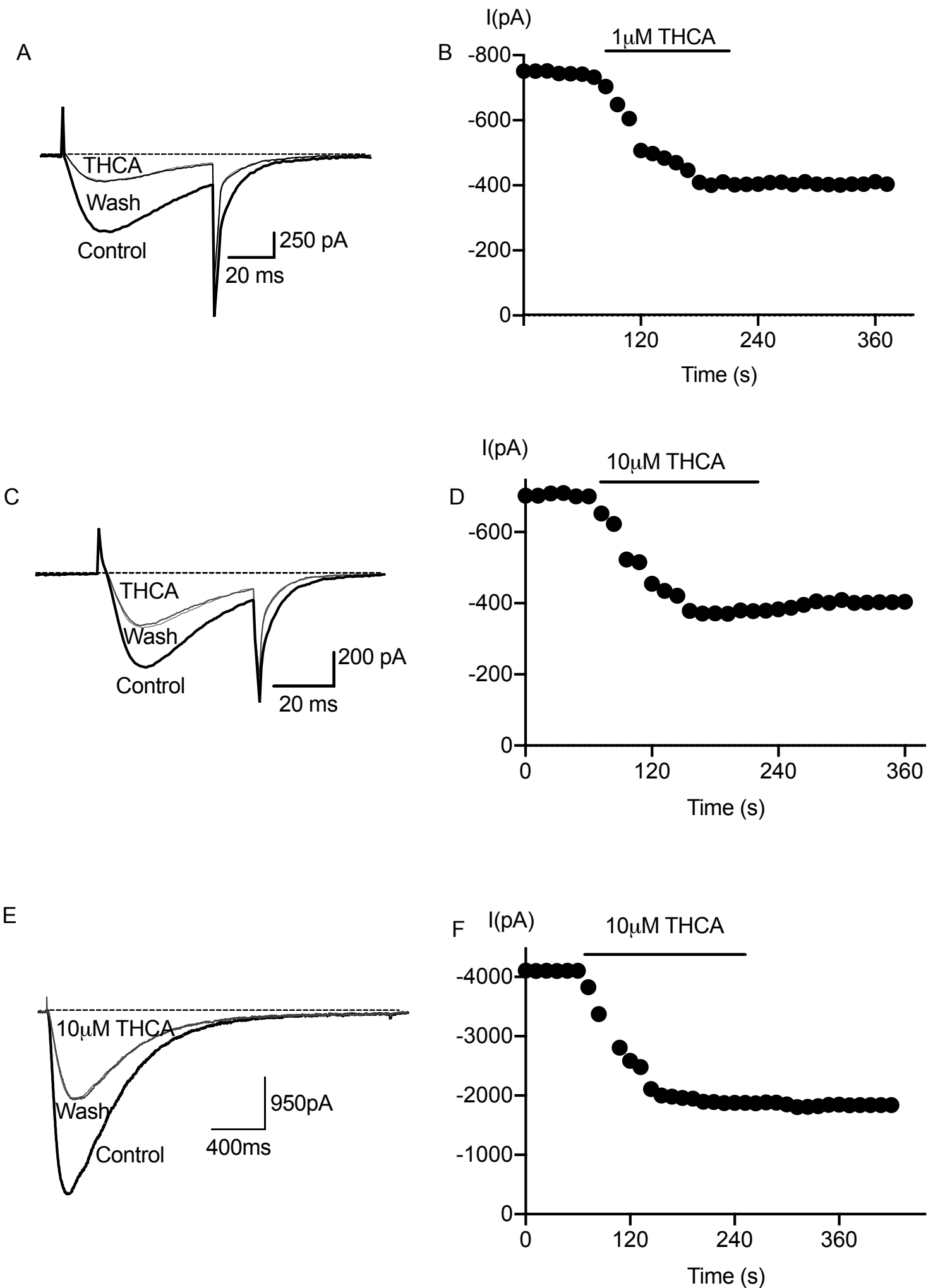
Figure 6. THCA and THC effects on time to peak of Cav3 channels. The plots illustrate the time to peak of current Cav3 before and after 5min superfusion of THC and THCA. THCA had no significant effects on time to peak of (A) Cav3.1, (B) Cav3.2 and (C) Cav3.3. No shift was seen to those in parallel THC experiments where solvent alone was super fused for (D) Cav3.1, (E) Cav3.2 and (F) Cav3.3. Each point represents the mean $\pm$ SEM of at least six cells (Unpaired t-test $P > 0.05$).

Figure 7. THCA and THC effects on Cav3 time constant of deactivation. Cells expressing Cav3 channels were stepped repetitively from a holding potential of -100 mV to test potentials between -75 and 50 mV. (A) THCA produced a significant change in time constant deactivation of Cav3.1 (ANOVA, $P < 0.0001$) across a range of potential membrane. (B) THC produced significant changes in time constant deactivation of Cav3.1 across a range of membrane potential (ANOVA, $P < 0.0001$). (C) 1 μ M THCA prolonged deactivation of Cav3.1 showing in example trace of tail current from I-V current relationships. (D) Example traces of tail current for Cav3.1 showed that 1 μ M THC slowed deactivation of Cav3.1. (E) Representative traces illustrated that THCA at 10 μ M did not affect Cav3.2 and (F) 10 μ M of THC slowed deactivation of Cav3.2.

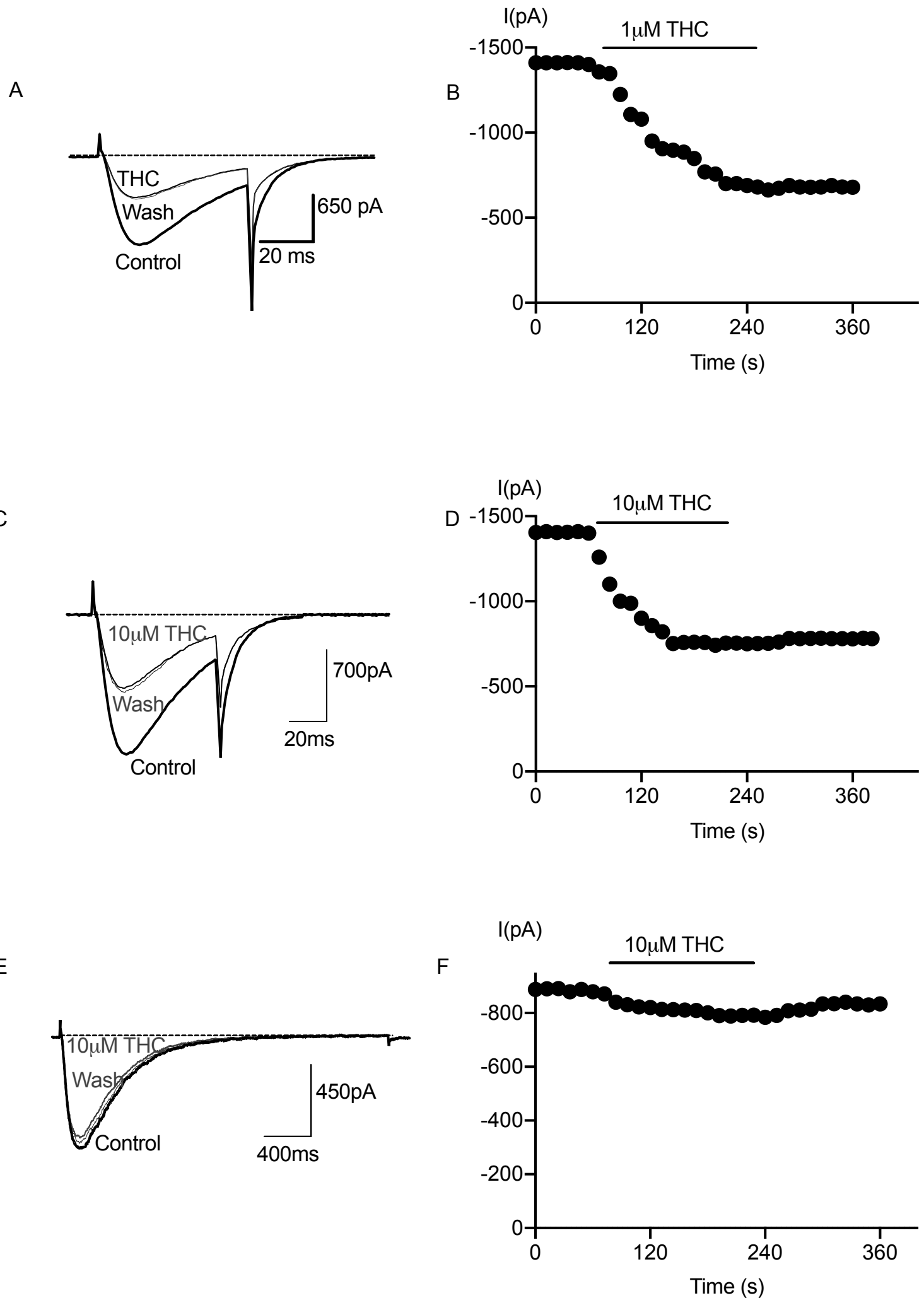
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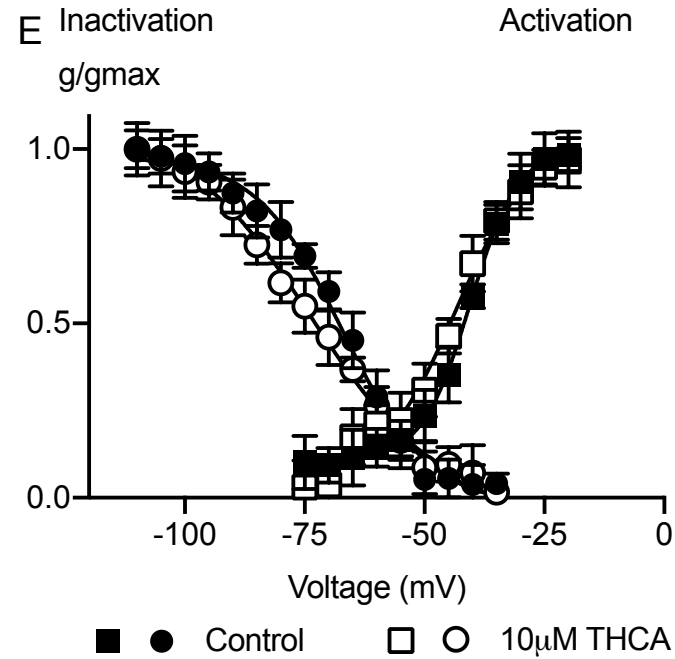
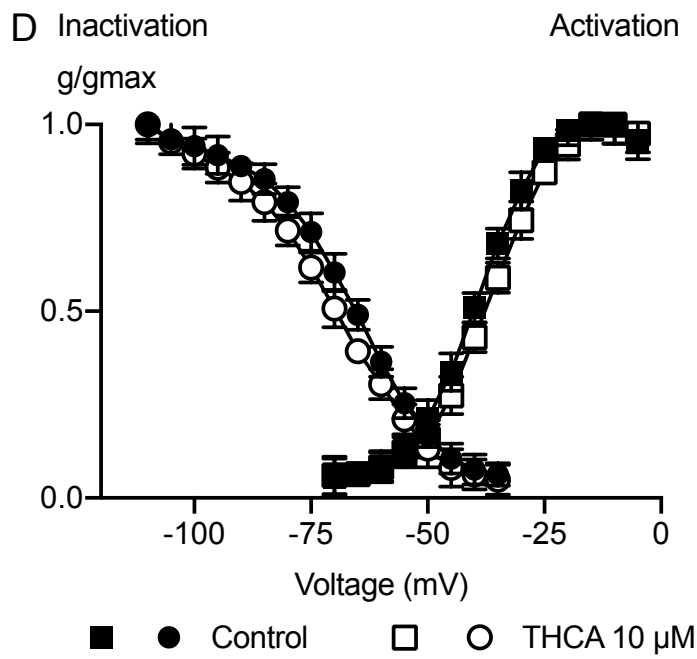
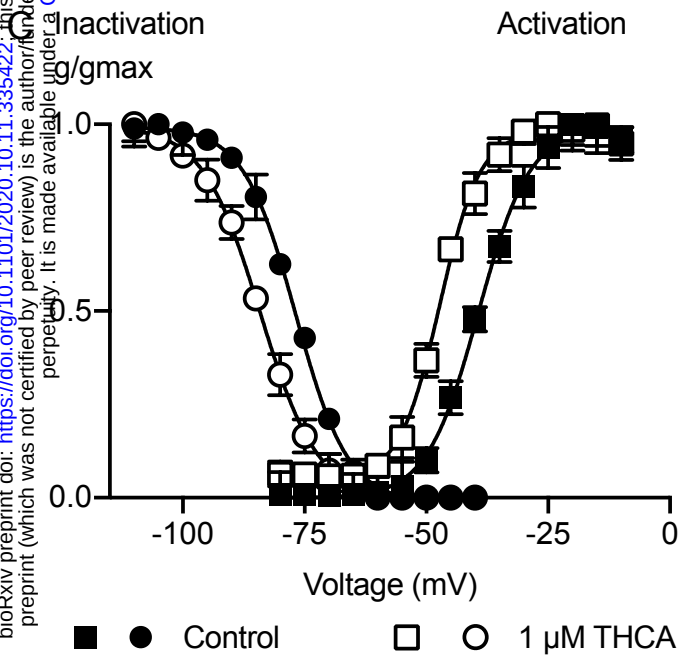
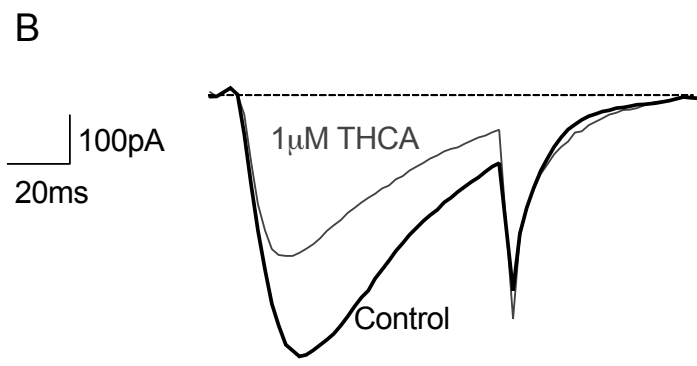
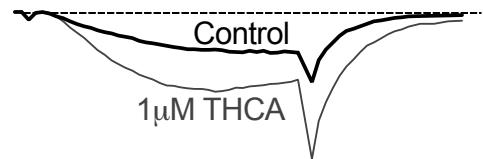
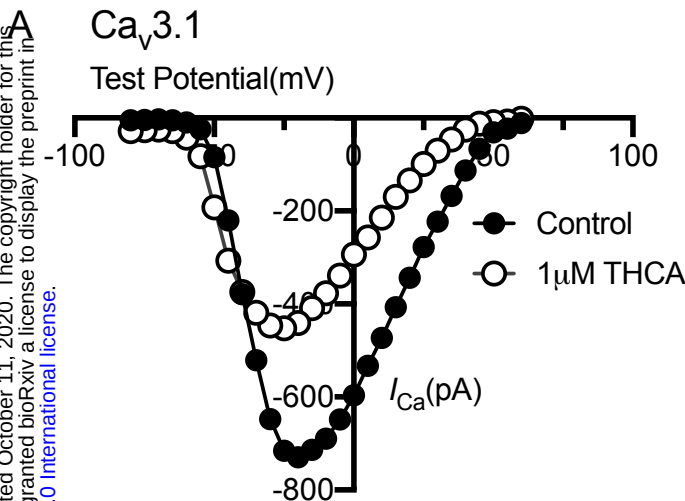
MIRLOHI Figure 2



MIRLOHI Figure 3



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$Ca_v3.1$

$Ca_v3.2$

$Ca_v3.3$

MIRLOHI Figure 5

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